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The Photoassimilation of Organic Compounds by Autotrophic Blue-green Algae

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SUMMARY

Four obligately photoautotrophic blue-green algae were shown to assimilate acetate. This reaction was light dependent and was greatly decreased in the absence of carbon dioxide. Acetate was incorporated mainly into the ethanol extractable (lipid) fraction of the organisms and into the protein fraction. Only four amino acids (glutamate, proline, arginine, leucine) were significantly radioactive as a result of the incorporation of (1-) or (2-)^{14}C-acetate. Partial degradations of amino acids derived from specifically labelled acetate and enzyme assays on crude cell-free extracts of *Anacystis nidulans* support the operation of established pathways for the biosynthesis of these amino acids. Growth of blue-green algae was not significantly affected by the presence of moderate concentrations of the sodium salts of the lower fatty acids, with the exception of propionate, low concentrations of which inhibited growth.

The blue-green algae are a ubiquitous group of procaryotic micro-organisms which grow photosynthetically and, unlike the photosynthetic bacteria, produce oxygen. Apart from studies on their photosynthetic activities and, with certain species, also on their nitrogen-fixing activities, surprisingly few biochemical investigations have been made on this important group of organisms. Although blue-green algae commonly occur in soil and waters containing organic matter, very few studies have been made on their ability to metabolize organic compounds. It is believed that most of the blue-green algae are obligate photoautotrophs. This seems well established for three pure cultures of blue-green algae studied by Kratz & Myers (1955*a*, *b*): namely *Anacystis nidulans*, *Anabaena variabilis* and *Nostoc muscorum* G. These organisms should therefore be useful for a study of the phenomenon of obligate photoautotrophy. It has been found that some obligately autotrophic photosynthetic bacteria can assimilate certain organic compounds (Sadler & Stanier, 1960; Hoare & Gibson, 1964). Accordingly a study has been made of the ability of some blue-green algae to assimilate some simple organic compounds. A preliminary account of part of these investigations has been published (Hoare & Moore, 1965; Hoare, Hoare & Moore, 1966*a*, *b*).

METHODS

Abbreviations used. ATP: adenosine triphosphate; CoA: coenzyme A; DCMU: 3-(3,4-dichlorophenyl)-1,1, dimethyl urea; NAD: nicotinamide adenine dinucleotide; NADP: nicotinamide adenine dinucleotide phosphate.

Organisms. Strains of *Anacystis nidulans*, *Anabaena variabilis* and *Nostoc muscorum* G were obtained from Dr J. Myers (Zoology Department of The University of Texas).

Anabaena flos-aquae A-37 was kindly provided by Dr R. G. Tischer (Mississippi State University). Cultures were maintained under constant illumination at 25–30° on agar slopes containing medium DM (see below) supplemented with ammonium chloride (0·1 g.).

Growth of organisms. Organisms were grown photosynthetically on the medium (DM) of van Baalen (1965*a*), which is a modification of the medium 'D' used by Kratz & Myers (1955*a*); after equilibration with air + 1–2 % (v/v) carbon dioxide the medium was at pH 7·6–7·8. For growth experiments 20 ml. medium in 22 × 175 mm. glass tubes were cotton-wool plugged and gassed continuously with a mixture of 1–2 % (v/v) CO_2 in air; the tubes were immersed in a water bath and were illuminated by a bank of fluorescent lights providing a light intensity of 1000 ft. candles and maintaining the cultures at 30°. Growth was determined by following extinction at 600 mμ in a Bausch & Lomb ('Spectronic-20') spectrophotometer. For larger scale growth, cultures were grown in the same medium in 2-l cylinders by using 10 ml. inoculum to 1500 ml. medium. Cultures were harvested after incubation for 40 hr in the light. In some cases cultures were grown in the continuous culture apparatus of Myers & Clark (1944), operated manually.

For photoassimilation experiments organisms were harvested under aseptic conditions by transferring cultures to sterile polyethylene bottles which were centrifuged for 15 min at 10,000 *g* at 20°, and washed three times in sterile medium DM. For enzyme experiments aseptic conditions were not necessary. After centrifugation and washing, the organisms were stored as a packed mass at −10°.

Determination of viable counts. The procedure was a slight modification of that used by van Baalen (1965*a*). Samples from cultures were harvested and the organisms washed three times and appropriately diluted in the same growth medium. Final dilutions were plated in 0·05 ml. of medium DM + 1 % agar on the surface of DM agar plates so that quadruplicates of three different dilutions could be placed on a single plate. The plates were inverted in a dessicator containing a small amount of water, gassed three times with 99 % (v/v) N_2 + 1 % (v/v) CO_2 and sealed. The system was illuminated by two 150 W. tungsten lamps and was maintained at about 35° with a fan. After 2–4 days of incubation colony counts were made under a binocular dissecting microscope. A standard curve relating number of viable organisms/ml. and extinction at 600 mμ with incubation time was determined.

Photoassimilation experiments. The photoassimilation of radioactive organic compounds was determined in the following manner. Organisms harvested and washed under sterile conditions were suspended in sterile growth medium to the appropriate concentrations and 20 ml. amounts were transferred aseptically to sterile glass tubes 22 × 175 mm. fitted with cotton-wool plugs and gassing tubes as in the growth studies. Reagent-grade organic acids (Na salts) were added and the tubes were gassed with 2 % (v/v) CO_2 in air, illuminated at 30° and equilibrated for 30 min. Sterile radioactive organic substrates (2 μC) were then added. Samples (1 ml.) were removed aseptically at intervals, filtered through 450 mμ pore-diameter Millipore filters and washed with 20 ml. 0·01 M-carrier organic substrates. The filters were mounted on aluminium planchettes with adhesive, dried and assayed for radioactivity with a Nuclear Chicago thin-window proportional flow counter.

Enzyme assays. Preparation of cell-free extracts. Most enzyme assays were done with crude (undialyzed) cell-free extracts made as follows. Stored packed organisms

2–3 g. wet weight were mixed with 10 g. washed No. 10A Ballotini glass beads and suspended in 15 ml. 0·05 M-potassium phosphate buffer (pH 7·0). The suspension was chilled on ice and treated with ultrasonics for 90 sec. with an M.S.E. sonic disintegrator with a $\frac{1}{2}$ in. diameter probe. The suspension was then centrifuged for 15 min. at 16,000 rev./min. in a Sorvall refrigerated centrifuge at +2° and the supernatant fluid centrifuged for 30 min. at 100,000 *g* in a Spinco Model L ultracentrifuge. The resulting clear blue extract was used for enzyme assays.

Protein estimations were done by determination of the extinction at 260 and 280 mμ (Warburg & Christian, 1942).

Specific enzyme assays were done as follows.

Acetic thiokinase (acetyl coenzyme A synthetase) was assayed by the procedure of Jones & Lipmann (1955). Reaction mixtures of 1·5 ml. total volume contained (μmoles): 0·08 coenzyme A, 10 ATP, 20 K acetate, 100 K phosphate (pH 7·5), 10 $MgCl_2$, 10 glutathione and 10 (neutralized) hydroxylamine. The reaction proceeded faster in the absence of potassium fluoride. Extracts containing 2–6 mg. protein were incubated with the above components for 30 min. at 35°. The reaction was stopped by adding 1·5 ml. ferric chloride reagent containing 10% (w/v) $FeCl_3.6H_2O$ and 3·3% (w/v) trichloroacetic acid in 0·66 N-HCl (Lipmann & Tuttle, 1945). Tubes were centrifuged and the extinction of the supernatant fluid measured at 540 mμ against a ferric chloride reagent blank.

Citrate synthase was assayed by determining citrate formation from oxaloacetate and acetyl coenzyme A. Reaction mixtures (5 ml.) contained (μmoles): 100 potassium oxaloacetate (freshly neutralized to pH 7·0); 8 acetylcoenzyme A; 50 cysteine; 100 K phosphate (pH 7·5); extract. Control tubes were without oxaloacetate. Tubes were incubated at 35° and samples (0·5 ml.) were withdrawn at different times for determination of residual acetylcoenzyme A and of citrate. Acetylcoenzyme A was determined by the hydroxamate procedure of Lipmann & Tuttle (1945). Citrate was determined by the colorimetric procedure of Taylor (1953). Acetylcoenzyme A was prepared by the acetylation of coenzyme A with acetic anhydride (Stadtman, 1957).

Citrate synthase was also demonstrated by coupling the acetic thiokinase system (in the absence of hydroxylamine) with oxaloacetate and determining the citrate formed. Formation of citrate from radioactive acetate was also determined in this way (details in the results section).

Isocitric-NADP dehydrogenase. This was assayed spectrophotometrically by following the reduction of NADP at 340 mμ. Reaction mixtures of 3 ml. total volume contained (μmoles): 0·5 potassium DL-isocitrate, 0·5 NADP; 10 $MgCl_2$; 100 KH_2PO_4 buffer (pH 7·0); extract. Control mixtures without isocitrate were used. Rates of NADP reduction were determined against a blank containing extract + buffer only. The stoichiometry of the reaction was determined by estimating the α-ketoglutarate formed as the 2,4-dinitrophenylhydrazone by the procedure of Friedmann (1957).

Transaminases. Transamination was demonstrated qualitatively. Reaction mixtures of 1 ml. total volume contained (μmoles): 25 L-amino acid; 50 potassium α-ketoglutarate; extract in 0·05 M-KH_2PO_4 (pH 7·0). Control tubes were included in which α-ketoglutarate and the different amino acids were incubated alone with the extract. Tubes were incubated 60 min. at 35° and were then deproteinized by treatment with 0·1 ml. glacial acetic acid + 0·5 ml. 95% (v/v) ethanol in water. Protein-free supernatant fluids were subjected to paper electrophoresis on Whatman 3 MM paper in a

pyridine + acetic acid buffer (pH 3·6) for 2 hr at 2000 V. (Dixon, Kauffman & Neurath, 1958) together with glutamate markers. Strips were dried and then dipped in ninhydrin (0·1 %, w/v, in acetone) to detect glutamate.

Glutamic dehydrogenase was tested in reaction mixtures of 3 ml. final volume containing (μmoles): 50 potassium L-glutamate; 5 NAD or NADP; 100 KH_2PO_4 (pH 7·0); extract. The reaction was also tested in the direction of glutamate synthesis as described in the Results section.

N-Acetyl glutamate kinase. Reaction mixtures of 1·5 ml. final volume contained (μmoles): 20 *N*-acetyl-L-glutamate; 10 ATP; 10 $MgCl_2$; 200 (neutralized) hydroxylamine; extract. Tubes were incubated at 35° and were deproteinized with the ferric chloride reagent of Lipmann & Tuttle (1945) and extinction of the ferric complex measured at 540 mμ as in the assay for acetic thiokinase.

α-N-Acetylornithine δ-transaminase was assayed by the procedure of Albrecht & Vogel (1964). Reaction mixtures of 1 ml. total volume contained (μmoles): 100 KH_2PO_4 (pH 8·0), 5 α-*N*-acetyl-L-ornithine; 10 α-ketoglutarate; 0·05 pyridoxal phosphate; extract. Controls were set up in which α-*N*-acetylornithine was omitted, and in which it was added at the end of the incubation period. Reactions were stopped by adding 0·3 ml. 6 N-HCl and the tubes heated 30 min. at 100°, cooled, 1 ml. of 3·6 M-Na acetate + 0·2 ml. 0·033 M-*o*-aminobenzaldehyde added and the extinction at 440 mμ measured after 15 min.

N-acetylglutamate-ornithine transacetylase. This enzyme was demonstrated qualitatively (details in the Results section).

Ornithine transcarbamylase. This was assayed by the procedure of Spector & Lipmann (1955). Reaction mixtures of 1 ml. total volume contained (μmoles): 150 tris (pH 8·0); 5 $MgCl_2$; 8 L-ornithine; 6 dilithium carbamyl phosphate; cell-free extract. Tubes were incubated for different times at 35° and the reaction stopped by adding 5 ml. 0·5 M-perchloric acid. Tubes were centrifuged and appropriate samples of the clear supernatant fluids were taken for citrulline estimation by the colorimetric procedure of Archibald (1944) as modified by Oginsky (1957).

RESULTS

Effect of organic compounds on growth of Anacystis nidulans. Preliminary experiments were done to examine the effect of sodium acetate on the rate of growth of *Anacystis nidulans*. A series of tubes containing 20 ml. medium DM supplemented with different concentrations of sodium acetate were inoculated with 0·1 ml. of a suspension of washed organisms adjusted to an extinction of 1·0 at 600 mμ, corresponding to 5×10^6 organisms/ml. Growth was followed by measuring the extinction at 600 mμ as a function of time. Typical growth curves are illustrated in Fig. 1. Acetate never stimulated growth, but concentrations of 0·01–0·04 M inhibited growth. Determination of viable counts showed that even at the higher growth inhibitory concentrations of acetate, the cultures were still viable after a 12-hr incubation period.

Similar growth experiments were done with other fatty acids. In each case highly purified substances were used as the sodium salts prepared by neutralization of freshly distilled fatty acids. The lower straight chain and branched saturated fatty acids were tested over the range 0·001–0·02 M. In most cases the results were very similar to those with acetate: namely growth was significantly inhibited only by relatively high concen-

trations of fatty acid. However, propionate was a very much more potent growth inhibitor, with significant growth inhibition at 10^{-4} M. and complete inhibition of growth by 5×10^{-4} M. Determinations of viable counts indicated that still higher concentrations of propionate killed the organisms. Low concentrations of propionate also inhibited growth of *Anabaena variabilis* and *Nostoc muscorum* G.

Several compounds were tested to determine whether the growth inhibition by propionate could be annulled. Acetate specifically annulled the growth inhibitory effects of propionate for *Anacystis nidulans*, *Anabaena variabilis* and *Nostoc muscorum* G. Other compounds tested over a range of concentrations (10^{-2} to 10^{-5} M) including butyrate, pyruvate, β-alanine, pantothenic acid, yeast extract (0·005–0·5 %, w/v) and casamino acids (0·005–0·5 %, w/v) did not annul the growth inhibitory action of propionate. Typical results are given in Table 1. Attempts to obtain reproducible growth rates with different concentrations of acetate and propionate intended to determine whether propionate might compete with acetate, were not successful. Qualitatively, growth inhibition by propionate of all three blue-green algae was consistently annulled by acetate.

Table 1. *Effect of acetate and propionate on growth of blue-green algae*

Organisms were grown at 30° in the light in 20 ml. medium DM with Na acetate and/or Na propionate at the final concentrations indicated; cultures gassed continuously with 2 % (v/v) CO_2 in air. Extinction at 600 mμ measured at intervals and growth rate constants (K) in $\log_{10}$ units per day determined according to Kratz & Myers (1955*a*).

	Concn of		
	Acetate (M)	Propionate (M)	K
Anacystis nidulans	0	0	0·91
	0	0·001	0
	0·005	0	1·05
	0·005	0·001	0·91
Anabaena variabilis	0	0	0·91
	0	0·001	0
	0·002	0	1·09
	0·001	0	0·97
	0·002	0·001	1·01
Nostoc muscorum G	0	0	0·70
	0	0·001	c
	0·001	0	0·56
	0·001	0·001	0·68

Photoassimilation experiments. The assimilation of acetate. Isotopic tracer studies with (1-) and (2-)^{14}C-acetate were carried out to determine if acetate was assimilated by growing cultures of blue-green algae. Preliminary tests with cell suspensions in growth medium showed that both (1-) and (2-)^{14}C-acetate were assimilated, and that the process was light dependent. The time course of photoassimilation of (2-)^{14}C-acetate followed over a 12 hr incubation period showed a uniform rate of assimilation over the initial 4 hr, and over 40 % of the added acetate was incorporated by the cell suspensions after 12 hr incubation (Hoare & Moore, 1965). In order to study in more detail the influence of different factors on the rate of photoassimilation of acetate, conditions were developed such that the rate of photoassimilation was proportional to

the cell concentration. A series of tubes was set up with a range of cell concentrations. (2-)^{14}C-acetate (0·01 M final concentration, 2 μC.) was added to each tube and samples (1 ml.) were withdrawn after 1, 2, 3 and 4 hr incubations and assayed for incorporated radioactivity. For each cell concentration the rate of photoassimilation of ^{14}C-acetate was determined. The results are given in Fig. 2. When the tubes were gassed with 1–2 % (v/v) CO_2 in air, the rate of acetate incorporation was proportional to concentrations up to 0·5 mg. dry weight organisms/ml. but decreased with higher concentrations. Increasing the carbon dioxide concentration to 4 % (v/v) increased the range over which the rate of acetate assimilation was proportional to cell concentration but significantly decreased the absolute rate. For all subsequent experiments suspensions of organisms were kept within the range 0·3–0·5 mg. dry weight/ml. and were gassed

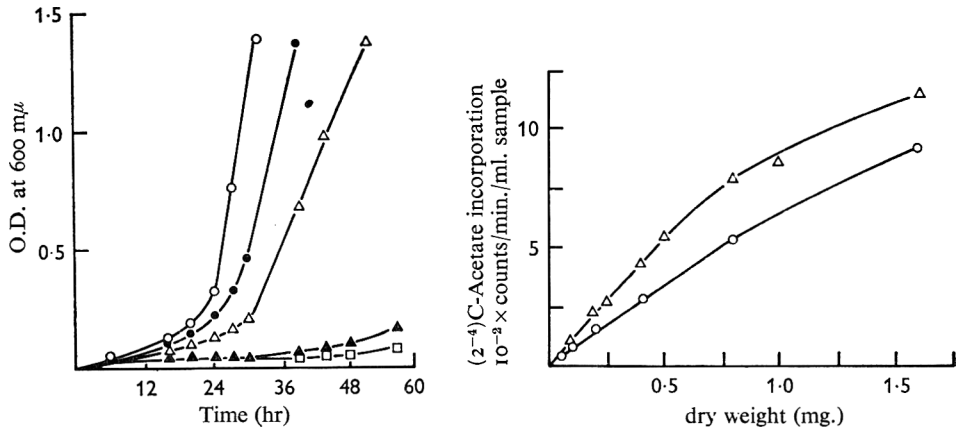


Fig. 1. The effect of acetate on the growth of *Anacystis nidulans*. 20 ml. tube cultures inoculated and aerated with 1 % carbon dioxide in air, incubated in the light at 30° as described in methods. Growth followed at 600 mμ, basal medium supplemented with different concentrations of acetate: ○—○ none, ●—● 10^{-2} M; △—△ 2×10^{-2} M; ▲—▲ 3×10^{-2} M, □—□ 4×10^{-2} M

Fig. 2. Effect of cell concentration on the rate of photoassimilation of acetate by *Anacystis nidulans*.

Cell suspensions (20 ml.) of different dry weight concentrations were incubated with sodium (2^{-})^{14}C acetate for periods up to 4 hr and samples (1 ml.) were withdrawn and assayed for radioactivity as described in Methods. Rates of incorporation are given as counts per minute per ml. suspension. △—△ aerated with 1 % carbon dioxide in air. ○—○ aerated with 4 % carbon dioxide in air.

with 1–2 % (v/v) CO_2 in air unless otherwise stated. The rate of photoassimilation of acetate was found to be carbon dioxide dependent. Cultures gassed with carbon dioxide free air, made by passing air through a tube packed with soda-lime and then through two Milligan gas washers containing N-sodium hydroxide, showed an exceedingly slow rate of acetate assimilation (Fig. 3). The gradual increase in rate of assimilation during the experiment in the cultures gassed with 1 % (v/v) carbon dioxide is probably due to cell growth.

The effect of different experimental conditions on the rate of photoassimilation of acetate by *Anancystis nidulans* is summarized in Table 2. Acetate assimilation was exceedingly sensitive to inhibition by DCMU (dichlorophenyl, dimethyl urea), which is a potent inhibitor of non-cyclic photophosphorylation and acts by blocking the

oxygen-evolving system. Acetate was assimilated at the same rate under 'semi-anaerobic' conditions when the cultures were gassed with a mixture (1+99 parts by volume) of carbon dioxide in nitrogen or in hydrogen. Carbon dioxide was still essential for optimal acetate assimilation under these 'semi-anaerobic' conditions. Cell suspensions in nitrogen-free media showed a diminished rate of acetate assimilation, but there was only a slight decrease in acetate assimilation after longer periods of incubation (4–8 hr) with cells suspended in magnesium or phosphate-deficient media. Sodium fluoroacetate at a final concentration of 10^{-3} M had little effect on the photoassimilation of acetate. Propionate which was found to be a potent growth inhibitor for the blue-green algae was shown to inhibit the photoassimilation of acetate, but at comparatively high concentrations.

Table 2. *Effect of experimental conditions on the rate of assimilation of acetate by Anacystis nidulans*

Suspensions of *A. nidulans* (1 mg. dry wt/ml.) in 20 ml. medium DM containing Na (2-)^{14}C-acetate (200 μmoles, 2 μC) incubated at 30° in the light and gassed continuously with the mixture indicated. Samples (1 ml.) taken after 1, 2, 3, and 4 hr. incubations for ^{14}C estimations to determine rates of acetate incorporation as described in Methods.

Experimental conditions	Acetate assimilation 10^{-2} × counts/min./mg. dry wt cells/hr
Expt. 1. Control: in air + 1 % (v/v) CO_2	34·2
+ $2{\cdot}5 \times 10^{-7}$ M-DCMU	33·0
+ 5×10^{-7} M-DCMU	12·6
+ $7{\cdot}5 \times 10^{-7}$ M-DCMU	9·7
+ 10^{-6} M-DCMU	6·5
Dark control	0·2
Expt. 2. Control: in air + 1 % (v/v) CO_2	33·0
in nitrogen + 1 % (v/v) CO_2	33·0
in nitrogen-deficient media (air + 1 % CO_2)	17·0
in hydrogen + 1 % (v/v) CO_2	33·0
Expt. 3. Control: in air + 1 % (v/v) CO_2	12·4
+ 2×10^{-2} M-Na propionate	2·1
+ 10^{-2} M-Na propionate	4·5
+ 5×10^{-3} M-Na propionate	9·7
+ 10^{-3}M-Na propionate	11·5

Photoassimilation of other organic compounds. Anacystis nidulans was also tested for its ability to assimilate a limited number of other simple organic compounds. Test conditions were similar to those employed with acetate, using ^{14}C-labelled compounds. Total incorporated radioactivity was determined after incubations in the light and in the dark. The results are summarized in Table 3.

Propionate incorporation was also tested, but at lower concentrations, since it was a potent growth inhibitor. Both (1-) and (2-)^{14}C-propionate were incorporated in a light dependent process and the rate of incorporation, which was linear for 2–3 hr, was significantly reduced if the cultures were aerated with carbon dioxide free air. Other organic compounds including butyrate, citrate, glutamate and succinate were not significantly incorporated by *Anacystis nidulans*.

Distribution of incorporated radioactive compounds. Distribution of incorporated

radioactivity in *Anacystis nidulans* was examined using the fractionation procedure of Roberts *et al.* (1955). Distribution of incorporated acetate was also examined in *Anabaena variabilis*, *Nostoc muscorum* G and *Anabaena flos-aquae* and part of these preliminary findings have already been reported (Hoare & Moore, 1965). The results with *Anabaena flos-aquae* which have not been previously reported are given in Table 4.

Table 3. *Photoassimilation of organic acids by Anacystis nidulans*

Washed suspensions (10 mg. dry wt organisms/10 ml. medium DM1 of photoautotrophically grown *A. nidulans* incubated in the light or in the dark with organic substrates (Na salts, 10 μmoles, 2 μC ^{14}C) gassed continuously with 1 % (v/v) CO_2 in air, 5 hr. at 30°. Total ^{14}C incorporation determined on samples as described in Methods.

Substrate		Total ^{14}C added 10^{-5} × counts/min.	Total ^{14}C assimilated 10^{-5} × counts/min.
Acetate	Light	30·9	20·5
	Dark	29·3	4·0
Succinate	Light	32·5	0·4
	Dark	31·3	0·1
Glutamate	Light	38·4	0·7
	Dark	35·0	0·5
Citrate	Light	38·9	0·7
	Dark	34·6	0·5

Table 4. *Distribution of incorporated ^{14}C-acetate by Anabaena flos-aquae*

10 ml. medium DM containing 26 mg dry wt organisms incubated 20 hr in the light at 30° with Na (1-)^{14}C-acetate (10 μmoles, 4 μC) gassed continuously with 1 % (v/v) CO_2 in air. Organisms harvested, washed and fractionated by the procedure of Roberts *et al.* (1955) and ^{14}C content of fractions determined.

Fraction	10^{-5} × counts/min.	%*
Whole culture (organisms + culture fluid)	57·0	—
1. Cold TCA soluble	0·47	3·2
2. 75 % ethanol soluble	8·52	58·9
3. Ethanol/ether soluble	0·65	4·5
4. Hot TCA soluble	0·34	2·3
5. Acid ethanol/ether soluble	0·52	3·6
6. Hydrolyzed residue	4·06	28·7
Total assimilated (sum of 1-6)	14·56	—

* % of total assimilated.

The 'residual protein' fractions were subjected to further examination. These fractions were hydrolysed in 6 N-HCl (18 hr at 100°) and the neutralized hydrolysates were examined by two-dimensional paper chromatography and radioautography as already described (Hoare & Moore, 1965). In all four blue-green algae, only four amino acids were significantly radioactive, and these were shown to be glutamic acid, proline, arginine and leucine. The protein hydrolysates showed a 'normal' distribution of about sixteen amino acids when the chromatograms were developed with ninhydrin. Separation of the amino acids was achieved on a micro-scale by a combination of high voltage paper electrophoresis and paper chromatography procedures. The

following more extensive examination of the isolated amino acids was carried out only with *Anacystis nidulans*.

Specific activities of isolated amino acids. The four radioactive amino acids and aspartic acid were isolated on a micro-scale. Specific activities were determined as follows. Radioactivity of the final amino acid solutions was determined on appropriate samples (in duplicate) in 15 ml. Bray solution (Bray, 1960), in an Ansitron liquid scintillation counter. Amino acid estimations were carried out by the colorimetric procedure of Yemm & Cocking (1954). Since proline gives a very weak colour in this procedure, the modified ninhydrin method of Chinard (1952) was used to estimate proline. The results are given in Table 5.

Table 5. *Specific activities of amino acids derived from (1-)^{14}C-acetate in Anacystis nidulans*

Amino acids isolated from a hydrolysate of *Anacystis nidulans* which had incorporated Na (1-)^{14}C-acetate for 18 hr in the light. Amino acids determined by the ninhydrin method of Yemm & Cocking (1954) and ^{14}C as described in Methods. Specific activity of added (1-)^{14}C-acetate $4{\cdot}8 \times 10^5$ counts/min./μmole.

Amino acid	Specific activity counts/min./μmole
Glutamic acid	$3{\cdot}8 \times 10^4$
Aspartic acid	$0{\cdot}06 \times 10^4$
Arginine	$3{\cdot}7 \times 10^4$
Leucine	$5{\cdot}4 \times 10^4$
Proline	$0{\cdot}83 \times 10^4$

Table 6. *Degradation of glutamic acid and leucine derived from (1-)^{14}C-acetate in Anacystis nidulans*

Amino acids, isolated and purified from a hydrolysate of *A. nidulans* which had incorporated Na (1-)^{14}C-acetate for 18 hr in the light, were degraded as described in Methods.

Compound	μmoles	Counts/min.	Specific activity counts/min./μmole
Glutamate	1,000	240,000	240
CO_2 (from γ-COOH)	470	110,000	234
α, γ diaminobutyrate	112	85	0·76
Leucine	11·9	80,320	6,740
CO_2	11·9	65,400	5,500

Degradations of isolated amino acids. Two of the amino acids, formed as a result of the photoassimilation of (1)-^{14}C-acetate by *Anacystis nidulans*, were degraded to locate the incorporated ^{14}C. Leucine was degraded by reaction with an excess of chloramine-T which decarboxylates the amino acid quantitatively. The reaction was carried out in a Warburg manometer vessel according to the procedure of Kemble & McPherson (1954) and as used previously by Hoare & Gibson (1964). Glutamic acid was degraded by a Schmidt reaction in which the amino acid was treated with hydrazoic acid resulting in decarboxylation of the terminal (C-5) or γ-carboxyl group (Cutinelli, Ehrensvard, Reio, Saluste & Stjernholm, 1951; Hoare, 1963). The residual α,γ-diaminobutyric acid was recovered from the reaction products as the dipicrate and was subsequently converted to the free amino acid. The results of the degradations are given in Table 6.

Enzyme activities in cell-free extracts of Anacystis nidulans. The results of the degradation of glutamate are consistent with the conventional pathway of glutamate synthesis from acetate via citrate. Accordingly evidence was sought for the presence, in cell-free extracts of *Anacystis nidulans*, of all the enzymes necessary to effect the incorporation of acetate into glutamic acid. Cell-free extracts were prepared as described in the Methods section, and the following enzymic activities were clearly established.

Table 7. *Acetyl CoA synthetase in cell-free extracts of Anacystis nidulans*

A. nidulans grown photoautotrophically on medium DM was harvested and cell-free extracts prepared as described in Methods. Extracts containing 4 mg. protein incubated at 35° for the times indicated in a reaction mixture of 1·5 ml. volume containing (μmoles): 0·08 CoA, 10 ATP, 10 K salt of fatty acid, 100 K phosphate (pH 7·5), 10 $MgCl_2$, 10 glutathione and 10 (neutralized) hydroxylamine.

Substrate	Component omitted	Incubation time (min.)	Hydroxamate formed absorbance at 540 mμ
Acetate	None	60	0·276
Propionate	None	60	0·193
Butyrate	None	60	0·040
Fluoroacetate	None	60	0·077
		90	0·093
		120	0·110
		200	0·140
Propionate	CoA	60	0·053
	ATP	60	0·038
	$MgCl_2$	60	0·124
	Glutathione	60	0·145

(i) *Acetic thiokinase (acetyl CoA synthetase).* Cell-free extracts catalysed an ATP and coenzyme A dependent formation of a hydroxamic acid from acetate. Propionate was also activated but no hydroxamate was formed with butyrate as substrate. Propionate appeared to compete with acetate in these reactions. Sodium fluoroacetate was only poorly activated by extracts of *Anacystis nidulans.* Typical results are summarized in Table 7.

(ii) *Citrate synthase.* Using cell-free extracts supplemented with all the co-factors necessary for optimal activity of the acetic thiokinase, but with the neutralized hydroxylamine omitted, it was possible to demonstrate the formation of citrate provided oxaloacetate was added. This was demonstrable in two ways. In the first procedure (2-)^{14}C-acetate was used as a substrate, whereby an oxaloacetate dependent incorporation of radioactivity into a non-volatile acidic fraction could be demonstrated. Radioactivity was extracted into ether from acid solution and two-dimensional chromatograms with carrier citrate showed coincidence between the carrier and radioactive areas on the chromatogram. Coincidence of added carrier citrate and radioactivity was also established by paper electrophoresis at pH 3·6. The chromatography system of Benson *et al.* (1950) was used. In the second procedure, citrate formed as a result of incubation under the conditions of the previous procedure, or alternatively using acetylcoenzyme A (prepared by the method of Stadtman, 1957) in place of the acetic thiokinase system, was measured directly by the colorimetric procedure of Taylor

(1953). Typical results are given in Table 8. Using acetylcoenzyme A as substrate attempts to establish a correlation between the amount of acetylcoenzyme A used, followed by measuring the absorption of deproteinized solutions at 260 mμ, or by hydroxamate formation, and citrate formation were not successful due to variable degrees of breakdown of acetylcoenzyme A by crude extracts in the absence of oxaloacetate.

Table 8. *Citrate synthase in cell-free extracts of Anacystis nidulans*

Complete reaction mixtures 2 ml. contained cell-free extract (2 mg. dry wt), 200 μmoles K (2-)^{14}C-acetate (17×10^5 counts/min.), 0·16 μmoles CoA, 20 μmoles ATP, 200 μmoles K phosphate (pH 7·5), 20 μmoles $MgCl_2$, 20 μmoles glutathione, and 20 μmoles K oxaloacetate. Tubes incubated at 35°, 100 μl. samples removed at times indicated, mixed with 100 μl. 2% (w/v) acetic acid, dried on planchettes and assayed for ^{14}C.

Incubation (min.)	(2-)^{14}C-acetate incorporation ($10^{-3} \times$ counts/min)		
	Complete	Coenzyme A omitted	Oxaloacetate omitted
20	22·7	4·9	4·4
40	45·7	7·6	9·3
60	70·1	9·1	9·9
90	84·0	8·1	10·1
120	128·5	13·9	19·7
180	166·4	18·4	19·7

Table 9. *Aconitase and isocitrate dehydrogenase in cell-free extracts of Anacystis nidulans*

Cell-free extract of *A. nidulans* (1·6 mg. dry wt) incubated at 35° in a reaction mixture of 3 ml. containing (μmoles): 0·5 K-DL-isocitrate, 0·5 NADP, 10 $MgCl_2$, 100 K phosphate (pH 7·0). For stoichiometry, lower concentrations of isocitrate were used as indicated.

(*a*) Stoichiometry D-isocitrate (μmoles)	NADP formed (μmoles)	α-ketoglutarate formed (μmoles)
0·06	0·06	0·05
0·12	0·10	0·09
0·18	0·17	0·14
0·24	0·21	0·20

(*b*) Reaction rates: Components	△OD at 340 mμ/min./mg. dry wt
Complete:	
DL-isocitrate and NADP	0·105
cis-aconitate and NADP	0·035
Citrate and NADP	< 0·005
DL-isocitrate and NAD	Nil
DL-isocitrate, NADP; Mg^{2+} omitted	0·020

(iii) *Aconitate hydratase* (*aconitase*) *and iso-citrate NADP dehydrogenase.* As outlined in Methods, aconitate hydratase was demonstrated indirectly by using cis-aconitate as substrate under the spectrophotometric assay conditions for iso-citrate NADP dehydrogenase. Iso-citrate dehydrogenase was readily demonstrated in extracts and was shown to be activated by magnesium, even in undialyzed extracts. The reaction was NADP specific, and stoichiometry was established between iso-

citrate disappearance (assumed to be complete when the reaction ceased with an excess of NADP), NADP reduced and α-ketoglutarate formed. Results are summarized in Table 9.

(iv) *Conversion of α-ketoglutarate to glutamate.* It was at first assumed that glutamate would be formed from α-ketoglutarate through the action of a pyridine nucleotide linked glutamate dehydrogenase. Numerous attempts to assay for L-glutamate dehydrogenase, using NAD or NADP as coenzyme, were unsuccessful. The reaction was tested in the synthetic direction by seeking a glutamate dependent oxidation of the reduced pyridine nucleotides, by spectrophotometric measurements at 340 mμ. It was also tested in the reverse direction by spectrophotometry at 340 mμ, and by seeking the formation of α-ketoglutarate by the 2,4-dinitrophenyl hydrazone procedure of Friedmann (1957). Whilst it is probably not justified to conclude that *Anacystis nidulans* lacks a glutamate dehydrogenase, alternative mechanisms for glutamate formation were sought. Qualitative tests, as outlined in Methods, readily established that glutamate was formed from α-ketoglutarate by transamination with appropriate L-amino acids. The most effective amino acids for the transamination reaction were the branched chain amino acids leucine, isoleucine and valine; phenylalanine and aspartic acid were less effective amino donors.

(v) *Conversion of glutamate to ornithine.* Evidence for the conversion of glutamate to ornithine by a transacetylation mechanism in a number of blue-green algae and photosynthetic bacteria was presented recently by Hoare & Hoare (1966). Extracts of blue-green algae were shown to contain a transacetylase which catalysed the reversible transfer of an acetyl group from α-*N*-acetyl-L-ornithine to L-glutamate, and to contain an *N*-acetyl glutamate phosphokinase which is specifically inhibited by L-arginine. These findings support the proposition that arginine is synthesized from L-glutamate via acetylated derivatives in some blue-green algae. Further evidence in support of the postulated enzymic pathway was sought by attempting to demonstrate the presence, in cell-free extracts of *Anacystis nidulans*, of other enzymes catalyzing reactions involving certain acetylated intermediates. The postulated sequence of reactions involving acetylated intermediates is as follows:

(i) L-glutamate + acetylcoenzyme A → *N*-acetyl-L-glutamic acid.

(ii) *N*-acetyl-L-glutamic acid + ATP → *N*-acetyl-L-glutamyl-γ-phosphate + ADP.

(iii) *N*-acetyl-L-glutamyl-γ-phosphate + NADPH + H^+ → *N*-acetyl-L-glutamyl-γ-semi-aldehyde.

(iv) *N*-acetyl-L-glutamyl-γ-semi-aldehyde + glutamate → α-ketoglutarate + α-*N*-acetyl-L-ornithine.

(v) α-*N*-acetyl-L-ornithine + glutamate → L-ornithine + *N*-acetyl-L-glutamic acid.

As pointed out by Hoare & Hoare (1966), this sequence of reactions for the biosynthesis of ornithine from glutamic acid involves a cycle of acetylated intermediates in which *N*-acetyl-L-glutamic acid is required in only catalytic amounts. Attempts to demonstrate an appreciable synthesis of *N*-acetyl-L-glutamic acid by the procedures of Maas, Novelli & Lipmann (1953) or of Hudock (1962) were not successful. It was, however, possible to demonstrate qualitatively the formation of *N*-acetyl glutamic acid using (2-)^{14}C-acetate as substrate in a system containing all reactants necessary for the conversion of acetate to acetylcoenzyme A supplemented with L-glutamic acid. Reaction mixtures of 1·5 ml. final volume contained (μmoles): 0·08 coenzyme A; 10 NaATP; 10 glutathione; 10 potassium acetate, (10 μC); 100 potassium phosphate

buffer pH 7·5; 10 $MgCl_2$; 5 sodium glutamate; cell-free extract containing 8 mg. protein. Tubes were incubated 60 min. at 35° and the reaction was stopped by immersing tubes in a boiling water bath for 5 min. Control tubes were run in which glutamate was omitted, and in which the complete reaction mixture was stopped at zero time. Ten μmoles carrier *N*-acetyl glutamate was added to the cooled tubes which

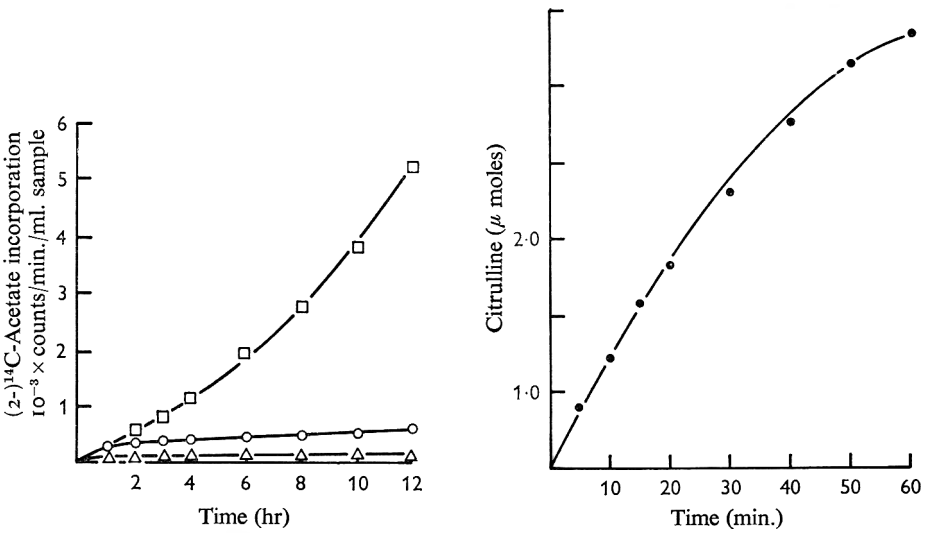


Fig. 3. Effect of carbon dioxide on the photoassimilation of acetate by *Anacystis nidulans*. Cell suspensions (0·38 mg. dry wt per ml.) were incubated as described with sodium (2-)^{14}C-acetate and samples (1 ml.) withdrawn at intervals for assay of incorporated radioactivity. □—□ control illuminated system aerated with 1 % carbon dioxide in air; ○—○ system illuminated and aerated with carbon dioxide free air; △—△ dark control aerated with 1 % carbon dioxide in air.

Fig. 4. Time course of citrulline formation from carbamyl phosphate and ornithine in cell-free extracts of *Anacystis nidulans*. Cell-free extract containing 2 mg. protein incubated in a final volume of 1 ml. under conditions given in the methods.

Table 10. *α-N-acetyl ornithine transaminase in extracts of Anacystis nidulans*

Substrate	Incubation (min.)	Absorbance at 440 mμ
α-*N*-acetyl-L-ornithine	15	0·06
	30	0·10
	60	0·15
	90	0·21

Cell-free extract of *A. nidulans* (2 mg. protein) incubated at 35° in a reaction mixture of 1 ml. containing (μ moles): 5 α-*N*-acetyl-L-ornithine, 10 α-ketoglutarate, 0·05 pyridoxal phosphate, 100 K phosphate (pH 8·0). Figures are corrected for 'controls' without α-ketoglutarate. Details given in Methods.

were then treated with 0·1 vols. 10 N-H_2SO_4, centrifuged, and the clear supernatants were extracted three times with 1·5 vol. chloroform + *n*-butanol (1 + 4 v/v). Combined butanol extracts were taken to dryness in a stream of air at 45–50° and the residues were applied to Whatman 3 MM paper and subjected to electrophoresis at pH 3·6 as previously described (Hoare & Hoare, 1966). The dried paper was then monitored for ^{14}C. Low but significant incorporation of radioactivity was observed in the *N*-acetyl

glutamic acid area (which could be located by spraying the paper with bromcresol green indicator) only in tubes incubated with the complete reaction mixture. However, acetate incorporation was estimated to be less than 5% of the acetate added to the system. Although variations in the experimental conditions were examined, no improvement in the net synthesis of *N*-acetyl glutamic acid was achieved and further attempts to establish the presence of the *N*-acetyl glutamate synthetase were abandoned.

The transamination of α-*N*-acetyl ornithine (reaction (iv) in the above reaction sequence) was demonstrated in cell-free extracts of *Anacystis nidulans* by the procedure of Albrecht & Vogel (1964). However, it was also found that ornithine participated in a γ-transamination reaction. The significance of the latter reaction is not clear (Table 10). Extracts of blue-green algae do not contain α-*N*-acetyl ornithine deacetylase.

(vi) *Citrulline formation from ornithine.* Citrulline was presumed to be an intermediate in arginine biosynthesis, therefore ornithine transcarbamylase activity was examined in extracts of *Anacystis nidulans* as described in Methods. Typical results are illustrated in Fig. 4. This reaction has also been reported in *Nostoc muscorum* by Holm-Hansen & Brown (1963).

DISCUSSION

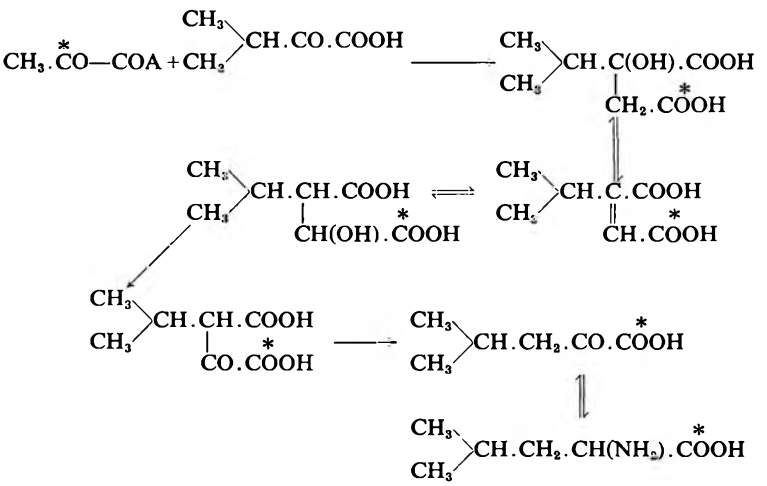
The most significant outcome of our limited survey of acetate assimilation by blue-green algae has been the discovery of a uniquely limited distribution of incorporated acetate into cell constituents. In all cases acetate was incorporated mainly into the lipid fraction and, to a smaller extent, into the cell proteins. The lipid fraction was not examined in detail. However, Nichols, Harris & James (1965) have recently examined the lipids of the blue-green algae *Anacystis nidulans* and *Anabaena variabilis* and have shown that acetate is incorporated into the fatty acids of the lipids: especially into phosphatidyl glycerol. Palmitate, stearate and oleate were also incorporated into lipids. Evidently blue-green algae are not impermeable to these long chain fatty acids.

Acetate incorporation into the protein fraction is of particular interest, since only four amino acids (glutamate, arginine, proline and leucine) were significantly radioactive following the assimilation of (1-) and (2-)^{14}C-acetate. This situation appears, so far, to be unique to the blue-green algae. The obligately autotrophic green sulphur bacteria of the genus *Chlorobium* incorporate acetate into all the amino acids of the cell proteins (Hoare & Gibson, 1964).

The degradation data for glutamate and leucine are consistent with established mechanisms for the biosynthesis of these amino acids. There is no need to invoke the operation of any novel mechanism of amino acid biosynthesis in blue-green algae. The conventional route for the biosynthesis of glutamate from acetate is supported by the demonstration, in cell-free extracts of blue-green algae, of all the enzymes necessary to catalyze the over-all reaction. The failure to detect a glutamate dehydrogenase is somewhat disturbing since this enzyme is important in the assimilation of inorganic nitrogen in most micro-organisms. It seems more probable that the transaminases which we have demonstrated qualitatively are involved in the terminal stages of the biosynthesis of other amino acids using glutamate as the amino donor. Biosynthesis of the branched chain amino acids (valine, isoleucine, and leucine) presumably

involves such transaminases in blue-green algae. Previous claims of the absence of fructose 1,6-diphosphate aldolase in blue-green algae (Richter, 1961; Fewson, Al-Hafidh & Gibbs, 1962) and the subsequent demonstration of the enzyme (van Baalen, 1965*a*; Willard, Schulman & Gibbs, 1965) make it clear that the failure to detect an enzyme in cell-free extracts should be treated with reservation.

Formation of the other three amino acids (i.e. leucine, proline and arginine) from acetate is believed to proceed by conventional routes. Thus leucine is believed to be formed by a condensation of acetylcoenzyme A with α-ketoisovaleric acid yielding α-isopropyl malic acid, with subsequent transformations according to the following scheme:



Apart from the presence of transaminases catalyzing the terminal step, no enzyme studies have been carried out on the mechanism of leucine biosynthesis, and support for the above scheme rests almost entirely on the isotope degradation data (Table 7). The above scheme results in the incorporation of the carboxyl (C-1) of acetate into the carboxyl group of leucine. Proline and arginine are believed to be derived from glutamic acid. Enzymes involved in proline biosynthesis have not been extensively examined in blue-green algae. In the course of our studies of *N*-acetylglutamate phosphokinase, it was found that extracts catalyzed a slower rate of formation of a hydroxamate from glutamate. However, it is not clear whether this is due to a glutamate γ-phosphokinase which would be involved in the biosynthesis of proline (Baich & Pierson, 1965) or due to a glutamine synthetase (Ravel, Humphreys & Shive, 1965). Evidence in favour of the mechanisms of conversion of glutamate to arginine is more substantial although still lacking in detail. This may be summarized as follows: (1) the specific activities of arginine and glutamate are very similar; (2) a number of enzymes catalyzing the interconversion of acetylated intermediates and the presence of ornithine transcarbamylase have been demonstrated in cell-free extracts of blue-green algae and (3) the *N*-acetyl-L-glutamate phosphokinase is specifically inhibited by arginine.

Since glutamate biosynthesis involves some of the reactions of the tricarboxylic acid cycle it is rather suprising that acetate is not incorporated into aspartic acid and amino acids derived therefrom. This may be compared with the studies of acetate incorporation by glucose grown *Escherichia coli* (Roberts *et al.* 1955). In numerous

experiments apartic acid was isolated from the proteins of blue-green algae which had assimilated (1-) or (2-)^{14}C-acetate, but its specific activity was exceedingly low. The results suggest that the flow of assimilated acetate proceeds more rapidly from α-ketoglutarate to glutamate than from α-ketoglutarate to succinate and round the tricarboxylic acid cycle to oxaloacetate from which aspartate is presumably derived. This further implies that the tricarboxylic acid cycle may be 'sluggish' in blue-green algae. Blue-green algae do respire at a slow rate, but little is known about their respiratory mechanisms (see, for example, Kratz & Myers, 1955*b*; Webster & Frenkel, 1953). Preliminary studies by Still & Wang (1965) on the role of the tricarboxylic acid cycle in the chemoautotrophic bacterium *Thiobacillus thioparus* merit comparison with the blue-green algae. Aseptic radio-respirometry techniques showed a number of organic compounds were utilized, although carbon dioxide was still the exclusive carbon source for proliferating cells. Most of the reactions of the tricarboxylic acid cycle were involved in the biosynthesis of the carbon skeletons of amino acids, but cells were unable to convert α-ketoglutarate to succinyl coenzyme A. Absence of this enzyme system in the blue-green algae would provide a reasonable explanation for the failure to get acetate incorporated into aspartate and would establish a close relationship between an obligate chemosynthetic bacterium and the obligately photoautotrophic blue-green algae.

Assimilation of organic compounds by blue-green algae was originally studied with the aim of understanding the general problem of obligate autotrophy. Obligate autotrophs as defined by organisms which appear to be unable to grow on organic compounds as sole or major source of cell carbon include some of the chemosynthetic bacteria, photosynthetic bacteria (viz. the genus *Chlorobium*) and probably most of the blue-green algae. Unfortunately comparatively few studies have been made of the assimilation of organic compounds by obligate autotrophs, and it is still far from clear why such organisms are unable to grow on organic compounds. In green sulphur bacteria of the genus *Chlorobium*, inability to grow on acetate as sole or major carbon source is probably attributable to the inability of these organisms to oxidize acetate, as was first suggested by Sadler & Stanier (1960). In Chlorobium, acetate assimilation is not only light dependent, but requires carbon dioxide and an inorganic reductant such as hydrogen sulphide which is the essential source of reducing power for cell synthesis. Formation of essential cell constituents from acetate in Chlorobium is then effected primarily through the pyruvate synthase reaction as was first indicated by the isotope incorporation studies of Hoare & Gibson (1964). Recent enzyme studies with *Chlorobium thiosulphatophilum* have established the importance of pyruvate synthase and other carboxylation reactions in this organism (Evans & Buchanan, 1965; Buchanan & Evans, 1965; Evans, Buchanan & Arnon, 1966; Buchanan & Evans, 1966).

Assimilation of organic compounds by blue-green algae has been studied by a limited number of investigators. Heterotrophic growth of blue-green algae in the dark was first clearly demonstrated by Harder (1917) with a strain of *Nostoc punctiforme* isolated from the rhizomes of the plant Gunnera. However, subsequent investigations have shown that the few blue-green algae which can grow heterotrophically in the dark grow under such conditions at exceedingly slow rates. Thus Allison, Hoover & Morris (1937) observed slow growth and nitrogen fixation by a strain of *N. muscorum* in the dark on a glucose medium, and more recently Fay (1965) observed growth and nitro-

gen fixation by *Chlorogloea fritschii* heterotrophically in the dark with sucrose as the most effective organic carbon source. Sucrose assimilation (and nitrogen fixation) were greater in the light than in the dark, but, in contrast to our findings on the photoassimilation of acetate, the photoassimilation of sucrose by *C. fritschii* was increased if carbon dioxide was omitted. Heterotrophic growth of *Tolypothrix tenuis* was first studied by Kiyohara, Fujita, Hattori & Watanabe (1960); this organism can respire on glucose and can assimilate glucose into a glucose containing polysaccharide (Cheung & Gibbs, 1966). The most thorough studies on the assimilation of organic compounds by blue-green algae were those of Allison *et al.* (1953), using radioactive tracer techniques to follow the assimilation of acetate by a strain of *N. muscorum*. Both carbon atoms of acetate were assimilated, and assimilation was greatly stimulated by light and the presence of carbon dioxide. Acetate was incorporated primarily into lipids and into free soluble glutamate and carboxylic acids; the proteins of the cells were not examined. Our studies of acetate assimilation are in accord with these findings. Furthermore, we have found light-dependent acetate assimilation to be inhibited by low concentrations of DCMU, suggesting that the photosynthetic oxygen evolving system is essential. We interpret this to imply that non-cyclic photophosphorylation is essential for acetate assimilation in blue-green algae. DCMU inhibition of the photoassimilation of acetate has been found in eucaryotic algae including species of the genera *Chlorella*, *Scenedesmus*, *Ankistrodesmus*, *Chlamydomonas*, *Chlorogonium* and *Euglena* (Wiessner, 1964).

Preliminary reports of acetate assimilation by *Anacystis nidulans* and *Anabaena variabilis* by Carr & Pearce (1966) and Pearce & Carr (1966) partly corroborate our findings.

From our very limited survey of the assimilation of organic compounds by blue-green algae, acetate is the most readily assimilated organic compound. This was also the case with the blue-green alga *Anabaena flos-aquae* studied by Moore & Tischer (1965), the chemosynthetic sulphur bacterium *Thiobacillus thiooxidans* studied by Butler & Umbreit (1966) and the chemosynthetic nitrifying bacterium *Nitrobacter agilis* studied by Delwiche & Finstein (1965) and by Ida & Alexander (1965). Recent preliminary studies by Kelly (1965*a*, *b*) of acetate assimilation by *T. neapolitanus*, in which acetate incorporation was thiosulphate-dependent and in which glutamate formation from acetate was inhibited by fluoroacetate, again indicate remarkably close similarities between obligate chemoautotrophs, at least as exemplified by thiobacilli, and obligate autotrophs, as exemplified by the blue-green algae.

The biochemical mechanism of the inhibitory effect of propionate on the growth of blue-green algae is still not clear. Propionate alone amongst a series of fatty acids inhibited growth. One possible explanation of this specificity and mechanism of growth inhibition would be that propionate 'drains off' coenzyme A which is essential for biosynthetic processes associated with cell growth. The acetothiokinase of *Anacystis nidulans* was found to activate propionate, but not other fatty acids. Reversal of propionate inhibition by acetate might then be due to the preferential activation of acetate in the presence of propionate, thus making acetylcoenzyme A (presumably normally generated from pyruvate) available for essential biosynthetic reactions. Growth inhibition by propionate and its reversal by acetate was observed in the bacterium *Streptococcus faecalis* by Hill (1952). Propionate inhibition of the growth of the eucaryotic alga *Haematococcus pluvialis* has been reported by Stross (1960).

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Delimitation of the Gametangia of *Rhizopus sexualis* (Smith) Callen: an Electron Microscope Study of Septum Formation

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SUMMARY

The development of the septa which cut off the gametangia of *Rhizopus sexualis* from the suspensor cells was studied by electron microscopy. The edge of the ingrowing septum extends by the incorporation of coalesced vesicles which were previously aligned in the plane of the developing septum. The membranes surrounding these vesicles fuse to form the plasmalemmas of the gametangium and suspensor cell and are eventually continuous with the plasmalemma of the original progametangium. New wall material is laid down between these plasmalemmas. Associated with this process is a considerable increase in complexity of the endoplasmic reticulum which forms broad, irregular bands on either side of the septum. Fine tubules, resembling plasmodesmata, pass through the wall at intervals. Thickening of the septum proceeds more rapidly on the gametangial side.

INTRODUCTION

Soon after a pair of progametangia of *Rhizopus sexualis* make contact at their apices, the terminal parts (gametangia) become cut off from the suspensor cells by the formation of transverse septa. This paper describes the process of formation of these septa as revealed by the electron microscope.

METHODS

The fungus was grown on cellophane laid on the surface of malt-agar plates. Young colonies with all stages of zygospore development present were fixed in 2% (w/v unbuffered $KMnO_4$ for 30 min. at room temperature, and washed several times in distilled water. The hyphae became brittle during fixation and the zygospores were readily detached from the mycelium during washing. Zygospores were then collected in centrifuge tubes, dehydrated, and embedded in Epon or Vestopal. Sections were cut at approximately 450–550 Å thickness.

RESULTS

The first sign of septum development detectable by light microscopy is a slight decrease in density of the cell contents on the inner side of the enveloping walls of the progametangium at the site from which the septum would be expected to develop. The electron microscope revealed, however, that in such a specimen a thin wall (about 1350 Å thick at the junction with the progametangial wall and tapering to about 385 Å thickness at the advancing inner edge) was already partially developed, extending inwards from the peripheral wall and leaving a central gap of about one third of the

total diameter of the progametangium (Fig. 1 *a*, *b*). At this stage the septum was not set at right angles to the original enveloping progametangial wall but formed an acute angle with it growing obliquely inwards in a direction away from the apex of the progametangium (Fig. 1 *b*; Pl. 1, fig. 1). Sections through the developing septum gave a different picture of the advanding edge according to whether they were radial or tangengial longitudinal sections of the zygospore initial (Fig. 1 *a–c*; Pl. 1, fig. 2; Pl. 2, figs. 3–5).

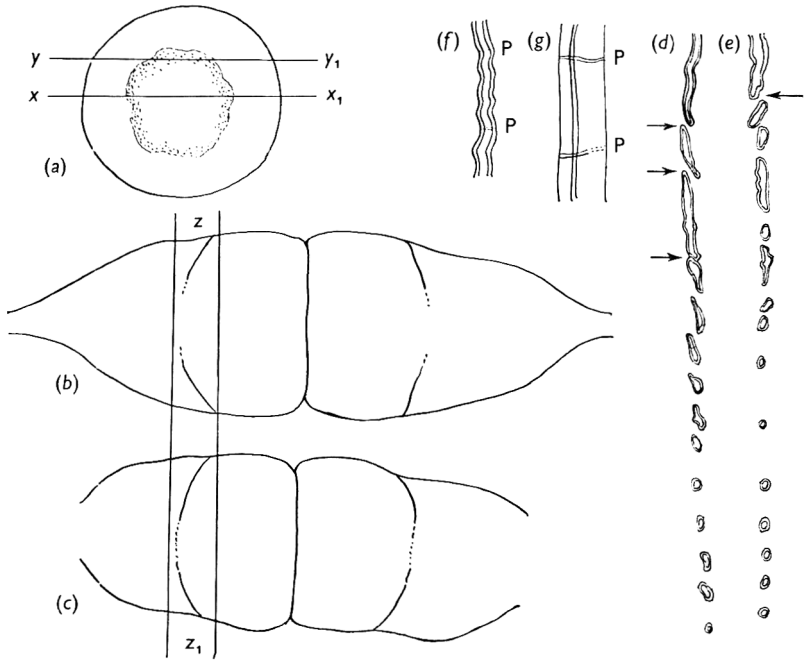

Fig. 1. *a–c*, Diagrammatic (× 500); *d–g*, drawn to scale from electronmicrographs (× 20,000). (*a*) Developing septum as it would be seen in optical surface view (i.e. in transverse section of zygospore shown as zone z–z_1 in Fig. 1 *b*, *c*), inner edge consisting of disconnected vesicles arranged in plane of septum. (*b*) Radial longitudinal section of zygospore through plane (x–x_1) of (*a*), showing developing septum set at an angle to progametangial wall and curving towards the centre of the progametangium, thus bringing the advancing edges into the same plane. A band of disconnected vesicles continues the plane of the septum inwards for a short distance. (*c*) Tangential longitudinal section of same zygospore through plane (y–y_1) of (*a*), showing disconnected vesicles extending across the gap between the advancing edge of the septum. (*d*, *e*). Edges of septum as seen in longitudinal radial and tangential sections respectively. Note overlapping of some of newly added, but as yet incompletely fused, sections of wall (indicated by arrows) resulting from imprecise alignment of contributing vesicles. (*f*) Section through older part of septum; irregularities due to overlapping of contributing segments now visible only as undulation of wall. (*g*) Older septum, secondary thickening beginning, undulations now barely visible. Note plasmodesmata (P).

The most recently formed part of the septum was very thin and undulating. It consisted of an inner electron-transparent zone or plate, sandwiched between two electron-dense layers (Pl. 2, figs. 3, 6). The line of the wall, as seen in radial section, was continued inwards for a short distance (Pl. 2, fig. 4) as a row of unconnected vesicles. Further towards the centre of the cell there was a broader band extending in the same plane as the advancing wall, but consisting of unaligned vesicles, circular or elongated as seen in section. These might have been spherical vesicles and tubules, respectively,

or part of a reticular network. Associated with these were several globules of regular outline, enveloped by a single membrane and containing an unknown substance or substances (Pl. 2, figs. 3, 4).

In tangential sections obviously cut near and just within the advancing edge of the septum (Fig. 1 *a*) the line of vesicles, as would be expected, was continuous across the central gap (Pl. 1, fig. 2). It can be seen that these 'vesicles' tended to coalesce and that this tendency was greatest adjacent to the developing septum (Pl. 2, fig. 3). Here they joined to form plates (often at first slightly overlapping like roof tiles, Fig. 1 *d*, *e*) which finally coalesced to give a continuous septum. It is clear that the septum developed by the addition to its edge of coalesced cisternae or vesicles which had already become aligned in the plane of the septum. This method of wall formation through the coalescence of vesicles was reflected in the wavy outline of the newly formed wall (as seen in Pl. 2, fig. 6; Fig. 1*f*), which persisted until the wall began to thicken. Finally the wall extended across the progametangium, leaving no central pore.

The membranes surrounding the vesicles which became incorporated in the septum became reorientated and formed two parallel layers comparable with and finally continuous with the original plasmalemma of the progametangium (Pl. 1, fig. 1). As the septum developed, wall material similar in appearance to that of the original progametangial wall was laid down between the two parallel plasmalemma membranes (Pl. 3, fig. 7), beginning at the peripheral zone (i.e. adjacent to the progametangial wall) of the new wall and proceeding inwards so that the developing septum was a narrow-based wedge, as seen in section. At a very early stage it could be seen that the new wall material was laid down immediately within the parallel plasmalemma membranes and that there was a thin electron-transparent central zone, corresponding to the original interior of the component coalesced vesicles and separating the two new layers of wall material (Pl. 2, fig. 6). At this early stage the two layers of new wall material were of approximately equal thickness. At intervals along the septum transverse fine tubules could be seen, recalling the plasmodesmata of higher plant cells (Pl. 3, fig. 7). These have already been reported in this fungus and in *Gilbertella persicaria* by Hawker, Gooday & Bracker (1966). At about this stage the endoplasmic reticulum, which had previously been sparsely but fairly evenly distributed throughout the progametangial cytoplast (as is typical of fungus cells; Hawker, 1965), became aggregated into complex masses along the septum on both sides of the newly formed wall (Pl. 3, figs. 8, 9) beginning in the angle between the septum and the original progametangial wall (Pl. 3, fig. 7). The septum then rapidly thickened by the deposition of new layers of wall material, presumably adjacent to the plasmalemma. This process, however, was much more rapid and continued longer on the gametangial side of the wall (Pl. 3, fig. 9), where it eventually formed part of the thick wall of the zygospore.

Despite this uneven rate of thickening of the wall layers on the gametangial and suspensor sides of the septum, no differences in the nature or amount of the adjacent bands of endoplasmic reticulum were detected. The plasmodesmata-like tubules could be seen passing across the entire width of the septum (Pl. 3, fig. 9) and may well function as conducting channels to allow passage of soluble materials through the developing wall.

The development of the two gametangia is not usually exactly synchronous in a conjugating pair, so that the same zygospore initial may show septa at different stages of development.

DISCUSSION

Detailed electron-microscope studies of septum formation are available for only a few organisms. Bracker (1966) described the formation of sporangiospores of *Gilbertella persicaria* (Eddy) Hesseltine. Here the spores were delimited and separated by the fusion of cleavage vesicles which developed between the spore initials. The membranes of these cleavage vesicles became the plasmalemma of the young spores. The spore envelope and, later, secondary wall layers, were formed outside the plasmalemma (i.e. on the side of the vesicle membrane which was originally innermost), as with the gametangial septum of Rhizopus. The characteristic dark dots associated with these membranes in Gilbertella were not seen in Rhizopus. Bracker reported that endoplasmic reticulum aggregates were often associated with the cleavage vesicles, but his photographs, which related to comparatively early stages of sporangiospore formation, do not show such large complex aggregations as those which developed along the gametangial wall of the Rhizopus zygospore.

Bracker pointed out that wall formation of ascospores (Moore, 1963; Moore & McAlear, 1962; Wilsenach & Kessel, 1965) differs in some essential points from sporangiospore formation; it also bears little resemblance to septum formation in the progametangium of Rhizopus. The closest parallel to the latter is the formation of septa in higher plant cells. Whaley, Dauwalder & Kephart (1966) illustrated the formation of a septum across the spindle plate of a dividing root cell of maize (*Zea mays* L.). Here the septum, in contrast to that of Rhizopus, begins at the centre of the cell and develops outwards, but it extends by the incorporation of coalesced vesicles in a manner essentially similar to that described above (p. 373). Frey-Wyssling, López-Saéz & Mühlethaler (1964) showed a similar type of wall development in *Phalaris canariensis* L.

In the higher plant, septum formation is associated with the presence of dictyosomes (Golgi apparatus). Mollenhauer, Whaley & Leech (1961) and Leech, Mollenhauer & Whaley (1963) considered that the Golgi bodies contribute not only to plate formation but also to later stages of wall development. No dictyosomes or similar regularly arranged plates of cisternae were seen during septum formation of Rhizopus. It was shown, however, that the vesicles which became integrated with the advancing edge of the septum arose in an area comparatively rich in irregular vesicles and cisternae, and that as the wall began to thicken a very great increase in endoplasmic reticulum in the form of tangled tubules occurred. Such complexes most probably play a part in wall formation similar to that of the dictyosomes. Porter (1961) in a discussion of the function of endoplasmic reticulum concluded that at the cell surface the units of the reticulum are short or circular, as seen in section, suggesting a tubular form rather than the typical lamellar form. He considered that the functional activity of these structures at the cell surface includes 'some exchange of metabolites and the production and deposition of materials in wall formation'. It is reasonable to conclude that the striking increase in complexity and amount of endoplasmic reticulum in irregular zones along both sides of the gametangial septum of Rhizopus is similarly associated with deposition of wall material and that exchange of metabolites between suspensor and gametangium may take place to a limited extent via the tubules seen to extend through the septum and which remain clearly visible and apparently unoccluded until at least a late stage in secondary wall thickening.

Wilsenach & Kessel (1965) considered that lomasomes (first thought to be peculiar to fungi; Moore & McAlear, 1961) play an important part in the formation of the ascospore wall in *Penicillium vermiculatum* Dangeard. These authors described the origin of lomasomes between the membranes of endoplasmic vesicles which were associated with the ascospore wall. Lomasomes have recently been seen in organisms other than fungi and have been shown to consist of aggregates of fine tubules (Hendy, 1966). They occur occasionally adjacent to the development gametangial septum of Rhizopus and may perhaps be interpreted as extracytoplasmic tubules essentially similar in function to those within the cytoplasm.

This study of zygospore development in Rhizopus is being continued by electron microscopy and by differential staining; it is hoped that it will further illuminate the various processes which lead to the production of mature zygospores.

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EXPLANATION OF PLATES

Key to symbols: S = suspensor cell, G = gametangium, PW = original progametangial wall, CW = new cross wall (septum), M = mitochondrion, N = nucleus, SV = storage vacuole, P = plasmodesmata, V = vesicle, ER = endoplasmic reticulum. Lines on figures represent 1 μ in each case.

Figs. 1–7 were fixed in 2% $KMnO_4$, 30 min., soaked in 0·5% Aq uranyl acetate 48 min., dehydrated, embedded in Epon; sections stained 10 min. in lead citrate (Reynolds, 1963). Fig. 8 was fixed in 2% $KMnO_4$, 30 min., dehydrated, embedded in Epon; sections stained lead citrate. Fig. 9. as Fig. 8, but embedded in Vestopal.

Plate 1

Fig. 1. Radial longitudinal section through incomplete septum. Note angle with progametangial wall, P.W.; continuity of plasmalemma of original cell with outer membrane of new wall; deposition of new secondary wall material (at part of septum adjacent to progametangial wall) between plasmalemma and central electron-transparent plate of septum; aggregation of endoplasmic reticulum (ER) in angle between septum and original wall; and two vesicles (V) aligned in plane of advancing edge of septum.

Fig. 2. Tangential longitudinal section through same developing septum (see Text-fig. 1). Note line of coalescing vesicles in plane of septum.

Plate 2

Fig. 3. A similar section to that of Fig. 2, showing details of the joining together of coalesced vesicles to form the advancing edge of the septum.

Fig. 4. A more nearly median section through the edge of the septum and part of the central gap showing a zone of irregular ER from which the wall-forming vesicles are budded off.

Fig. 5. Part of fig. 4 enlarged.

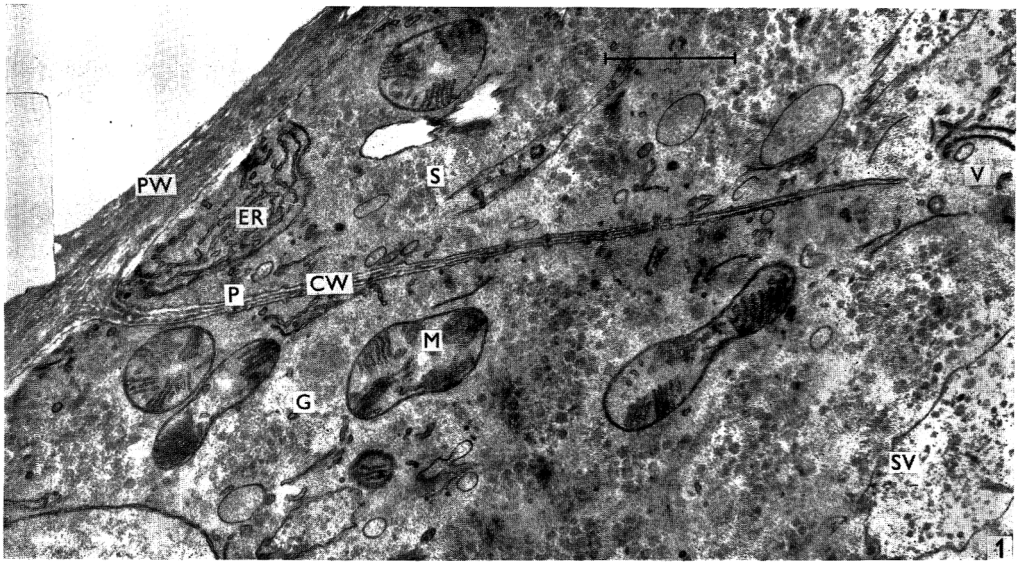
Fig. 6. Part of the newly developed septum, before secondary thickening, showing plasmalemma, two adjacent 'grey' zones and the central electron transparent one. Note occasional 'plasmodesmata' (P).

Plate 3

Fig. 7. Section through junction of septum and lateral wall showing increased thickness at outer part due to secondary deposition of new wall material. Note also plasmodesmata (P).

Fig. 8. Similar section of septum at slightly older stage, showing unequal thickening, greater on gametangial side.

Fig. 9. Later stage, showing pronounced thickening on gametangial side of septum. Plasmodesmata clearly seen as fine tubules passing through wall.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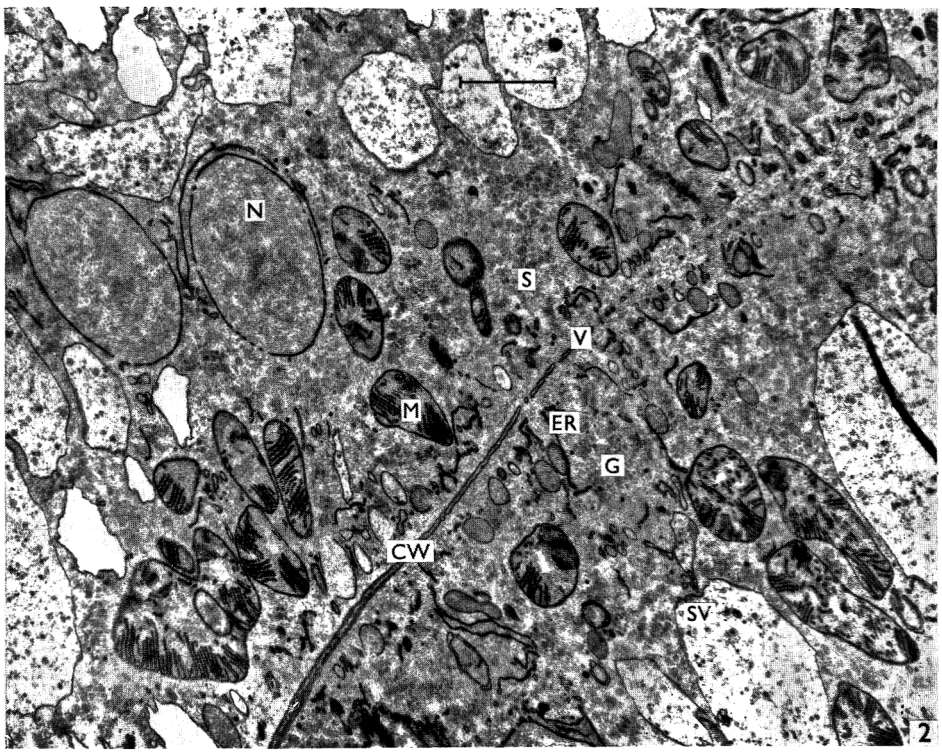
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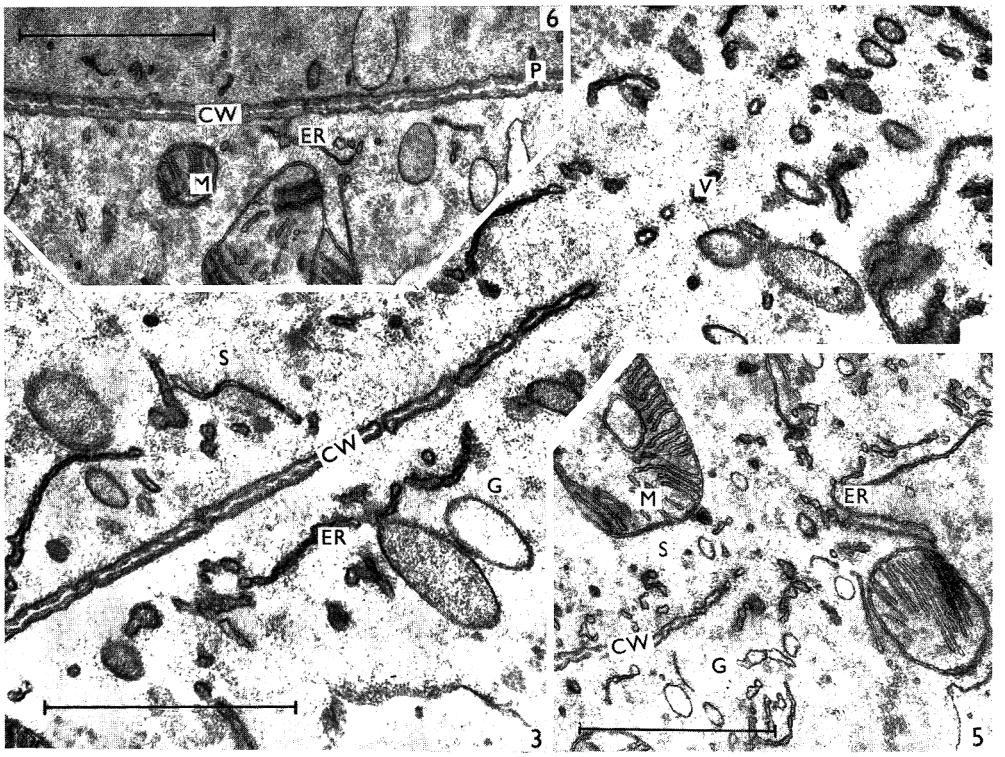
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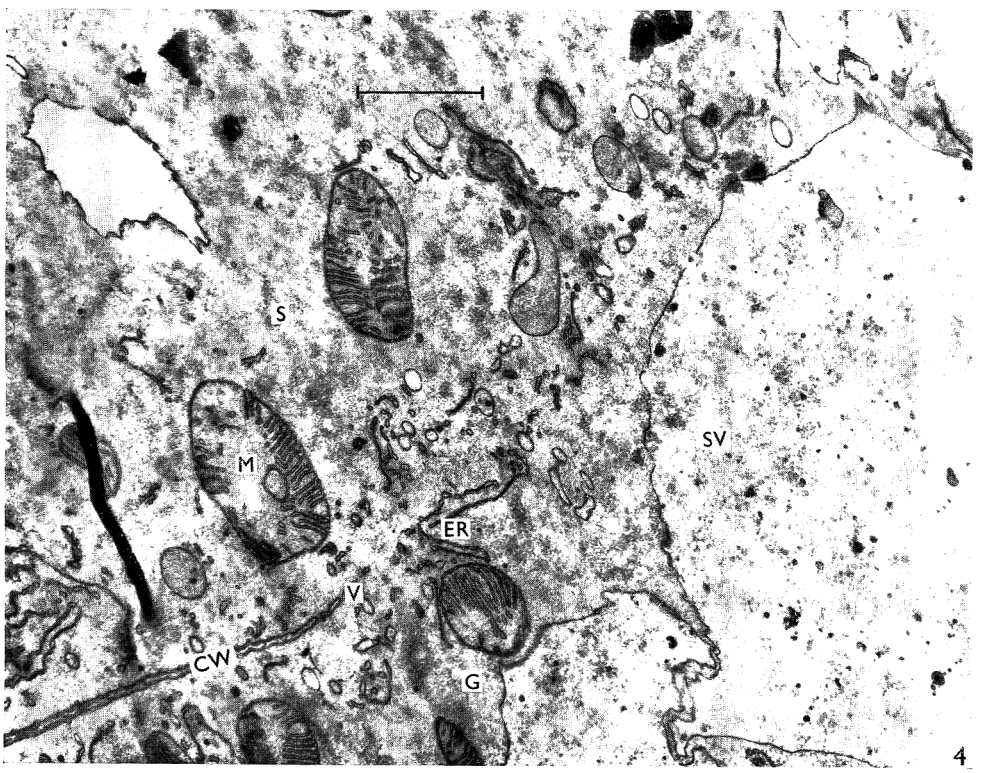
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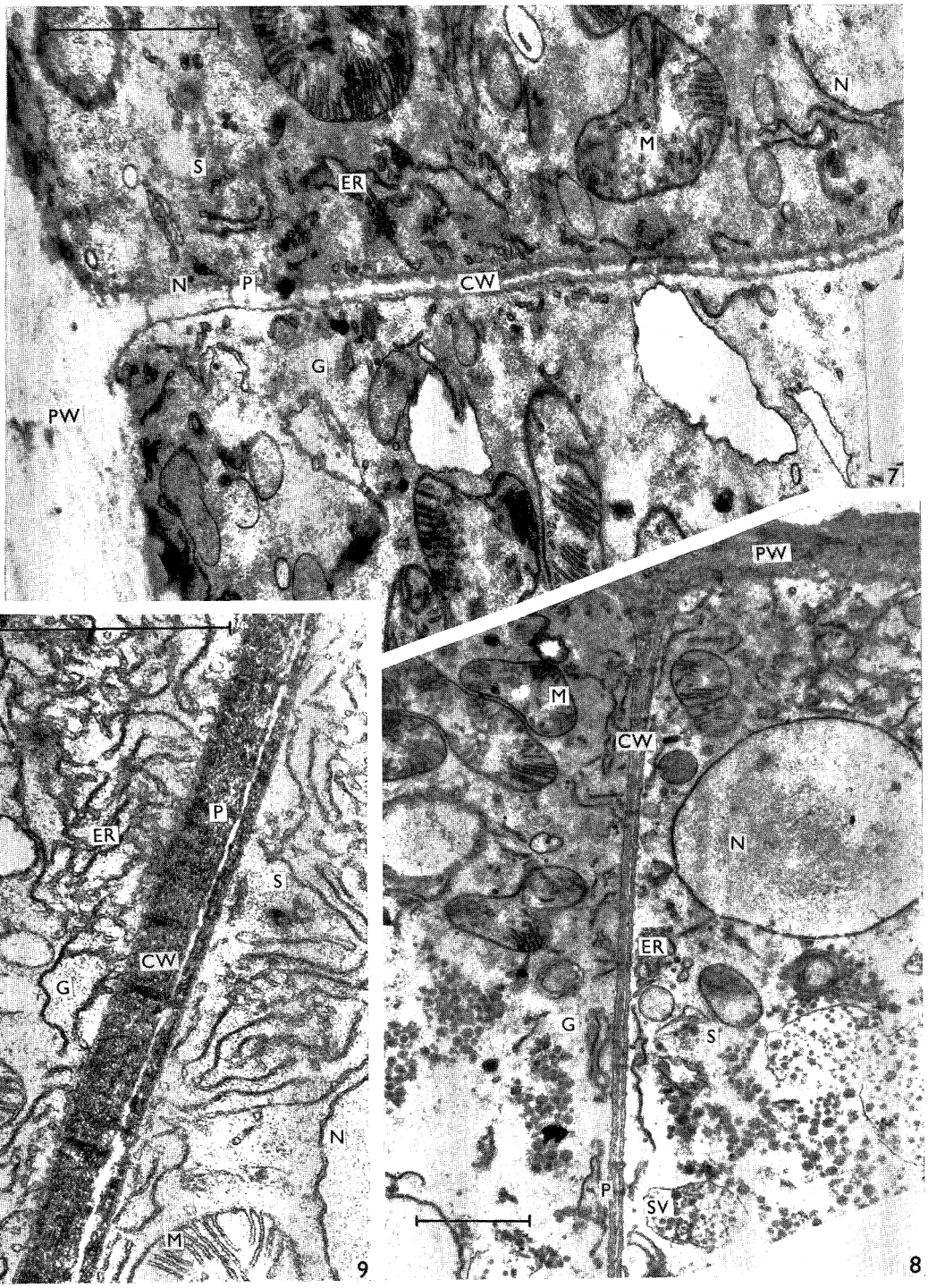
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Cell Wall Composition in *Mycobacterium lepraemurium*

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SUMMARY

Cell-wall fractions from *Mycobacterium lepraemurium* were examined chemically and immunologically. The major sugar and amino acid components detected were arabinose and galactose, alanine, glutamic acid, α,ϵ-diaminopimelic acid (DAP) and hexosamine, but substantial amounts of aspartic acid, glycine, valine, serine, threonine, leucine and *iso*-leucine were also present in preparations not extracted with neutral lipid solvents and alkaline ethanol. However, after such extraction and treatment with proteolytic enzymes, alanine, glutamic acid and DAP (1·5:1·0:1·0) were present in much larger amounts than any other amino acids (not more than 0·15), indicating that these three are the mucopeptide constituents. Cell-wall agglutination tests indicated the presence of an antigen shared with other mycobacteria.

INTRODUCTION

Examination of cell wall fractions prepared from a number of strains of mycobacteria have shown a common pattern of components which these organisms share with strains of nocardia and some corynebacteria (Cummins & Harris, 1958; Cummins 1962). This common pattern is characterized by the presence of the sugars arabinose and galactose, and of alanine, glutamic acid and DL-diaminopimelic acid as mucopeptide amino acids. In addition, it has been noted (Cummins, 1962) that organisms whose cell walls show this pattern have a cell-wall antigen in common.

Mycobacterium lepraemurium is the causative organism of a chronic infection in wild rats known as rat leprosy. The organism has not been grown in ordinary bacteriological medium; however, it has been grown in tissue culture (Rees & Garbutt, 1962). *M. lepraemurium* has a generation time of about 10 days in tissue culture and *in vivo*. Therefore it seemed of interest to examine cell-wall fractions from this very slowly growing and obligate intracellular species of mycobacterium to see whether its cell wall was of the same general type as that of all other mycobacterial species examined.

METHODS

Preparation of Mycobacterium lepraemurium suspensions. The Douglas strain of *M. lepraemurium* (Balfour-Jones, 1937) was used; suspensions of the organism were prepared by homogenizing heavily infected liver from mice injected intravenously 4–6 months previously with *M. lepraemurium* (Rees, Valentine & Wong, 1960). The liver homogenates were prepared in 0·01 M-phosphate buffer (pH 7·1) and partially

purified suspensions of organisms obtained by repeated centrifugation and washing in buffer.

Electron microscopy. Samples were suitably diluted with distilled water and thin layers allowed to dry on electron microscope specimen supports covered with carbon films. The preparations were shadowed with platinum before examination in the electron microscope.

Preparation of cell walls. This was done either by the method of Cummins & Harris (1956) (omitting ribonuclease treatment) or by disintegration in a Mickle shaker followed by centrifugation in a sucrose gradient in which the walls formed a layer above 40 % (w/v) sucrose.

Extraction with neutral solvents. This was done at 37°. The material was first extracted twice with ethanol + ether (1 + 1, v/v), and then twice with chloroform + methanol (1 + 1, v/v), the total extraction period being several weeks. During this time the extraction mixtures were shaken occasionally by hand.

Extraction with alkaline ethanol. Absolute ethanol containing 0·5 % KOH was used, and the extraction was continued for 2–3 days at 37°, with occasional shaking. The mixture was centrifuged, and the extracted, deposited material washed twice with absolute alcohol and then three times with distilled water.

Chromatography. Material was hydrolysed in 6N-HCl for 18 hr at 105°, the liquid filtered and the filtrate evaporated to dryness at 100° over a water bath. The soluble material was taken up in a small quantity of distilled water. To identify the amino acids two-dimensional ascending chromatograms were run in 10 in. squares of Whatman no. 1 paper. The first solvent was butanol + acetic acid + water (120 + 30 + 50, by vol.) and the second phenol + ammonia + water (phenol 80 + water 20, v/v; + 1 ml. sp.gr. 0·880 ammonia per 200 ml.). The spots were made visible with 0·2 % ninhydrin in 95 % (v/v) acetone in water.

For the separation of diaminopimelic acid (DAP) isomers descending chromatograms were run using the methanol + pyridine + HCl + water solvent (Hoare & Work, 1957).

For sugars the method was that of Cummins & Harris (1956), except that one-dimensional descending chromatograms were used, the solvent being ethyl acetate + pyridine + water (9 + 2 + 2 by vol.).

Quantitative estimation of amino acids. This was done by the method of Atfield & Morris (1961) with high-voltage paper electrophoresis. The results were expressed as molar ratios, taking DAP = 1·0.

Cell-wall agglutination tests. The method was that of Cummins & Slade (1961) except that an MSE sonic disintegrator was used to redisperse the cell wall fragments.

RESULTS

Chemical analyses were done on samples of *Mycobacterium lepraemurium* cell walls prepared in four different ways (see Table 1). Quantitative amino acid analyses were made on all four samples, but qualitative examination for amino acids and sugars were also done on sample B, as was the determination of the type of DAP present.

Electron micrographs of the fractions analysed showed them to be very largely composed of cell walls, as can be seen in Pl. 1, fig. 1, which shows a preparation made from sample C. A preparation from sample D was also examined (Pl. 1, fig. 2). The

cell walls of sample C were generally almost intact, while in sample D they were mostly fragmented and showed a greater tendency to aggregate. However, considering that sample D had been prepared from sample C by procedures which resulted in an 85% loss of weight (see Table 1) there is surprisingly little difference to be noted in the appearance of the cell-wall fragments in the two samples.

Table 1. *Methods of preparation of Mycobacterium lepraemurium cell-wall fractions*

Sample	Method of preparation
A	Formalin-killed suspension disintegrated, deposit treated with trypsin and pepsin, extracted with 0·5% KOH in ethanol for 24 hr at 37°; finally washed with ethanol and distilled water
B	Organisms allowed to autolyse for 1 month at 37°, disintegrated, and cell-wall fraction separated by centrifugation in sucrose gradient
C	Fresh suspension disintegrated, cell walls separated by centrifugation in sucrose gradient: no other treatment except for washing
D	Prepared from part of sample C by thorough extraction at 37° with neutral lipid solvents (ethanol+ether 4 weeks, chloroform+methanol 6 weeks), followed by 0·5% KOH in ethanol (3 days at 37°). Finally the residue was washed with absolute ethanol and then distilled water, and treated with trypsin and pepsin. Final yield = 15%: i.e. 13·4 mg. of sample C yielded 2·0 mg. of sample D.

Table 2. *Qualitative composition of Mycobacterium lepraemurium cell walls; sample B*

Amino acids		Sugars and amino sugars	
Ala, Glu, DAP (DL and/or DD-)	+++	Arabinose	+++
		Galactose	++
		Mannose	trace
Asp, Gly, Ser, Threonine, Valine, Leucines	+	Hexosamines	+
Lysine, Arginine	trace		

Qualitative analyses

The results of chromatography of acid hydrolysates of *Mycobacterium lepraemurium* cell walls is shown in Table 2. The principal amino acids found were alanine, glutamic acid and DAP, the latter the DL-isomer (or DL+DD), and the principal sugars identified were arabinose and galactose, with a trace of mannose. This pattern of sugar and amino acid components is that to be expected in a mycobacterium (Cummins & Harris, 1958; Cummins, 1962). The identity of the hesoxamines is not known for certain, since the chromatographic method used did not separate glucosamine and muramic acid, but it may be assumed that both are present.

Quantitative amino acid analyses

The high-voltage electrophoretic method used enabled analyses to be done on samples of 1 mg. or less. The results obtained with samples A–D are recorded in

Table 3; these results are the mean of two estimations in each case. It is obvious from an examination of these results that the method of preparation of cell-wall fractions has a very considerable influence on the total amino acid content and also on the molar ratios of the different amino acids present, especially the latter. The most interesting comparison is between samples C and D, since the latter was prepared from the former by a rather extensive extraction with lipid solvents followed by treatment with proteolytic enzymes. This resulted in a very considerable loss of weight: for example, 13·4 mg. of sample C after treatment gave only 2·0 mg. of sample D as the final product, a yield of about 15 %. It might be expected that such treatment would remove all amino acid-containing material except mucopeptide. The results suggest that this had in fact happened to a large extent, since over 80 % of the total amino acid content of sample D was made up of alanine, glutamic acid and diaminopimelic acid. In view of this it seems reasonable to suppose that these are the mucopeptide amino acids in *Mycobacterium lepraemurium*. In the starting material C, however, which had no treatment except separation in a sucrose gradient followed by washing in distilled water, the picture was very different; appreciable amounts of aspartic acid, glycine, leucines, valine and serine, were present, with rather smaller amounts of 7 other amino acids. The pattern is similar in samples A and B, although the amounts of non-mucopeptide amino acids are lower.

Table 3. *Mean molar ratios of amino acids in cell-wall fractions prepared from Mycobacterium lepraemurium in different ways*

Constituent	A	B	C	D*
Diaminopimelic acid	1·0	1·0	1·0	1·0
Glutamic acid	1·5	2·0	3·0	1·0
Alanine	2·0	3·0	3·5	1·5
Aspartic acid	0·6	0·7	2·0	0·1
Glycine	0·8	0·7	2·0	0·15
Leucine isomers	Leuc + isoleuc 0·65	Leuc + isoleuc 1·0	Leuc 1·3 Isoleuc 0·7 Allo isoleuc 0·7 } 2·7	Leuc + isoleuc 0·15
Valine	0·3	0·4	1·5	0·1
Serine	0·25	0·3	1·4	0·1
Threonine	0·2	0·2	0·95	0·05
Lysine	0·15	0·15	0·5	Trace
Histidine	Not detected	Trace	0·3	Not detected
Phenylalanine	Not detected	0·1	1·0	0·1
Tyrosine	Trace	Trace	0·2	Not detected
Arginine	0·2	0·25	0·8	Trace
Methionine sulphone	Not detected	Not detected	0·2†	Trace
Approx. % amino acids	21	14	25	15

* DAP = 1·0.

† Confirmed as methionine sulphone by nitroprusside test.

Serological tests with Mycobacterium lepraemurium cell-wall preparations

The results of cell-wall agglutination tests are given in Table 4, and show clearly that *Mycobacterium lepraemurium* contains a cell-wall antigen shared with other mycobacteria, since suspensions of cell walls prepared from it agglutinated with anti-smegmatis serum in the same way as cell-wall fractions from the BCG and avian strains of *M. tuberculosis*. In fact, *M. lepraemurium* cell-wall suspensions agglutinated

to the same titre as those of the avian strain. Antiserum 583G, which had been prepared against *M. lepraemurium* itself, seemed to contain only a little antibody against the common mycobacterial antigen.

Agglutination tests were not possible with many of the samples of cell walls prepared from *Mycobacterium lepraemurium* suspensions because of auto-agglutination in saline; broadly speaking it was only when the bacteria were extracted with neutral solvents and then with alkaline ethanol that the cell-wall fractions made from them were sufficiently stable in saline to be used in agglutination tests, i.e. samples A and D. Table 1. This parallels similar findings for other mycobacteria (see Cummins & Harris, 1958; Cummins, 1962). However, during the present work it was noticed that cell-wall suspensions prepared in a sucrose gradient after disintegration of freshly made suspensions of *M. lepraemurium* were stable in saline and gave a titre against *M. smegmatis* antiserum similar to that recorded in Table 1 for suspension of extracted cell walls.

Table 4. *Agglutination tests with cell-wall suspensions from Mycobacterium lepraemurium and some other mycobacteria*

Serum	Cell-wall suspension† from	20	40	80	160	320	640	SAL
Antiserum SMEG R11/62	*M. smegmatis**	++	++	++	++	+	±	−
	M. tuberculosis BCG	++	++	++	++	+	+	−
	M. tuberculosis, avian type	++	++	++	+	−	−	−
	M. lepraemurium (sample D)	++	++	++	+	Tr	−	−
Antiserum *M. lepraemurium* 583G	*M. tuberculosis* BCG	+	±	−	−	−	.	−
	M. lepraemiruum (sample D)	±	Tr	−	−	−	.	−
Normal rabbit serum	*M. lepraemurium* (sample D)	−	−	.	.	.	.	−

* Homologous suspension.

† The cell-wall suspensions used in these tests were prepared from organisms extracted by neutral solvents, followed by 0·5% KOH in ethanol.

. = not done.

Because of the limited amount of material available it was not possible to do full absorption tests. However, in a single test, 1 ml. of a 1/80 dilution of *Mycobacterium smegmatis* serum was absorbed with 2 mg. of *M. lepraemurium* cell walls. On testing the absorbed samples against *M. smegmatic* cell-wall suspension it appeared that all antibodies had been removed.

DISCUSSION

These results provide clear evidence that the cell walls of *Mycobacterium lepraemurium* closely resemble those of other mycobacteria, both chemically and serologically. The presence of arabinose and galactose as principal cell-wall sugars, and of alanine, glutamic acid and DAP as mucopeptide amino acids, coupled with evidence that the walls contain a high proportion of lipid (loss of weight on extraction with neutral lipid solvents) are all typical of this group of organisms. Unfortunately it was not possible to analyse the lipids extracted, as it would have been of interest to know whether or not they contained mycolic acid, and also whether the chloroform-soluble

fractions had any adjuvant activity of the type described by White, Coons & Connolly (1955) in Wax D from other mycobacteria.

As mentioned above, the considerable differences in amino acid patterns between samples C and D make it clear that alanine, glutamic acid and DL-diaminopimelic acid are the mucopeptide amino acids in this organism. However the rather large number of amino acids in sample C indicate the presence of other peptide material in the unextracted wall.

If it is assumed that in samples A, B and C a certain amount of alanine and glutamic acid are also 'non-mucopeptide', it appears possible that there is a peptide in these cell-wall samples, not associated with mucopeptide, which is largely composed of glutamic acid, alanine, aspartic acid, glycine, leucines, valine and serine. It seems that thorough treatment with lipid solvents, followed by proteolytic enzymes, is necessary to remove this peptide, which is perhaps associated with lipid; but these treatments were not fully investigated in all combinations. It is interesting to compare these results with those obtained by Belknap, Camien & Dunn (1961) for other mycobacteria. These workers examined 4 strains, 2 of *Mycobacterium tuberculosis*, one of *M. ranae*, and an unclassified 'Battey' strain, and found the major components in each case were alanine, glutamic acid and diaminopimelic acid. They also found appreciable amounts of aspartic acid, glycine, serine, leucines, threonine and valine, although not all of these were present in every strain. The cell-wall material prepared by Belknap *et al.* was made from organisms which had been extracted rather briefly with acetone and ether and then dried; after disintegration, the cell-wall fractions were treated with trypsin and pepsin.

Aspartic acid and glycine have also been found as the principal 'non-mucopeptide' amino acids in the peptido-glycolipids of Wax D in avian, bovine and saprophytic strains of mycobacteria (Jollès, Samour & Lederer, 1963). Misaki, Yukawa, Tsuchiya & Yamasaki (1966), who have made detailed studies of the composition of the cell wall of *Mycobacterium tuberculosis* BCG, considered that glycine was a mucopeptide constituent in the strain they examined because it was the only amino acid (other than alanine, glutamic acid and diaminopimelic acid) which was not removed by treatment with 'pronase'. In their strain, glycine was present in pronase-treated walls to the extent of 0·33 (diaminopimelic acid = 1·0). Similar studies on cell walls of *M. phlei* by Takeya, Hisatsume & Inoue (1963) showed 'basal layer' (= mucopeptide) amino acids to be alanine, glutamic acid and diaminopimelic acid in ratio 1·9:1·7:1·0, with no other amino acid present in more than 20% of the amount of diaminopimelic acid. Both these groups of workers found arabinose and galactose to be the principal sugars present in the strains they examined.

The total amino acid content of the different fractions, also shown in Table 2, probably varies with a number of factors, the principal ones being the amounts of lipid and non-mucopeptide protein present. These latter factors might be expected to act in opposite directions, since a large amount of protein would raise the amino acid content and a large amount of lipid would make it relatively smaller; but in the absence of more definite information about the exact amounts of protein and lipid present the figures for total amino acid content are not very informative. However, the decrease from 25% amino acids in sample C to 15% in sample D presumably represents a considerable removal of non-mucopeptide protein.

In previous work (Cummins, 1962) all strains of mycobacteria, corynebacteria and

nocardias examined which had arabinose and galactose as characteristic cell-wall sugars showed cross agglutination when suspensions of their cell walls were tested against antisera to *Mycobacterium smegmatis*. From the results obtained with *M. lepraemurium* cell walls (Table 4) there seems to be no doubt that this antigen is present in them also.

It was somewhat unfortunate that the only *Mycobacterium lepraemurium* antiserum available appeared to contain very little antibody to the cell-wall antigens, but the results of cell-wall agglutination tests with *M. smegmatis* antiserum seemed quite conclusive of the presence in *M. lepraemurium* cell walls of the common mycobacterial antigen, since in the tests the titre at which *M. lepraemurium* cell walls reacted was at least as high as that for *M. avium* (Table 4). Moreover, the absorption test, using *M. lepraemurium* cell wall to absorb *M. smegmatis* antiserum, provides confirmatory evidence of the presence of the common antigen in the cell walls of *M. lepraemurium*.

One of use (C. S. C.) wishes to thank the Medical Research Council for grants for equipment and technical assistance.

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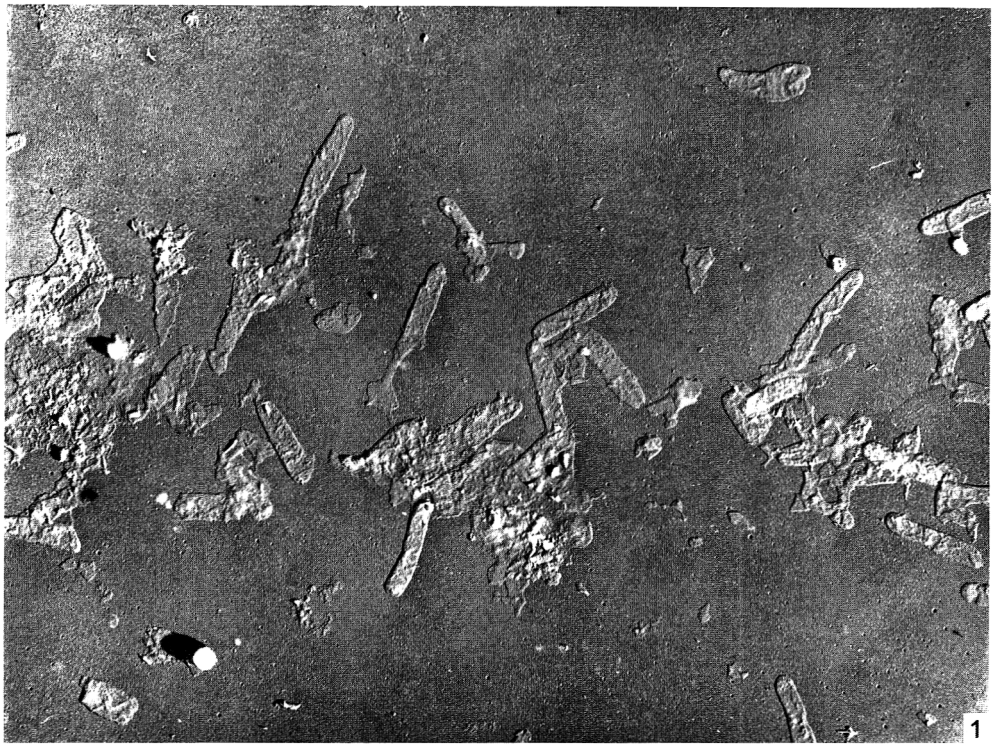
EXPLANATION OF PLATE

Electron micrographs of *Mycobacterium lepraemurium* cell-wall fractions. (Pt shadowed.) × 7,300.

Fig. 1. Sample C.

Fig. 2. Sample D, prepared from sample C by thorough extraction with lipid solvents (see text for details.



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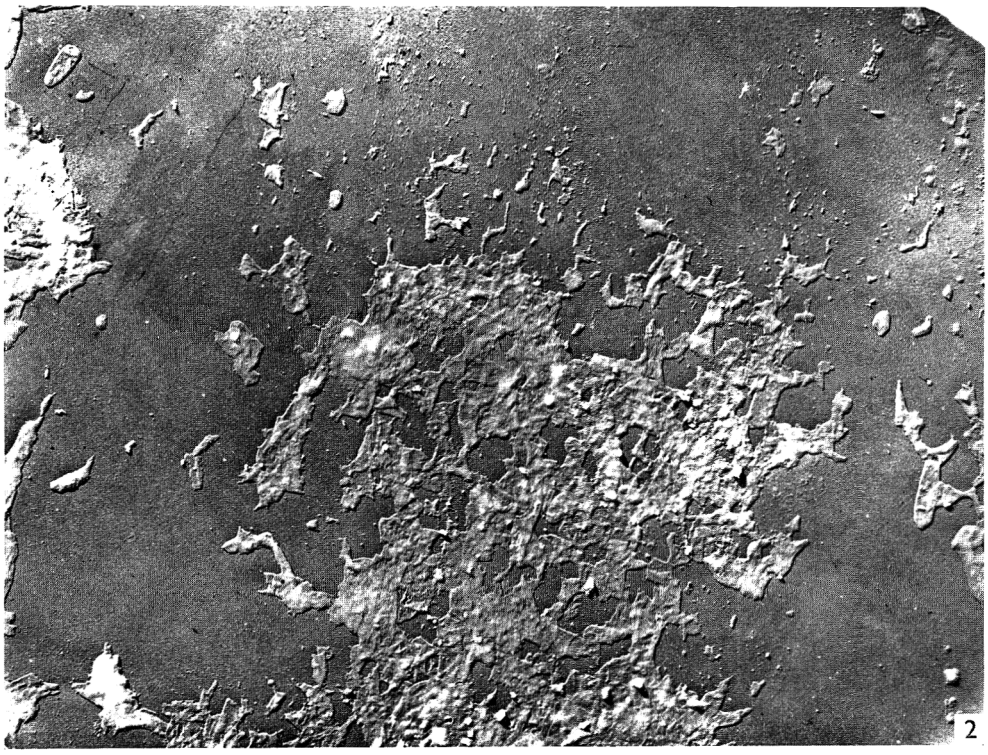

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With 1 *plate*
Printed in Great Britain

Effect of Clover Phyllody Virus on Nodulation of White Clover (*Trifolium repens*) by *Rhizobium trifolii* in Soil

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SUMMARY

When grown in sterilized soil inoculated with an effective Rhizobium strain which produced many large pigmented nodules on virus-free plants, plants infected with clover phyllody virus (CPV) produced mainly small white nodules characteristic of an ineffective reaction. Mainly small nodules were also given by virus-free plants exposed to mixtures of effective and naturally ineffective Rhizobium strains, which separately gave predominantly large and predominantly small nodules, respectively, indicating that the ineffective strain had a high competitive ability for invasion sites on the roots.

When the CPV-infected plants were removed and fresh seed sown in the soil, germination was poor and the seedlings produced predominantly small nodules. The rhizobia seemed modified to a less effective form which produced mainly small nodules and competed successfully with unmodified bacteria. Yields of clover in swards may therefore be decreased by CPV infection, not only through a direct effect on plant growth but also through effects on the soil Rhizobium population, and hence on the growth of infected plants and neighbouring virus-free plants.

INTRODUCTION

Joshi, Carr & Jones (1967) reported that white clover plants infected with the persistent jassid-transmitted clover phyllody virus CPV (= strawberry green-petal virus; Frazier & Posnette, 1957; Chiykowski, 1962; Posnette & Ellenberger, 1963), produced a high proportion of small and non-pigmented nodules characteristic of decreased effectiveness in fixing nitrogen when inoculated in agar culture with a normally highly effective strain of *Rhizobium trifolii* derived from a single bacterial colony. Rhizobia transferred from infected to healthy plants again elicited this partially ineffective response, possibly because of a genetic change induced in the bacterium and not because it acted as a vector of the virus.

Although the experiments were made with artificial media, our earlier unpublished observations and those of Vanderveken (1964) suggest that similar effects occur under natural soil conditions. An important practical point to consider is whether, when released into the soil, rhizobia induced to partial ineffectiveness through having invaded virus-infected plants compete better than fully effective ones for invasion sites on the roots of healthy hosts, with ensuing general decrease of the efficiency of nitrogen fixation throughout the sward. Nicol & Thornton (1942) showed that some Rhizobium strains were better competitors than others, and although their competitive ability was generally independent of the degree of effectiveness, certain strains were found to be both ineffective and highly competitive. Fred, Baldwin & McCoy (1932)

demonstrated that plants already infected with one Rhizobium strain resisted infection by another strain to a greater degree than un-nodulated plants. Nutman (1952) showed that a substance which inhibited further nodulation was produced in the nodule apex.

The results of experiments comparing the growth and nodulation of CPV-infected and healthy white clover, grown consecutively in sterilized soil inoculated with effective and ineffective strains of Rhizobium both separately and together, are reported in this paper. Throughout, the term 'healthy' implies only freedom from infection with CPV.

METHODS

Wooden boxes 18 × 12 × 8 in. deep were treated with 2% formaldehyde solution to destroy naturally occurring rhizobia, and were filled from the same batch of John Innes no. 1 compost sterilized previously by autoclaving for 90 min. at 121°. They were then allowed to stand for 2 weeks before inoculation. An effective W4 culture and an ineffective W22 culture of *Rhizobium trifolii*, both from the W.P.B.S. collection and each re-isolated recently from a single colony, were streaked on plates of yeast mannitol agar. Organisms were removed from 10-day plates by scraping the surface with a smooth-edged microscope slide, and transferred to demineralized water. Following homogenization in a blender, the concentration of the two suspensions, as measured by their turbidity in an EEL nephelometer, was equalized by adding a further quantity of demineralized water. A 40 ml. portion was sprayed from a fine atomizer on to the separated top inch of soil removed from each box, with frequent mixing, and the soil was then replaced in an even layer. Eight boxes received the effective W4 strain, four the ineffective W22, and four a mixture of 20 ml. W4 and 20 ml. W22 suspension.

Young actively growing stolon apices, each bearing a single apical growing point and an incipient root not yet broken through the outer tissues, were detached from plants of two white clover clones (nos. 33, 45), selected from the variety S. 100 on the basis of their marked reaction to CPV. Some of these plants were healthy and others infected with our moderately virulent CPV isolate F. After thorough washing in sterile water they were planted in the boxes, each genotype occupying alternate rows of six plants and each box receiving 24 cuttings. Four of the eight boxes inoculated with the effective Rhizobium strain were planted with healthy and four with CPV-infected cuttings. Boxes containing the ineffective strain and those with a mixture of effective and ineffective strains were all planted with healthy cuttings. The boxes were arranged in a 4 × 4 Latin square under mist propagation for 10 days, and were then removed in the same order to the glasshouse bench. Healthy cuttings in a box without added rhizobia had very few nodules by the end of the experimental period, indicating the adequacy of the sterilization procedures. Data from plants in this box did not feature in the main analyses. At the end of June, when this first half of the experiment had run for 3 months, plants were harvested, their shoot dry-weight and root nodulation recorded.

Immediately after harvest, all roots were separated from shoots, chopped into small pieces and returned to the soil of their respective boxes with thorough mixing of the upper layer, so that rhizobia present in the nodules would be released as the roots decayed. Each box was then sown with surface-sterilized seeds of the white clover variety S. 100, with the intention to thin the number of seedlings down to 24. How-

ever, germination was abnormally low in boxes which had previously contained CPV-infected plants, and these boxes were resown after 6 days with excess seed. Although germination was again poor, there were now sufficient plants to enable thinning to the 24 required. Because the weather was now favourable, all boxes were moved outside for this second half of the experiment until harvest in mid-September.

At each harvest, all plants from each box were separated into root and shoot, the shoot portions were oven-dried at 80° for 6 hr and the mean dry weight per plant recorded. Root portions from four randomly selected plants were examined in fresh condition and the mean number of nodules per plant in each of the visually estimated classes (large, medium, small) determined. The roots were then air-dried at normal laboratory temperature (17°) for 2 days and their weight recorded, so that the nodule data could also be expressed as the number of nodules/g. root. All data were subjected to analysis of variance; the statistical technique used for the nodule counts was such that the interaction of treatment with nodule size was a function of the effect of treatment on the frequency of distribution over the three classes of nodules.

RESULTS

Growth and nodulation of uninfected and CPV-infected cuttings

Immediately before harvesting the plants of the first half of the experiment there were marked differences in growth between the four treatments. Healthy plants inoculated with the effective Rhizobium strain were robust and dark green; those inoculated with a mixture of effective and ineffective rhizobia were smaller and paler green; those with ineffective rhizobia only were smaller still and distinctly yellowish, giving an impression of nitrogen starvation. CPV-infected plants were, as usual, very small, with chlorotic leaves. The healthy plants with effective rhizobia had produced large, mostly elongated, pink or pinkish-white nodules confined mainly to the upper root region, typical of an effective reaction. Healthy plants with ineffective rhizobia had abundant small white ineffective nodules distributed over the entire root, while those with the mixture of effective and ineffective rhizobia also bore many small ineffective type nodules and the few larger nodules produced were confined mainly to the lateral roots. CPV-infected plants, although inoculated with the effective Rhizobium strain, nevertheless bore mainly small round white ineffective-type nodules, although a few were larger and pinkish white.

The analyses of variance showed that the four treatments differed significantly in shoot dry weight ($P = 0{\cdot}01$), and total number of nodules per plant ($P = 0{\cdot}05$) and per g. air-dried root ($P = 0{\cdot}01$). The effect of the four treatments on nodule size distribution was highly significant ($P = 0{\cdot}001$) both per plant and per g. root.

Figure 1 presents the mean data for shoot dry weight and for nodule number and size distribution which confirmed the visual observations. The histograms show a close similarity between the nodules on a per unit weight of root basis, indicating a constancy of root development for all except the CPV-infected plants, and that most of the variation was in the degree of nodulation. However, CPV-infected plants had smaller roots than healthy ones; hence the smaller differential here between the per plant and per unit root weight data. There was a significant correlation between shoot dry weight and the number of large nodules produced ($r = 0{\cdot}66$; $P = 0{\cdot}01$) by either CPV-infected or healthy plants.

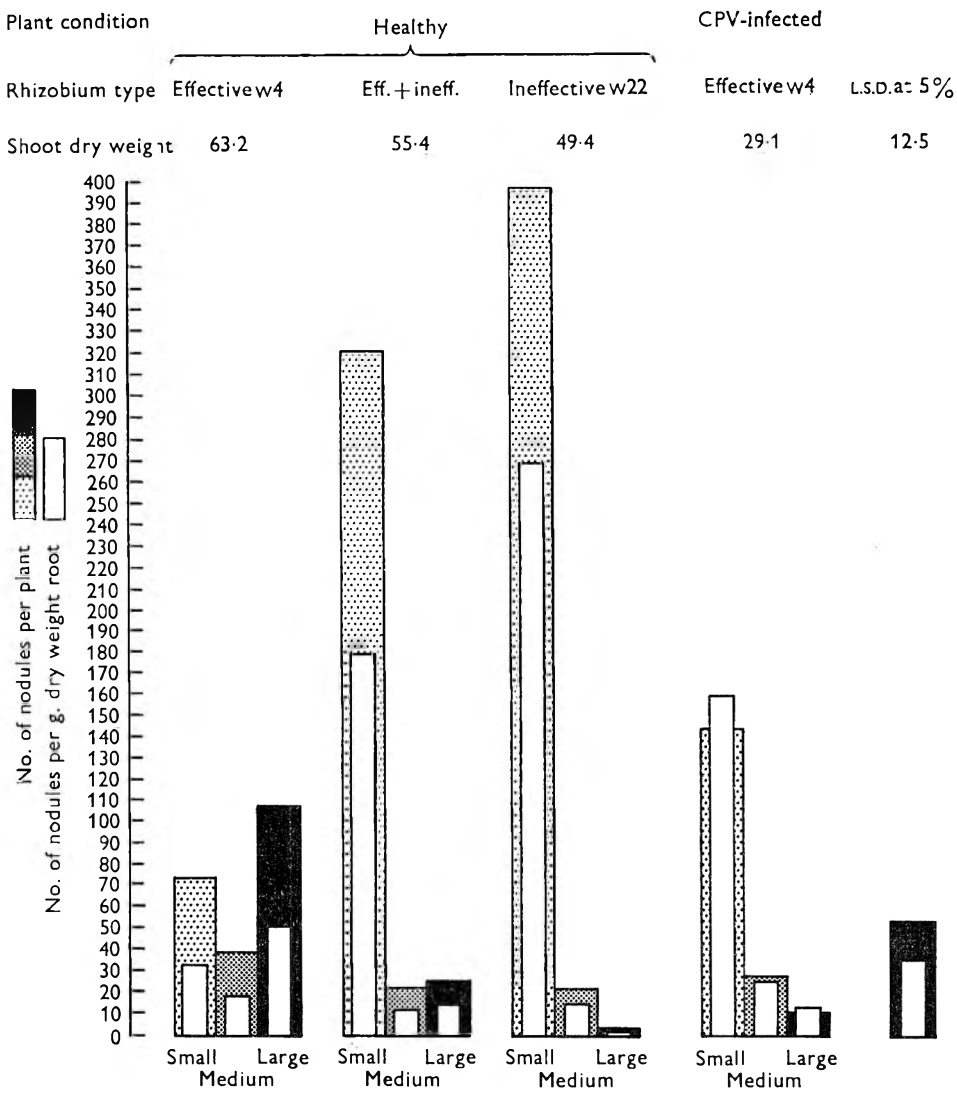

Fig. 1. Mean dry weight and nodulation characteristics of healthy and CPV-infected clones of white clover grown in soil inoculated with effective w 4 and ineffective w 22 strains of *Rhizobium trifolii*.

Growth and nodulation of healthy seedling plants raised in soil from the previous treatments

Plate 1, shows the growth and general vigour of the seedling plants in the four different treatments immediately before harvest. Those raised in boxes previously containing healthy cuttings nodulated by the effective Rhizobium strain were clearly the most vigorous, the darkest green, and had begun to flower; those in the healthy/ineffective boxes were stunted, pale yellowish green, and very few had reached the flowering stage. Seedling plants in the healthy/mixed effective and ineffective boxes

were intermediate between these extremes in growth and colour, although there was considerably more plant-to-plant variation; none of these plants had flowered. Plants in boxes which had previously contained CPV-infected cuttings in combination with the effective Rhizobium strain were little better in either growth or colour than those in the healthy/ineffective boxes and none had reached the flowering stage.

Initial plant condition	Healthy			CPV-infected	
Initial Rhizobium type	Effective w4	Eff. + ineff.	Ineffective w22	Effective w4	L.S.D. at 5%
Percentage germination	84·7	70·9	68·8	22·9	31·3
Shoot dry weight	39·1	22·2	6·3	10·8	11·3

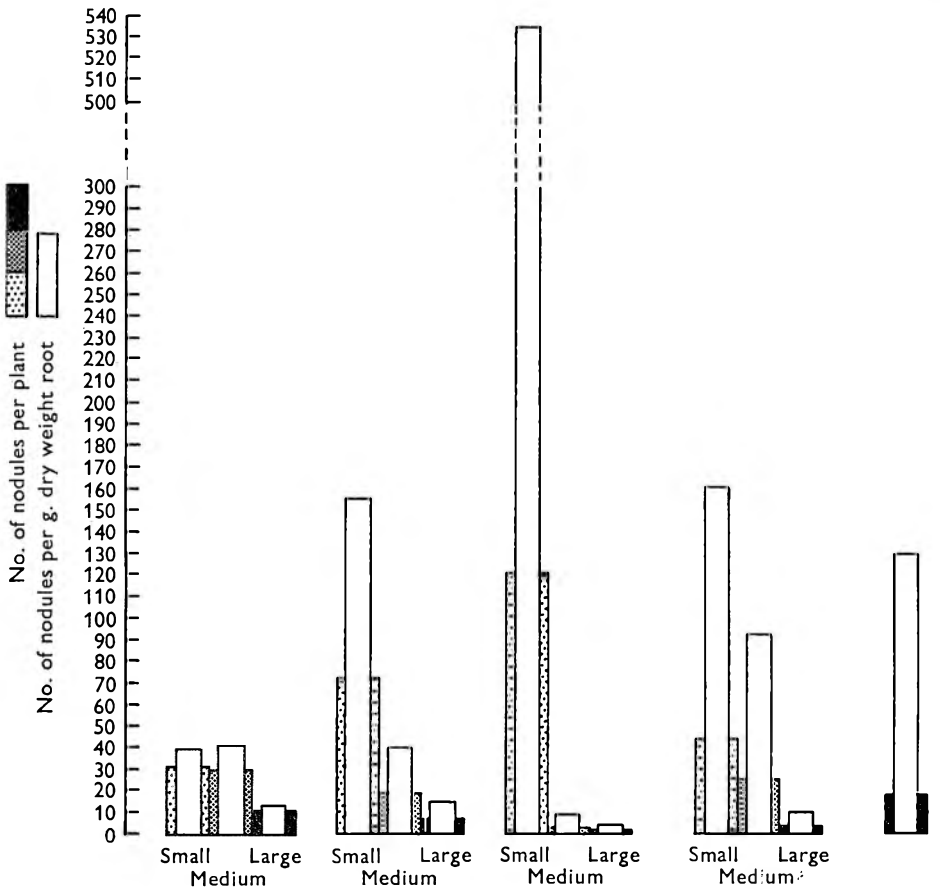

Fig. 2. Mean percentage germination, dry weight and nodulation characteristics of healthy white clover plants reared from seed in soil previously inoculated with effective w 4 and ineffective w 22 strains of *Rhizobium trifolii*, and in which healthy and CPV-infected white clover clones were grown.

The analyses of variance showed that previous treatment had influenced significantly the shoot dry weight ($P = 0{\cdot}01$), the total number of nodules produced/g. air-dried root ($P = 0{\cdot}05$), and the distribution of nodules among the three classes (large,

medium, small) both per plant ($P = 0{\cdot}001$) and per g. root ($P = 0{\cdot}01$). Differences in total number of nodules per plant just failed to reach significance at $P = 0{\cdot}05$. As in the first half of the experiment, the number of large nodules produced was correlated with the shoot dry weight, but with an even higher degree of significance ($r = 0{\cdot}91$; $P = 0{\cdot}001$).

The mean data for shoot dry weight, percentage germination, and number of nodules of different sizes are presented in Fig. 2. The data for shoot dry weight clearly confirm the visual observations. The virus-free seedlings raised in soil that had previously contained CPV-infected cuttings in association with a normally highly effective Rhizobium strain did not yield significantly more than those in soil previously containing healthy cuttings nodulated by an ineffective strain. Figure 2 shows also that the pattern of nodulation was very similar whether calculated on a per plant or a per unit weight of root basis. However, because the seedlings were both smaller and younger than the cuttings harvested in the first half of the experiment, their root systems were smaller; hence the number of nodules per plant was generally less than the number/g. air-dried root. The least differential between the per plant and per unit weight data was evident in the healthy/effective combination, due to the proportionately greater amount of root produced. Even the healthy/effective treatment did not produce the expected frequency of large nodules, because the seedlings were harvested before potentially large nodules had reached their maximum sizes. Despite this, the frequency of small nodules increased significantly from the healthy/effective, to the healthy/mixed effective and ineffective, and healthy/ineffective treatments. However, the CPV-infected/effective plants did not produce significantly more small nodules than the healthy/effective ones. The original analysis of variance did not reveal any significant difference between treatments in the production of large nodules, but a χ^2 test of the ratio of large:medium:small nodules produced per plant showed the distribution to vary significantly between treatments ($P = 0{\cdot}001$). Partitioning χ^2 between individual comparisons showed that, although the ratio of large:medium:small nodules produced by the CPV-infected/effective treatment differed significantly from the ratios for all other treatments, and particularly from the healthy/effective combination, it approached most closely to that of the healthy/mixed effective and ineffective. Thus, the nodulation characteristics of healthy seedling plants grown in soil previously inoculated with an effective Rhizobium strain and planted with CPV-infected cuttings were very similar to those obtaining when the soil was inoculated with a mixture of effective and ineffective strains and planted originally with healthy cuttings.

DISCUSSION

These experiments show that, as in agar culture (Joshi *et al.* 1967), so also in soil, white clover plants infected with clover phyllody virus (CPV) produced mainly small white nodules even though nodulated by an effective strain of *Rhizobium tirfolii* which normally produced many large pigmented nodules. Although CPV-induced ineffectiveness did not lead to an increase in the total number of nodules per plant, as is common when a naturally ineffective Rhizobium strain is involved, this was due entirely to the poor root development of the virus-infected plants; the number of nodules per unit weight of root was greatly in excess of the number developed on healthy plants. An important criterion of effectiveness in terms of plant growth is,

evidently, the number of large nodules produced irrespective of the number of small nodules: even in the first half of the experiment, where available soil nitrogen was undoubtedly high, a reasonably good correlation was obtained between shoot weight and the number of large nodules; and in the second half, where less soil nitrogen would have been available, the correlation was even higher.

Presumably rhizobia from the CPV-infected plants were released into the soil when the nodules decayed, and were then able to compete with the existing Rhizobium population for sites on the host roots. The suggestion is that, in the second half of the experiment, when healthy plants in soil formerly occupied by CPV-infected plants developed mainly small white nodules, the healthy plants were nodulated preferentially by rhizobia which had become modified to ineffectiveness by passage through CPV-infected plants. This was similar to the result obtained when the effective W4 strain and the naturally ineffective W22 strains of *Rhizobium trifolii* competed for nodule sites on the normal virus-free host. If this inference is correct, then it is evident that in soil as in agar culture ability to produce many large nodules is not immediately restored when previously effective rhizobia transfer from CPV-infected plants to healthy ones, and the contention of Vanderveken (1964) that it is restored is not substantiated by these results.

One factor not investigated here, which might complicate the interpretation of these results, is the changing nitrogen status of the soil with the various treatments. Although the same batch of compost was used throughout, and all treatments were presumably of equivalent nitrogen content initially, it is obvious that by the time seeds were sown at the start of the second half of the experiment the available nitrogen concentration might have varied widely between treatments. Current techniques of soil-nitrogen determination are not sufficiently critical to be of great value in this type of experiment, nor would it have been possible, even if they were, to apportion the end-results between the various factors leading to an increase or decrease of soil-nitrogen content.

One unexplained result was the poor germination of seed in soil previously containing CPV-infected plants. It seems unlikely that this was due to a nutritional deficiency. Possibly such plants exude substances inhibitory to germination.

Clearly the hitherto unsuspected effect of CPV on nodulation even of healthy clover plants in the sward, in addition to the more direct action of virus on host, might well be responsible for a considerable and largely non-measurable decrease of yield. The possibility of amelioration, not only through breeding white clover varieties resistant to the virus, but also by selection at the symbiont level, seems worthy of investigation.

We are indebted to Professor P. T. Thomas, Director of the Welsh Plant Breeding Station, for facilities and helpful advice and criticism, to Dr G. ap Griffith for advising against determining soil-nitrogen content, Mrs E. Horzelska for help in preparing the manuscript and Mr H. Richards for the photograph.

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EXPLANATION OF PLATE

Healthy seedling plants of the white clover variety S. 100 immediately before harvest, growing in boxes of soil previously containing: top left, uninfected plants with effective w4 strain of *Rhizobium trifolii*; top right, uninfected with mixed effective w4 rhizobia and ineffective w22 rhizobia; bottom left, uninfected with ineffective w22 rhizobia; bottom right, CPV-infected with effective w4 rhizobia.

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The Sulphur Metabolism of *Pityrosporum ovale* and its Inhibition by Selenium Compounds

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SUMMARY

A medium was developed for the more rapid culture of *Pityrosporum ovale*. [^{35}S]Methionine was utilized preferentially as sole sulphur source from which cysteine/cystine were synthesized. Growth was less on cysteine, negligible on cystine and nil on inorganic sulphite and sulphate. Selenium disulphide, amorphous sulphur, stable colloidal (red) selenium and selenourea were extremely toxic, entering the organism by diffusion. Tellurium and selenomethionine entered the organism, the latter by active transport, but were not toxic for up to 1 week of incubation although synthesis of [^{75}Se]selenomethionine was demonstrated. Selenite and metallic (black) selenium were not toxic and did not enter the organism. Possible mechanisms of toxicity are discussed.

INTRODUCTION

Schultz & McManus (1950) measured the growth of 80 strains of yeast with sulphate, cystine, methionine or glutathione as sole sulphur source in a defined medium and divided the strains into classes: (i) those which utilized methionine and cystine and (ii) those which utilized methionine but not cystine. One strain was found which utilized all the compounds tested except sulphate equally. Margolis & Block (1958) measured the relative growth of one strain from each class on 30 sulphur compounds and found that most of those yeasts which did not utilize cystine did utilize cysteine. Skerman & Singleton (1964) reported another strain of yeast which did not use inorganic sulphate although full growth was obtained with cysteine or methionine, and 85% growth with cystine. This yeast was a strain of *Debaromyces kloeckeri*, an inhabitant of sub-Antarctic soil rich in organic sulphur compounds.

Wood & Perkinson (1952) grew yeasts on $Na_2{}^{35}SO_4$ and found most of the activity in the methionine and cystine of the cell-wall protein, with a small portion in the cysteine as part of the cytoplasmic glutathione. In similar experiments a very small degree of activity was reported also in biotin, thiamin, coenzyme A and *S*-adenosylmethionine (Maw, 1963; Pigg, Spence & Parks, 1962). This paper describes the sulphur metabolism of *Pityrosporum ovale*, a lipophilic yeast which is found on human scalps, usually associated with dandruff (Spoor, Traub & Bell, 1954), and its growth inhibition by certain sulphur and selenium compounds.

METHODS

Growth conditions

Pityrosporum ovale 9265/3, originally isolated in this laboratory from a human scalp, was indistinguishable morphologically and biochemically from type cultures 3073 and 3074 (London School of Hygiene and Tropical Medicine) and 1878, 4163, 5332, 5333 and 5334 (Centraalbureau voor Schimmelcultures, Delft). This organism was maintained on Littman ox-gall agar slopes (Oxoid). Subculture into Littman ox-gall broth at 37° gave luxuriant growth in shake culture. Organisms were washed in Ringer solution and an even suspension prepared in a Jencon homogenizer. The suspension was adjusted to give 50% transmission in an EEL absorptiometer, diluted 100 times and 0·1 ml. added as inoculum to media.

Cultures can be maintained in a defined medium containing a fatty acid, asparagine and oxaloacetate, yielding about 3 mg. dry wt organisms/ml. in 4–6 weeks (Benham, 1941, 1945). Much better growth was obtained in the present work in a medium containing: 2 mg. palmitic acid, 8 ml. 0·0224 M-NH_4Cl, 15 ml. of the salts solution and 2 ml. of the growth factor solution of Schultz & McManus (1950), 0·2 ml. of the trace element solution of Skerman & Singleton (1964), 3 ml. phosphate buffer (pH 5·0; Schultz, McManus & Pomber, 1949), 1 ml. of an aqueous solution of sulphur source (as below); total volume 19·2 ml., pH 5·0. To this was added 1 g. egg-yolk lecithin (95–100% pure, British Drug Houses Ltd.) or 5 ml. of 6% (w/v) aqueous solution of sodium taurocholate (67% pure, Difco Laboratories) which served to emulsify the palmitic acid and to provide other unidentified water-soluble growth factors. Compounds for testing as sole sulphur source were prepared in water, with the addition of HCl when necessary (e.g. for cystine) at a concentration of 1 mg. S/ml. and sterilized by filtration (Postgate, 1963).

For estimation of amounts of growth, organisms were harvested after 3 days in shake culture at 37°, washed three times with 10 ml. 0·9% (w/v) NaCl solution, three times with 10 ml. water and stood twice in 10 ml. chloroform + methanol + conc. HCl (50 + 50 + 1, by vol.) for 1 hr at 25° to remove external lipid. The organisms were then suspended in a minimum of chloroform + methanol (1 + 1, v/v) and dried to constant weight.

Metabolism of ^{35}S- and ^{75}Se-amino acids

[^{35}S]L-cystine.2HCl, [^{35}S]L-cysteine.HCl, [^{35}S]L-methionine, [^{75}Se]L-selenomethionine and $Na_2{}^{35}SO_4$ (carrier free) were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England, and sterilized by filtration except the sulphate which was heated at 100° for 10 min. (Pasternak, 1962). One ml. of a solution containing 30–40 μc of ^{35}S or ^{75}Se compound together with 50 μg. of inactive compound was added to the growth medium as sole sulphur source.

Organisms were harvested after incubation at 37° for 24–48 hr in shake-culture and washed three times with 10 ml. 0·9% (w/v) NaCl, three times with 5 ml. 1% K_2CO_3 to remove adherent fatty acids and three times with 5 ml. water. The removal of all extracellular ^{35}S was checked by counting a portion of each type of pooled washings in an end-window counter. The washed organisms were disintegrated in a Mickle homogenizer, because of their very thick cell walls (Swift & Dunbar, 1965), made to 5·0 ml. and ^{35}S counted in a 0·5 ml. portion of the suspension. For direct comparison of the

uptake of β-[^{35}S]methionine and γ-[^{75}Se]seleno-methionine, 0·5 ml. samples of the suspension were stood at 25° for 12 hr with 0·1 ml. M-hyamine hydroxide in methanol, 10 ml. scintillant added (4 g. BBOT (Ciba) in 500 ml. toluene + 500 ml. methanol) and the activity counted in an IDL liquid-scintillation counter. An internal standard in 0·1 ml. methanol was added and the solution counted again to allow calculation of the efficiencies of counting.

For thin-layer chromatography of incorporated radioactive amino acids, the remaining suspension of washed organisms was oxidized with 1 ml. formic acid + 30 % (w/v) H_2O_2 (9 + 1, v/v) for 1 hr at 25° (Eastoe & Courts, 1963). The reagent was removed by freeze drying, 1 ml. 6 N-HCl added and proteins hydrolysed in vacuum in a sealed tube at 100° for 24 hr (Crestfield, Moore & Stein, 1963). The hydrolysates were freeze-dried, the residue dissolved in a minimum of 10 % (v/v) isopropanol in water and examined by thin-layer chromatography on Silica Gel G (Merck), with *n*-propanol + 34 % (w/v) ammonia (70 + 30, v/v) for one-dimensional chromatography (James & Morris, 1964). For two-dimensional separation *n*-butanol + acetic acid + water (4 + 4 + 1, by vol.) was followed by chloroform + methanol + 17 % (w/v) ammonia (2 + 2 + 1, by vol.). Amino acids were located with ninhydrin and autoradiography was done by using Kodak 0·800 Super-Speed orthochromatic plates for at least 5 days, development being with Ilford PQ Universal Developer and fixing with 'Amfix' (May and Baker Ltd., Dagenham, Essex, England).

Addition of inhibitors. Selenious acid, sodium selenite, DL-seleno-methionine (Calbiochem Ltd.) and seleno-urea were added as aqueous solutions. Selenium disulphide (British Drug Houses Ltd.) was deposited inside the culture flasks from a fresh solution in hexane (10 μg./ml.), the solvent being evaporated off; amorphous sulphur was similarly deposited in culture flasks as a solution of flowers of sulphur in CS_2 (Horsfall, 1956). An attempt was made to deposit red amorphous selenium, which exists in the same puckered rings of 8 atoms as amorphous sulphur, into flasks in a similar manner; but black crystalline 'metallic' selenium (British Drug Houses Ltd.), which exists in regular spirals of atoms, was insufficiently soluble in CS_2, chloroform or ether. Red amorphous selenium was eventually prepared by dissolving selenious acid in water and adding N-$SnCl_2$ solution (115 g. $SnCl_2 2H_2O$ in 170 ml. 12 N-HCl made to 1 l. with water; Treadwell & Hall, 1952). The precipitate was washed with water and suspended in ethanol but, when placed in the growth medium, it rapidly reverted to the black allotrope. Elemental tellurium, which has no allotropic forms and exists as hexagonal rhombohedra isomorphous with rhombohedral sulphur, was added to the medium as an even suspension in benzene.

RESULTS

Maximum growth (6 mg. dry wt organism/19·2 ml. medium) was found with methionine and thiazolidine-4-carboxylic acid as sole sulphur sources. Less growth was found with cysteine (4 mg.), methione sulphoxide (3·5 mg.) *S*-methylcysteine (3 mg.) and cystine (2·5 mg.). Growth was slight with homocysteine (1 mg.) or homocystine (1 mg.). There was no growth with sulphate, sulphite or taurine and in the control without sulphur source. The preference for methionine, cysteine and cystine, in that order, was confirmed by measurement of the uptake of the [^{35}S]amino acids (Table 1). Most amino acids are believed to be taken into the micro-organisms

by active transport although differences in findings with different organisms raises the possibility that more than a single mechanism may be found to operate (Holden, 1962). Autoradiography of cell hydrolysates showed the metabolism of methionine to cysteic acid and of cysteine to methionine sulphoxide. Very little metabolism of incorporated cystine occurred. *Pityrosporum ovale* is thus the third yeast to be recognized as unable to utilize inorganic sulphate. It is similar to the strain of *Debaromyces kloeckeri* from sub-Antarctic soil in that its natural habitat is rich in organic sulphur compounds. *Pityrosporum ovale* is thus in the class of yeasts defined by Schultz & McManus (1950) as able to synthesize cystine from methionine but not the reverse, its ability to utilize cysteine agreeing with the findings of Margolis & Block (1958) that most of the yeasts in this class can do so.

Table 1. *Uptake of* [^{35}S]*-amino acids by different strains of Pityrosporum ovale*

Organism grown in 19·2 ml. standard medium in shake culture at 37° for 3 days (amounts of amino acids expressed as μg. sulphur/culture).

Sole sulphur compound added to medium	Sulphur content of medium at start (μg.)	Strains: 9265/3*	3073†	3974†	1878‡	4162‡
		Sulphur taken up (μg.)				
Methionine	10·0	4·6	3·1	2·6	2·4	1·7
Cysteine	10·0	1·7	1·3	0·6	2·4	1·7
Cystine	2·4	0·2	0·1	0·2	1·1	0·8

* From Unilever Research Laboratory, Isleworth, Middlesex.
† From London School of Hygiene and Tropical Medicine.
‡ From Centraalbureau voor Schimmelcultures, Delft.

Selenium disulphide. Selenium disulphide is a potent fungicide (Nordlander, 1929) and has been incorporated into anti-dandruff shampoos for this reason (Thorne, 1963). The yield of *Pityrosporum ovale* grown with methionine as sole sulphur source and the uptake of [^{35}S]-methionine were very sharply inhibited, i.e. an 'all-or-nothing' effect, when SeS_2 was added to the medium. The concentration of SeS_2 at which this cut-off occurred varied in the region of 10–20 μg. SeS_2/19·2 ml. medium, depending on very slight differences in the number of organisms added in the inocula. Thin-layer chromatography followed by autoradiography showed that the methionine incorporated into the organisms was still being metabolized to cysteine. Just before the sharp cut-off in methionine incorporation, e.g. at 16 μg. SeS_2, there was a gradual increase in the amount of methionine incorporated, but no increase in the yield of organisms. This accumulation of methionine may represent an attempt by the organisms to overcome the toxic effect of the inhibitor. The very few organisms that it was possible to harvest after the sharp cut-off, e.g. at 17 μg. SeS_2, were markedly red in colour.

Selenium disulphide is prepared by fusing sulphur and selenium in the molecular proportions 2 + 1 although these two elements are miscible in all proportions. There is no evidence that any compound with the structure SeS_2 is present in the final product (Matson, 1956); X-ray diffraction patterns show that some SeS is present and the rest of the material is present as solid solutions of elemental selenium and sulphur in the amorphous form. There are some sulphur rings-of-8, some selenium rings-of-8 and

some mixed rings-of-8 of the formula Se_nS_m where $n+m = 8$. These are all stable lipid-soluble forms which are able to penetrate into the organisms (Horsfall, 1956).

Sulphur. Amorphous sulphur in the rings-of-8 form was a very good inhibitor of the growth of *Pityrosporum ovale* although the cut-off in yield was not so sharp and occurred gradually over the range 35–50 μg. S/19·2 ml. medium.

Selenium. Previous work in this laboratory showed that a carefully prepared stable colloid of red selenium (Scandurra, Picani & Cavallini, 1964) strongly inhibited the growth of *Pityrosporum ovale*. The red selenium described here did not inhibit growth at 1000 μg./19·2 ml. medium; this observation is explained by non-penetration into the organisms of the black allotrope which was rapidly produced from the red allotrope in the medium.

Tellurium. Successful penetration of tellurium into *Pityrosporum ovale* was indicated by the black appearance of the organisms but no significant inhibition of growth occurred up to 1000 μg./19·2 ml. medium, confirming that tellurium is much less toxic to fungi than is sulphur (Hochster & Quastel, 1963).

Seleno-urea. Seleno-urea inhibited the growth and uptake of [^{35}S]methionine by *Pityrosporum ovale* in the same manner as selenium disulphide except that the cut-off was not so sharp, starting at about 20 μg./19·2 ml. medium. Inhibited organisms were red, again showing the uptake of selenium. Seleno-urea is lipid-soluble, as are selenium disulphide, amorphous sulphur, red selenium and tellurium, and their mode of penetration into the organism is believed to be identical with that of other fungicides, i.e. simple diffusion through the lipoprotein cytoplasmic membranes, those with the highest lipid-water partition coefficient penetrating fastest (Albert, 1960).

Table 2. *Yields of Pityrosporum ovale and amounts of methionine and seleno-methionine incorporated*

19·2 ml. shake culture of the standard medium with supplements indicated were incubated 1 week at 37°.

Sole sulphur source added to medium in 1·0 ml. solution	μmoles incorporated into cells	Amount of organism (mg. dry wt)
100 μg. DL-methionine + 13·2 μg. [^{35}S]L-methionine	0·309 methionine	8
100 μg. DL-seleno-methionine + 12·5 μg. [^{75}Se]L-seleno-methionine	0·253 seleno-methionine	9
100 μg. DL-methionine + 13·2 μg. [^{35}S]L-methionine + 100 μg. DL-seleno-methionine	0·210 methionine	9
100 μg. DL-methionine + 100 μg. DL-seleno-methionine + 12·5 μg. [^{75}Se]L-seleno-methionine	0·170 seleno-methionine	8

Selenite. Selenite up to 760 μg. SeO_2/19·2 ml. medium did not inhibit the growth of *Pityrosporum ovale* nor the uptake of [^{35}S]methionine, which is consistent with the non-utilization of sulphate and sulphite for growth and the failure to incorporate [^{35}S]sulphate. Sulphite and selenite compete for specific sites on the cell membrane which are believed to form a complex with the substance before its active transport into the cell (Shrift, 1961); it is concluded that *P. ovale* lacks these specific active sites.

Seleno-methionine. Pityrosporum ovale grew satisfactorily on seleno-methionine as

sole 'sulphur' source for 1 week (Table 2), the weight of organisms obtained being the same as for methionine. The amount of seleno-methionine incorporated into the organisms was slightly less than the amount of methionine; the incorporation of either was inhibited by the presence of the other and both were incorporated when present together. Methionine and seleno-methionine are known to behave as a competitive pair in a similar manner to sulphate/selenate and sulphite/selenite (Shrift, 1961). Some organisms have become adapted to growth on seleno-methionine, e.g. *Chlorella vulgaris* (Shrift & Spoul, 1963), when selenium analogues of organic sulphur metabolites are used as substrates by the enzymes of the sulphur metabolites, and altered proteins are formed which function normally, except that organisms grown on seleno-methionine are larger and more dense, because of interference with cell division. A similar phenomenon probably occurred here with *Pityrosporum ovale*; cell division probably eventually ceased when sufficient cell-wall protein contained seleno-cystine instead of cystine. The effects of the inhibitors tested are summarized in Table 3.

Table 3. *Effect of inhibitors on growth and metabolism of Pityrosporum ovale*

Compound	Effect on yield of organism	Effect on uptake of [^{35}S]methionine	Mode of uptake into organism	Mode of inhibition
Selenium disulphide	All-or-nothing effect	All-or-nothing effect	Diffusion	Direct action on SH groups of cell walls, sudden failure of cell division
Stable colloidal red selenium	All-or-nothing effect	Not measured	Diffusion	
Seleno-urea	Sharp inhibition	Sharp inhibition	Diffusion	
Amorphous sulphur	Inhibition	Not measured	Diffusion	
Unstable red selenium	None	None; reverts to black allotrope	None	Not toxic
Black selenium	None	None	None	
Tellurium	None	Not measured	Diffusion	
Seleno-methionine	None	Reduced	Active transport	Accumulation of seleno-cystine in cell wall and eventual failure of cell division
Selenite	None	None	None*	Both mechanisms when taken up by cell

* Known to be by active transport in those organisms which incorporate selenite.

DISCUSSION

Accumulation of red amorphous selenium granules inside fungi (Rosenfeld & Breath, 1962) and bacteria (Weiss, Ayres & Kraft, 1965) growing on selenium compounds is a well-known phenomenon. For selenite the suggested mechanism is a spontaneous reaction with, for example, 32 molecules cysteine to form seleno-dicystine which decomposes to 16 molecules cystine and red Se_8. For S_8 (and Se_8) which penetrate into the cell intact, Owens (1960) suggested ring fission to form H_2S and an S_7 free radical which could undergo many possible reactions, e.g. combination with 2 SH groups forming a sulphur bridge of the nature R—S—$(S)_x$—S—R.

Sulphydryl groups in the cell walls are important in cell division in yeasts (Nickerson & van Rij, 1949), protein disulphide reductase of the mitochondria controlling a local change of disulphide bonds to SH groups at the position of budding, so allowing the cytoplasm to 'balloon' out from the cell (Nickerson & Falcone, 1954). Organic mercurial fungicides combine with the SH groups of the cell, especially of the cytoplasmic membrane, and an all-or-nothing effect results with the sudden failure of the semi-permeable membrane (Passow, Rothstein & Clarkson, 1961). Failure of organisms to divide is another typical example of the operation of all-or-nothing effects, which are known to occur when a group of ligands acting in unison are essential for a particular cell function.

The fungitoxic effect of selenium disulphide, stable colloidal red selenium, amorphous sulphur and seleno-urea on *Pityrosporum ovale* is probably exerted by irreversibly changing the free SH groups of the cell wall into polysulphide bonds by the production of bridges, thus preventing cell division suddenly when enough such unions have been made. The eventual toxicity of seleno-methionine would be due to the production of seleno-cystine in the cell-wall proteins, which, when present in sufficient quantity, will stop cell division suddenly. Selenite appears to have a dual toxic function (in those organisms which can utilise sulphite), some selenium going through the normal pathway of sulphur metabolism to seleno-cystine and some forming red selenium and uniting SH groups.

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Cultural Characters and Serological Relationships of Some Mycoplasmas Isolated from Bovine Sources

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SUMMARY

Cultural characteristics and serological relationships of mycoplasmas from the bovine vagina, mastitic milk, and other sources were studied. Parasitic strains could be distinguished from saprophytic strains in that they required incorporation of horse serum into the PPLO agar for growth, produced surface crystallization on PPLO agar and did not ferment carbohydrates. All strains were haemolytic for red blood cells of the guinea pig, rabbit, sheep, cow and horse. Satellite growth enhancement was seen only with the strains associated with mastitis.

Rabbit antisera to each mycoplasma were tested against homologous and heterologous strains for precipitins and for growth inhibitory antibodies. Four serotypes were distinguished among the bovine isolates by agar-gel double-diffusion technique. Growth inhibitory antibodies were detected in homologous antisera against only four of the strains studied.

INTRODUCTION

Recent reports from England of bovine mastitis due to mycoplasmas (Davidson & Stuart, 1960; Stuart *et al.* 1963; Bruford *et al.* 1965), from the United States (Hale, Helmboldt, Plastridge & Stula, 1962; Carmichael *et al.* 1963; Fincher, 1964; Stern & Harris, 1964; Jasper, Jain & Brazil, 1966) and from Israel (Bar-Moshe, 1964) have provided a detailed description of the disease, but only limited descriptions of serological, morphological and biochemical characteristics of the agents involved. The recognition of mycoplasma as a causative agent of bovine mastitis, arthritis, respiratory and genital infections has stimulated frequent culturing of milk and other body fluids or tissues for mycoplasma. The resultant frequency of recovery of mycoplasma from clinical or normal conditions in cattle requires a determination of distinguishing characteristics by which the organism can, at least in part, be identified and classified. In the present work some of the cultural, morphological, biochemical and immunological characteristics of certain mycoplasmas from bovine mastitic milk, the reproductive tract, and other sources were determined in a search for reliable criteria for classification of mastitis pathogens and other isolates from bovines.

METHODS

Mycoplasma strains. The mycoplasmas used in this study include seven isolates from California (Table 1). Four of these isolates (01, 1042, 1182, 1336) were from milk from cows with mastitis in the reported epizootic (Jasper *et al.* 1966); the isolate 1063

was from a bulk tank milk sample from another location (Jasper *et al.* 1966); and the two vaginal isolates (1367, 1370) were from a dairy herd with an infertility problem. These isolates were compared with four bovine, a human and a saprophytic strain obtained from other investigators.

Table 1. *Source of Mycoplasma strains*

Mycoplasma	Recovery site	Source
01, 1042, 1182, 1336	Bovine mastitic milk	Isolated in our laboratory (Jasper *et al.* 1966)
1063	Bulk tank milk	Jasper *et al.* (1966)
1367, 1370	Bovine vagina	Recently isolated in our laboratory
Strain '56R'	Bovine mastitic milk	Dr L. E. Carmichael, Cornell Univ., Ithaca, N.Y.
Strain 'WIDANKA F'	Bovine mastitic milk	Dr L. E. Carmichael
Connecticut strain	Bovine mastitic milk	Dr R. S. Hirth, Univ. Connecticut, Storrs, Conn.
M. bovigenitalium strain PG-11	Bovine vagina	Dr J. G. Tully, NIH, Bethesda, Md.
M. pharyngis strain PATT	Normal human	Dr W. A. Clyde, Jr., Univ. North Carolina, Chapel Hill, N.C.
M. laidlawii	Chicken sinus	Dr H. E. Adler, Univ. California, Davis, California

Basic cultural procedures. Agar plates used for surface inoculations contained 20 ml. Bacto PPLO agar (Difco) supplemented with 1 % (w/v) yeast autolysate and 15 % (v/v) horse serum. Agar plate cultures were routinely incubated at 37° in an atmosphere containing approximately 10 % (v/v) CO_2+90 % (v/v) air in a moist chamber, and were read after 3 days. Stock cultures of mycoplasmas (broth cultures prepared from an isolated colony of a mycoplasma grown on PPLO agar) were maintained in tubes containing a slope of 2 ml. of PPLO agar made with 5 % (v/v) horse blood overlaid with 5 ml. standard broth medium (SBM). The SBM consisted of Bacto PPLO broth (Difco) containing 0·5 g. glucose, 1 g. yeast autolysate, 20 ml. horse serum, and 100,000 units penicillin G (potassium) per 100 ml., adjusted to pH 7·8. Serial transfers of stock cultures were usually made after storage at 4° for 3–4 weeks, but occasionally at shorter intervals. Transfers for the various experiments were made after 3–5 days growth in SBM. Broth cultures were incubated in air.

Colonial and cellular morphology. Gross examination of growth on PPLO agar plates and in broth medium was made daily for 3 days. Cellular morphology was studied by light microscopy on Wright–Leishman-stained (Jasper *et al.* 1966) coverslip smears prepared from sediments obtained from SBM cultures centrifuged at 12,000*g* for 30 min. Wet preparations were also examined under light and phase-contrast microscopes

Morphological characteristics of isolated colonies on agar plates were usually recorded after 3 days at 37°, but were sometimes supplemented by further observations after a week at room temperature. Unstained colonies and colonies stained with Dienes stain (Madoff, 1959) were examined under a microscope at × 100 magnification.

Differences in growth of mycoplasma on plates incubated in air and in air containing 10 % (v/v) CO_2 were recorded.

Haemolysis. Haemolytic activity of mycoplasma for washed red blood cells of the cow, sheep, horse, rabbit and guinea pig was determined on PPLO agar medium (Tully, 1965).

Serum requirement. Plates prepared from PPLO agar containing horse serum in 0 2·5, 5, 10 and 15 % (v/v) concentrations were streaked with a 3-day growth of mycoplasma in SBM. Growth was recorded after 3 days at 37°, and, depending on the amount of the growth, arbitrary values of 1+, 2+, 3+ or 4+ were assigned.

Satellite growth. Two-tenths ml. of a 3-day broth culture of a mycoplasma was evenly spread with a glass-rod on duplicate PPLO agar plates until dry. Then a narrow streak of a 24 hr culture of a coagulase-positive, $\alpha\beta$ haemolytic staphylococcus was made across the plate. One set of the plates was incubated in air, and the other in air + 10 % (v/v) CO_2 in a moist chamber at 37°. After 3 days the plates were examined grossly and under a dissecting microscope for enhancement of growth near the staphylococcal streak (Morton, Smith & Leberman, 1949; Dinter, Danielsson & Bakos, 1965).

Biochemical reactions. The basal medium used for sugar fermentations consisted of PPLO broth containing 1 % (v/v) ethanolic phenol red (1 %, w/v, solution) as an indicator. Test sugars were added in 0·5 % (w/v) final concentration, adjusted to pH 7·6, and the medium sterilized by autoclaving. After cooling to 52°, the medium was supplemented with 20 ml. sterile heated horse serum (56° for 30 min.) and 100,000 units of penicillin G (potassium)/100 ml., and dispensed in tubes in 5 ml. amounts. Before inoculations for sugar fermentation studies, at least three serial transfers of each mycoplasma were made in SBM containing heated horse serum, but without yeast autolysate and glucose. The difficulty experienced in adapting some of the organisms to grow without yeast autolysate was overcome by further serial subculturing in this medium at 3-day intervals. Each set of sugar tubes received 0·2 ml. broth culture of each mycoplasma, and change in colour and pH value (Beckman pH Meter, Model 72, Beckman Instrument Co., Palo Alto, Calif.) was recorded after incubation at 37° for 7 days. Growth of organisms in sugar tubes was confirmed by plating a loopful of medium from the glucose tube after 3 days and 7 days of incubation. Two sets of uninoculated control sugar tubes were included in each series.

Gelatin liquefaction was tested in tubes containing 10 ml. of SMB and a small sterile piece (15 × 6 × 2 mm.) of gelatin + charcoal gel (Kohn, 1953). The tubes were inoculated with 0·2 ml. of a mycoplasma culture, and were read along with control tubes after 7 days at 37°.

Catalase production was determined on PPLO agar medium (O'Berry, Bryner & Frank, 1966). Methylene-blue reduction was tested in SBM and the time of complete decolorization of the medium recorded (Tully, 1965).

Serological procedures. Growth-inhibitory antibodies were tested by a modified filter-paper disc method (Clyde, 1964). A sterile 6 mm. diam. Bacto concentration disc (sterile blank) (Difco) was soaked in the test serum, lifted, gently touched to the edge of the tube to drain the hanging drop, and placed on an agar plate which had been evenly spread with 0·1 ml. of a 3-day growth of mycoplasma. The zone of growth inhibition was measured after incubation for 3 days at 37°.

The mycoplasma antigens (for use in gel-diffusion precipitation tests and immunization of rabbits) and immune rabbit sera were prepared essentially according to the techniques used for porcine mycoplasmas (Dinter *et al.* 1965). Five hundred ml. of PPLO broth were inoculated with 10 ml. of a 3-day growth of a mycoplasma and

incubated at 37° for 3 days. The growth was harvested by centrifuging the culture for 30 min. at 12,000*g* in a refrigerated centrifuge. The sediment was washed 3 times with sterile buffered isotonic saline, resuspended in 5 ml. of sterile distilled water, distributed in 0·5 ml. amounts in small screw-capped tubes, and stored in a solid CO_2 chest at approximately −20°. This suspension of mycoplasma in distilled water (concentrated antigen) was frozen and thawed 5 times before use as an antigen in gel diffusion tests.

For the production of immune rabbit sera, 1 ml. of the concentrated antigen was diluted to 5 ml. with sterile distilled water. Four ml. of this diluted antigen was mixed thoroughly with an equal volume of Freund complete adjuvant (Difco). Two ml. of this adjuvant vaccine was given intramuscularly into each leg of a rabbit. After 5 weeks, 1 ml. of the diluted antigen (stored at −20°) without adjuvant was injected intravenously. Rabbits were bled a week after the intravenous injection, and collected sera were stored frozen in small amounts without added preservative.

Rabbit sera were absorbed with lyophilized PPLO broth containing 20% (v/v) horse serum by thoroughly mixing 200 mg. of the dry powder into 1 ml. immune serum. The absorption was continued for 2 days at 4°, and sera cleared by centrifugation at 1000*g* for 10 min. were used in the gel-diffusion test.

A microtechnique was adopted for absorption of antisera with homologous and heterologous mycoplasma antigens in order to conserve antigen. Small quantities, about 0·01–0·02 ml., of broth-absorbed antiserum and concentrated antigen, in ratios of 1:1 and 1:2, were transferred by separate Pasteur pipettes into small screw-capped tubes. Absorption proceeded at 4° for 2 days. The antiserum+antigen mixture was then used as an antiserum, and was tested against the different antigens by gel diffusion. The wells were filled 2 or 3 times with antiserum+antigen mixture, depending upon the dilution factor of the absorbed serum.

Ouchterlony's double-diffusion precipitation test was performed in Special Agar–Noble (Difco) gel on glass slides (LKB Operation Manual 1-6800A-E 11, LKB 6800A Immunodiffusion Equipment, LKB Produkter AB, Stockholm 12, Sweden). The reactants were allowed to diffuse at room temperature for 2–5 days, and sometimes at 4° for 2 days and the precipitation patterns recorded. The number and density of precipitation lines formed were found to vary somewhat with the length of time allowed for diffusion. Sharper lines were obtained at 4°. Photographs were taken without staining.

RESULTS

Cultural characters

A 3-day growth of mycoplasma in PPLO broth was easily recognized by a uniform diffuse turbidity of the medium, a thin fragile pellicle and a slight fine sediment. When horse blood agar slopes overlaid with PPLO broth were used, a change in colour of the medium from pale yellow to light green occurred. After storage at 4° for 2–3 weeks, clearing of the slopes, starting from top to bottom, was observed with the vaginal isolates (1367, 1370), but not with the others. These strains and *Mycoplasma bovigenitalium* did not grow as well in broth as did the other strains. A 3-day broth culture of *M. bovigenitalium* contained about 10^6 colony-forming units/ml. as compared to 10^8–10^9 for the mastitis strain 01.

Incorporation of horse serum in PPLO agar was essential for growth of all strains except *Mycoplasma laidlawii* which grew well, and strain 1063 which grew poorly,

without horse serum. Growth was better on medium containing 10–15% (v/v) horse serum than with 2·5–5·0% (v/v) horse serum. Growth on agar was always better in air + 10% (v/v) CO_2 than in air alone. The amounts of growth in air varied between strains and sometimes within strains.

Colonial and cellular morphology

Examination of wet preparations by phase-contrast microscopy and of Wright–Leishman-stained coverglass smears prepared from SBM culture sediment revealed myriads of pleomorphic organisms consisting of tiny spherules, ring forms approximately 1 μ diam., cocco-bacillary rods, and short smooth or beaded filaments. These were indistinguishable from those seen in mastitic udder secretions (Jasper *et al.* 1966).

Two colony forms were observed on agar plates for each strain of mycoplasma studied: the larger typical colonies (0·3 mm. average diam.) having a distinct raised centre and a thin peripheral zone, and the smaller granular colonies (0·02 mm. average diam.) without a peripheral zone. However, in the case of strain 1063 and *Mycoplasma laidlawii*, only the granular colonies were seen after 3 days of incubation, the larger colonies appearing after an additional week of incubation at room temperature. Surface 'crystallization' occurred near mycoplasma colonies of all strains examined except with strain 1063 and *M. laidlawii*. It was more prominent and could be recognized grossly on relatively freshly poured PPLO plates or in the presence of heavy growth.

Haemolysis

All of the mycoplasmas were haemolytic for the red blood cells of each species tested. The most extensive haemolysis occurred with rabbit and guinea-pig red cells, in which case the margins of haemolytic zones were hazy. Clear haemolysis with sharp borders occurred with sheep, cow and horse red cells, sheep cells giving the sharpest reactions.

Satellite growth

Growth enhancement due to the presence of staphylococcal colonies was not observed on plates incubated in air + 10% (v/v) CO_2 but was quite evident on plates incubated in air alone. Satellite growth enhancement was seen only with the strains associated with mastitis, but not with strains 1063, 1367, 1370, *Mycoplasma bovigenitalium*, *M. pharyngis* or *M. laidlawii*. Mycoplasma colonies in the vicinity of the staphylococcal colonies were more numerous and distinctly larger (2–4 times) than the more distant colonies. Consequently, this effect was usually easily visible grossly.

Biochemical reactions

Change in colour from red to yellowish orange (phenol-red indicator) was not observed in most of the sugar fermentation tubes. Therefore a decrease in pH value by 1·0 unit or more from that of the control tubes was considered to be evidence of carbohydrate fermentation. *Mycoplasma laidlawii* fermented starch, glycogen, dextrin, maltose, galactose, fructose and glucose, and strain 1063 fermented starch, dextrin, glycogen, maltose and glucose. All the other mycoplasma strains were regarded as non-fermenters. Gelatin was liquefied after 14 days only by strains 1367, 1370, *Mycoplasma bovigenitalium*, and *M. pharyngis*. All strains were catalase-negative. Tests for methylene-blue reduction were variable and not considered to be reliable.

Serological relationship

Growth-inhibitory antibodies were detected in homologous antisera only to *Mycoplasma pharyngis*, *M. bovigenitalium*, strain '56R' and strain 1367 (Table 2). Growth inhibition was evident only against homologous strains and no cross-inhibition was observed.

Precipitating antibodies were detected in all homologous immune rabbit sera (Table 2). For comparative purposes, gel-diffusion patterns were arranged so that each of the PPLO broth-absorbed homologous antisera in a central cup was tested against all the mycoplasma antigens. These homologous and heterologous precipitation studies resulted in precipitin patterns which allowed some degree of antigenic grouping (Table 2). For the first six mycoplasmas listed in Table 2, each antiserum usually formed 2–3 precipitation lines against homologous and heterologous antigens within the group (Fig. 1). At least one distinct dense band and two light bands were common to these six mycoplasmas as shown by the pattern of fusion. Cross reactions with the other strains were not seen.

Table 2. *Antigenic relationship of various mycoplasma studied by gel-diffusion precipitation test*

Mycoplasma antigen	No. of precipitation lines with rabbit antisera to												
	California 01	California 1042	California 1182	California 1336	N.Y. 'WIDANKA F'	Connecticut	N.Y. '56 R'	Tank milk 1063	*M. laidlawii*	California 1367	California 1370	*M. bovigenitalium* strain PG-11	*M. pharyngis* strain PATT
California 01	2–4	2–3	2–3	2–3	3	1–2	0	0	0	0	0	0	0
California 1042	1–2	1–3	1–3	2–3	1–3	1–2	0	0	0	0	0	0	0
California 1182	2	2–3	2–3	2–3	2	1–2	0	0	0	0	0	0	0
California 1336	2–3	2–3	3	3	1–2	1–2	0	0	0	0	0	0	0
N.Y. 'WIDANKA F'	2	2–3	2	2	2–3	2	0	0	0	0	0	0	0
Connecticut	2–4	2–3	2	2–3	2–3	2	0	0	0	0	0	0	0
N.Y. '56 R'	0	0	0	0	0	0	2–3*	0	0	0	0	0	0
Tank milk 1063	0	0	0	0	0	0	0	1	1	0	0	0	0
M. laidlawii	0	0	0	0	0	0	0	0	2	0	0	0	0
California 1367	0	0	0	0	0	0	0	0	0	2*	2	2	0
California 1370	0	0	0	0	0	0	0	0	0	2	3	3	0
M. bovigenitalium strain PG-11	0	0	0	0	0	0	0	0	0	2	3	3*	0
M. pharyngis strain PATT	0	0	0	0	0	0	0	0	0	0	0	0	1*

* Growth-inhibiting antibody present.

The presence of common antigens was also evident in the group composed of vaginal strains 1367, 1370, and *Mycoplasma bovigenitalium* (Table 2). Similar precipitation lines were obtained with antisera to *M. bovigenitalium* and strain 1370 (Fig. 2–4). These two mycoplasmas shared one diffuse broad band and two distinct narrow bands, whereas strain 1367 lacked the antigen causing the diffuse broad band. Antiserum to 1367 formed two distinct lines with homologous antigen and with strain

1370, but only one distinct line with *M. bovigenitalium*. Cross-reactions with isolates from milk and with other strains were not observed.

Antiserum to strain 1063, an isolate from bulk tank milk, formed a precipitation band with homologous antigen, and did not cross-react with *Mycoplasma laidlawii* (Table 2). However, antiserum to *M. laidlawii* produced two broad bands of precipitation with the homologous antigen and shared a faint thin precipitation line with strain 1063. Antisera to strain '56R' and *M. pharyngis* formed a distinct precipitation line only against homologous antigens, and did not cross-react (Table 2).

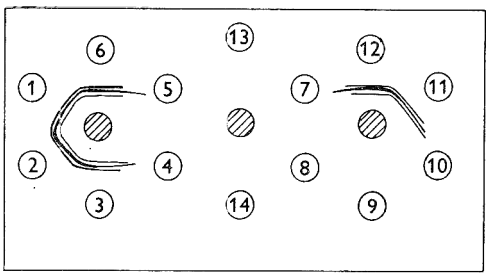

Fig. 1

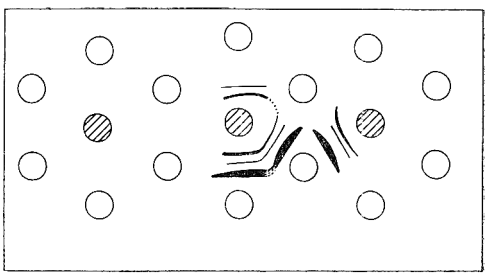

Fig. 2

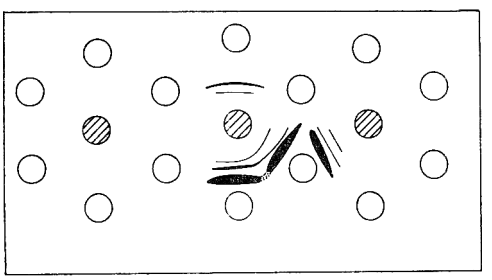

Fig. 3

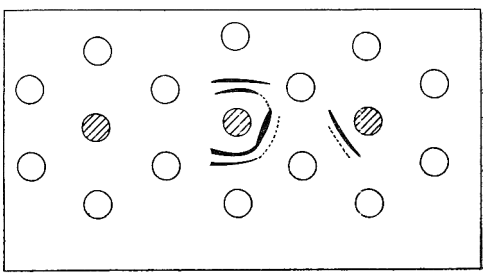

Fig. 4

Figs. 1, 2, 3, 4. Gel-diffusion precipitation patterns drawn from photographs taken after 40 hr at 4°. Broken lines represent faint precipitation lines. The origin of each strain is indicated in Table 1. The outer wells were filled with the mycoplasma antigens arranged in the same numerical order in each case as is indicated on Fig. 1 and as follows: Well 1, strain 1042; 2, strain 1182; 3, strain 1336; 4, strain 1063; 5, *M. laidlawii*; 6, strain 01; 7, sediment from uninoculated PPLO broth (control); 8, *M. bovigenitalium* strain PG-11; 9, *M. pharyngis* strain PATT; 10, N.Y. strain '56R'; 11, N.Y. strain 'WIDANKA F'; 12, Connecticut strain; 13, strain 1367; 14, strain 1370. The centre wells received different rabbit immune sera as follows: Fig. 1, antiserum to strain 1042; Fig. 2, antiserum to *M. bovigenitalium* strain PG-11; Fig. 3, antiserum to strain 1370; Fig. 4, antiserum to strain 1367.

To substantiate these serological relationships among the various mycoplasmas, the antisera were absorbed with selected mycoplasma antigens and again tested for homologous and heterologous precipitins. This study, although limited, showed the following. (1) All antisera absorbed with homologous antigens no longer formed precipitation lines with their respective antigens. (2) In the case of the first 6 mycoplasmas listed in Table 2: (*a*) precipitation lines were no longer formed against the heterologous antigens after absorption with homologous antigen; (*b*) absorption of each of these six antisera with strain 01 antigen removed precipitins for all homologous and heterologous antigens; (*c*) cross-absorption of each of these six antisera with other antigens, namely,

strain '56R', strain 1063, *Mycoplasma laidlawii* and *M. bovigenitalium* did not remove precipitins. (3) Cross-absorption of antiserum to strain '56R' with strain 01 and *M. bovigenitalium* antigens did not remove precipitins. (4) Homologous absorption of antiserum to *M. laidlawii*, removed cross-reacting antibody for strain 1063. (5) Homologous absorption of antisera to vaginal strains 1367 and 1370, and to *M. bovigenitalium* removed precipitating antibodies against both the heterologous and homologous strains. (6) Cross-absorption of antisera to vaginal isolates 1367 and 1370 and to *M. bovigenitalium* with strain 01 and '56R' antigens did not remove precipitins. Thus the mycoplasmas isolated in our laboratory and bovine strains from other sources appeared to fall into 4 serotypes (Table 3).

Table 3. *Serotypes of bovine mycoplasma strains based on precipitation patterns obtained by gel diffusion test*

Serotype	Mycoplasma strains
1	01, 1042, 1182, 1336, 'WIDANKA F', 'Connecticut strain'
2	'56 R'
3	1367, 1370, *M. bovigenitalium* strain PG-11
4	1063

DISCUSSION

Although cultural, morphological and biochemical characteristics of mycoplasmas are not species-specific, a broad grouping into parasitic and saprophytic organisms was apparent on the basis of sugar fermentation and horse serum requirements (Freundt, 1958). The requirement of horse serum for growth on PPLO agar and the inability to ferment carbohydrates indicated that all our mycoplasma isolates associated with mastitis and with the vagina were parasitic. Recent experiments with the milk strain 01 and the vaginal strain 1370 have shown that both are pathogenic when injected into the bovine udder.

Strain 1063, an isolate from bulk milk, shared some cultural and biochemical characteristics common to saprophytic *Mycoplasma laidlawii*. These include limited carbohydrate fermenting capacity, growth (although poor) on PPLO agar without serum, small granular colonies after 3 days growth and no surface crystal formation. Gel-diffusion studies with immune sera against other mycoplasmas differentiated strain 1063 from those causing mastitis, but revealed an antigen possessed by *M. laidlawii*. Upon injection into one quarter of an experimental cow, strain 1063 did not cause mastitis or establish infection. It is therefore probable that this strain entered the bulk tank milk from outside contamination rather than from udder infection, although mastitis-causing strains have been repeatedly demonstrated in the bulk tank milk of an infected herd (Jasper *et al.* 1966).

Satellite growth enhancement of mycoplasmas by a staphylococcus was seen only aerobically. Growth was enhanced for all mycoplasmas associated with mastitis whereas the vaginal, human and saprophytic strains studied were not influenced. This interaction between the staphylococcus and mycoplasma is poorly explored and needs further investigation.

Poor growth of vaginal isolates in PPLO broth as compared to that of other strains suggests a need for some additional growth factors or better cultural conditions. One other isolate from a bovine oviduct was lost during subculture, apparently because of

inadequate growth conditions. Deoxyribonucleic acid was found essential for primary isolation of *Mycoplasma bovigenitalium* (Edward, 1954) and might prove to be helpful if routinely incorporated as a growth supplement in PPLO medium. Some of our recent experiments indicate that addition of Bacto supplement B (Difco) to PPLO broth markedly enhanced growth of vaginal mycoplasmas as well as of other strains.

Growth inhibition by specific immune serum was found to be reliable for identification and differentiation of human mycoplasmas because of the low order, or absence, of cross-reactivity as seen in complement-fixation tests (Clyde, 1964). Growth-inhibitory antisera against many human (Clyde, 1964), porcine (Dinter *et al.* 1965) and bovine strains (Carmichael *et al.* 1963) have been produced successfully. We did not obtain growth-inhibitory antisera against the mycoplasmas studied with the exception of *Mycoplasma bovigenitalium*, *M. pharyngis*, strain '56R' and strain 1367; however, precipitating antibodies were detected in all antisera. Others have obtained growth-inhibitory antisera against *M. bovigenitalium*, strain '56R', strain 'WIDANKA F' and the Connecticut strain by giving graded doses of antigens twice weekly over a period of 2–4 months (Carmichael *et al.* 1963). These variations in developing growth-inhibitory antisera against different strains of mycoplasmas are not fully understood at present, but may depend upon the techniques and duration of immunization, and the time of harvesting serum during the course of immunization. Production of growth-inhibitory antibodies is generally known to be difficult (Morton, 1966).

Unabsorbed immune rabbit sera gave faint precipitation lines when tested against PPLO broth as an antigen, indicating development of some non-specific precipitating antibodies to ingredients of the culture medium. These lines were no longer evident after the absorption of sera with lyophilized PPLO broth. Therefore, the precipitin patterns obtained with absorbed homologous and heterologous antisera tested against various mycoplasma antigens were considered specific, and not associated with the PPLO broth medium.

On the basis of the gel-diffusion patterns obtained with absorbed homologous and heterologous antisera, and the cross-absorption studies, the mycoplasmas isolated in our laboratory and the bovine strains from other sources could be placed in four serotypes (Table 3). The findings that mycoplasmas isolated from mastitic milk in California are related to the Connecticut strain and to a New York strain (strain 'WIDANKA F'), and that the strain '56R' and *Mycoplasma bovigenitalium* are distinct serotypes, confirmed earlier observations with growth-inhibiting antisera (Carmichael *et al.* 1963). Our classification of these strains of bovine origin into four distinct serotypes has since been confirmed by Dr R. H. Leach (personal communication).

With the exception of strain 56R, isolated from a single case of mastitis in New York (Carmichael *et al.* 1963) all studies reported on isolates from mastitis in the United States have indicated that a single and distinct strain is involved. The nomenclature previously suggested, *Mycoplasma agalactiae* var. *bovis* (Hale *et al.* 1962) does not appear to be appropriate since no close relationship to *Mycoplasma agalactiae* has been demonstrated. We therefore suggest that the term '*Mycoplasma bovimastitidis*' be considered for this serotype.

Although some variation existed in the number of precipitation lines obtained with homologous and heterologous antisera to six mycoplasmas of sero-type 1, at least 2–3 antigenic components common to all six strains were usually recognized. With a more sensitive diffusion technique, such as immunoelectrophoresis and starch-gel electro-

phoresis, it might be possible to find and resolve some minor antigenic components specific to a particular strain or common to different strains.

The isolation of two vaginal mycoplasmas (strain 1367, 1370) having antigenic components common to *Mycoplasma bovigenitalium* was unexpected. *M. pharyngis*, an organism commonly cultured from the normal human oropharynx (Clyde, 1964), was antigenically unrelated to the bovine mycoplasmas tested. However, the spread of human mycoplasmas to cattle is an interesting possibility and may prove of significance if some of the human mycoplasmas are found to be carried by or pathogenic to cattle.

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Microbial Oxidation of Hydrocarbons. Oxidation of *p*-Isopropyltoluene by a *Pseudomonas* sp.

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SUMMARY

Whole organisms and soluble extracts of a *Pseudomonas* sp. were examined for their ability to oxidize suspected intermediates in the oxidation of *p*-isopropyltoluene. A comparison of the relative activities of glucose-grown and *p*-isopropyltoluene-grown bacteria and their extracts indicated that *p*-isopropylbenzyl alcohol, *p*-isopropylbenzaldehyde and *p*-isopropylbenzoic acid are intermediates in the oxidation of the hydrocarbon. *p*-Isopropylbenzoic acid was formed during growth on *p*-isopropyltoluene and this was also shown to be a product of the oxidation of *p*-isopropyltoluene, *p*-isopropylbenzyl alcohol and *p*-isopropylbenzaldehyde by soluble extracts prepared from *p*-isopropyltoluene-grown bacteria. The enzymes which catalyze the oxidation of *p*-isopropyltoluene were repressed by glucose and induced by growth on either *p*-isopropyltoluene or *p*-isopropylbenzoic acid.

INTRODUCTION

In 1957 Dagley & Patel described the oxidation of *p*-hydroxytoluene by a *Pseudomonas* sp. and indicated that the route of degradation proceeded via *p*-hydroxylbenzyl alcohol to the aldehyde and then *p*-hydroxybenzoic acid. Such an attack on the methyl group of an aromatic compound was also suggested by the work of Davis & Raymond (1961). The Nocardia described in their report was incapable of growth on *p*-isopropyltoluene but could convert this compound to *p*-isopropylbenzoic acid while growing at the expense of hexadecane. Yamada, Horiguchi & Takahashi (1965) described the formation of *p*-isopropylbenzoic acid from *p*-isopropyltoluene by several strains of Pseudomonas. The pathway involved in the oxidation of *p*-isopropyltoluene has never been verified by either induction studies or the use of cell-free extracts, although from the work mentioned it is probable that the methyl group serves as the initial point of enzymic attack. The work reported here was initiated to gain further insight into the mechanism of *p*-isopropyltoluene oxidation by microorganisms.

METHODS

Chemicals. Terpene-free *p*-isopropyltoluene was purchased from the Matheson Company (East Rutherford, N.J.). *p*-Isopropyl alcohol, *p*-isopropylbenzaldehyde and *p*-isopropylbenzoic acid were purchased from the Eastman Kodak Company (Rochester, N.Y.). *p*-Isopropylbenzaldehyde was further purified by formation of its bisulfite addition compound which was hydrolysed with dilute base immediately

prior to its use. *p*-Isopropyltoluene and *p*-isopropylbenzyl alcohol were washed with dilute base prior to their use. Co-factors and 2,3,5-triphenyltetrazolium chloride were purchased from the Sigma Chemical Company (St Louis, Mo.)

Identification of organism. The organism used in this study was a Gram-negative, polarly flagellated rod. It formed a green water-soluble pigment during growth on *p*-isopropyltoluene or glucose. The organism did not grow on glucose or *p*-isopropyltoluene in the absence of oxygen. In accordance with these criteria it was identified as a member of the genus *Pseudomonas*.

Growth of organism. The pseudomonad was grown in a mineral-salts medium, sterilized by autoclaving for 15 min. at 15 lb./in.^{2}, of the following composition (g./l.): $(NH_4)_2SO_4$, 2·0; $MgSO_4.7H_2O$, 0·2; Na_2HPO_4, 3·0; KH_2PO_4, 2·0; $CaCl_2$, 0·01; Na_2CO_3, 0·1; $FeSO_4.7H_2O$, 0·005; $MnSO_4$, 0·002. A seed inoculum for a 3 l. fermenter was prepared by inoculating a 250 ml. Erlenmeyer flask containing 50 ml. mineral salts medium with a loopful (0·01 ml.) of organisms which had been grown and maintained on solidified medium. *p*-Isopropyltoluene (0·2 ml., 174 mg.) was added directly to the flask. The hydrocarbons were sterilized by filtration. The flask was shaken at 30° for 48 hr to yield a population with an extinction of 4·0 when measured at 400 mμ. All extinction determinations in this study were made with a Spectronic 20 colorimeter (Bausch and Lomb, Rochester, N.Y.). Twenty ml. of this seed inoculum was added to 3 l. of mineral-salts medium contained in a 5 l. fermenter. This was incubated at 30° in a New Brunswick fermenter (Model FS-305, New Brunswick Scientific Co., New Brunswick, N.J.) and was continuously agitated by means of an immersed propeller which revolved at a speed of 700 rev./min. Air was bubbled through the culture at a rate of 400 ml./min. *p*-Isopropyltoluene (10·0 ml., 8·6 g.) was added to the culture with the inoculum. After 24 hr the culture was harvested by centrifugation at 6000*g* for 10 min. at 3°. The bacteria were washed with cold distilled water and resuspended to a concentration of 1 g. wet weight bacteria in 4 ml. water. The yield varied from 6 to 12 g. wet wt organisms/l. medium.

Preparation of extracts. Cell-free extracts were prepared by ultrasonic disintegration (20 kc./sec. for 5 min. at 14°) of suspensions of organisms (1·0 g. wet wt/4·0 ml. water) in a Branson Sonifer Model S-75 (Heat Systems Co., Long Island, N.Y.). The extract was clarified by centrifugation for 30 min. at 34,000*g* at 3°.

Chromatography. The solvent systems utilized for thin-layer chromatography were: A, butanol + ethanol + ammonia (8 + 1 + 3 by vol.); B, butanol + acetic acid + water (6 + 2 + 2 by vol.). Thin-layer chromatograms were sprayed with 0·03 % (w/v) aqueous solution of methyl red buffered with 0·05 M-tris (pH 7·2) or with ammoniacal silver nitrate. Pre-coated sheets of silica gel were used for identification of the enzyme-produced intermediates. The sheets were purchased from Distillation Products Industries (Rochester, N.Y.)

Gas-chromatographic analyses were done: A, with a 10 ft. × 5 mm. internal diameter stainless steel tube packed with 30 % (w/v) silicone rubber coated on Diatoport-S-60-80 mesh; B, with a 6 ft. × 5 mm. internal diameter stainless steel tube packed with 6 % (w/v) diethylene glycol succinate (Lac 728) on Chromosorb W, 80 mesh. The helium flow rate was 30 ml./min. and the temperature was maintained at 250°. A thermal conductivity detector system was employed. Acids were detected as their methyl esters. All columns and support materials were purchased from the F and M Scientific Corp., Avondale, Pa.

Assay methods. Glucose was assayed with glucose oxidase and peroxidase by the method of Bergmeyer & Bernt (1963). The enzymes were purchased from Calbiochem (Los Angeles, Calif.).

The amount of *p*-isopropylbenzoic acid formed during growth on *p*-isopropyltoluene was determined as follows. Samples were withdrawn from the culture medium and centrifuged to remove the bacteria. The clarified supernatant fluids (1·0 ml.) were acidified with 0·1 ml. 10 N-H_2SO_4 which resulted in the formation of a white precipitate of *p*-isopropylbenzoic acid. A clarified culture of the same density of organisms grown with glucose as the sole carbon source remained clear upon the addition of an equal amount of acid. Since no other product accumulated during growth on *p*-isopropyltoluene, the opacity, measured at 400 mμ of, the clatrified supernatant fluid which occurred after the addition of acid was used as a measure of the amount of *p*-isopropylbenzoic acid formed. The quantity of acid formed was determined by comparison with a standard solution and is expressed as μg./mg. dry wt organisms. The solvent used for the standard solution was the mineral salts medium previously described.

Protein was determined by the method of Itzhaki & Gill (1964).

The dye 2,3,5-triphenyltetrazolium chloride was used as an electron acceptor in all enzyme assays unless otherwise noted. The reduction of the dye was nil in the absence of either enzyme or cell material or in the presence of boiled extract incubated with substrate. The extinction coefficient of the coloured formazan at 500 mμ remained constant from 0 to 55% absorbancy and proceeded linearly with respect to both increasing time and enzyme or cell concentration. The absorbance $\div$ 3·64 = number of μmoles of substrate oxidized. The assay system contained in 1·0 ml.: tris buffer (pH 8·0), 33 μmoles; washed bacteria; substrate, 33 μmoles; and 2,3,5-triphenyltetrazolium chloride, 1 μmole. Tubes were incubated for 20 min. at 25° and the reaction interrupted by the addition of 3·0 ml. of 95% ethanol. The solutions were clarified by centrifugation and the extinction of the coloured formazan formed was determined at 500 mμ. This assay was used to obtain the results shown in Tables 1–5.

For the induction studies in which *p*-isopropyltoluene was the inducer (Table 1) organisms were grown for 18 hr in the mineral-salts medium supplemented with a limiting concentration of glucose (0·1%). Glucose (0·1%) plus *p*-isopropyltoluene (0·1%) were then added to the culture. The culture was shaken at 30° and subsequent changes in turbidity were followed together with the ability of washed cells, suspended in water, to oxidize *p*-isopropyltoluene, *p*-isopropylbenzyl alcohol and *p*-isopropylbenzaldehyde and with the appearance of *p*-isopropylbenzoic acid in the medium. The first sample (2 hr) was taken before glucose exhaustion (corresponding to 2 hr in Fig. 1).

For the study in which the oxidative capacities of *p*-isopropyltoluene-grown and *p*-isopropylbenzoate-grown bacteria are described (Table 2), the organisms were harvested after growth (18 hr) on the carbon source indicated (0·2%), resuspended in distilled water and assayed for their oxidative capacities using tetrazolium chloride as an electron acceptor. Tubes were incubated as previously described at 25° for 10 min.

Organisms grown on the surface of solidified mineral medium (0·2% agar) for 17 hr in the presence of possible inducers (Table 3) were suspended in distilled water and assayed for their oxidative capacities as previously described. Solid compounds

(0·2%) were incorporated in the medium. When liquid hydrocarbons or their derivatives were used, the cultures were incubated in atmospheres saturated with their vapours.

For the experiment in which the product formed by incubating *p*-isopropyltoluene with the cell-free extract was characterized, the reaction mixture contained: tris buffer (pH 8·0), 330 μmoles; substrate, 1·0 m-mole; ethanol, 200 μmoles; cysteine, 200 μmoles; extract containing 50 mg. protein and distilled water. Total volume, 20 ml. The reaction was incubated in air for 18 hr, acidified and extracted as described in Methods.

For the experiment in which the product formed by incubating *p*-isopropylbenzyl alcohol and *p*-isopropylbenzaldehyde with the cell-free extract was characterized, the reaction mixture contained: tris buffer (pH 8·0), 330 μmoles; substrate, 1·0 m-mole; NAD, 20 mg.; extract containing 50 mg. of protein and distilled water. Total volume, 20 ml. The mixtures were incubated under N_2 for 3 hr, acidified and extracted as previously described.

RESULTS

Studies with whole organisms

During growth of the organism on *p*-isopropyltoluene, a compound was formed which could be precipitated from the clarified culture by adding mineral acid to pH 1·0. This compound was isolated and identified as *p*-isopropylbenzoic acid, as follows. The suspension was extracted with an equal volume of diethyl ether and the ethereal solution was re-extracted with aqueous N-NaOH. Ether extraction was repeated after acidification of the aqueous solution. The compound was recovered from this ethereal solution by evaporation to dryness, the residue was then dissolved in ethanol and crystallized by adding water. The white crystalline product melted at 116–117° and did not depress the melting point of authentic *p*-isopropylbenzoic acid. Chloroform solutions of the unknown compound and of *p*-isopropylbenzoic acid had identical i.r. spectra. The compound and its methyl ester were chromatographically indistinguishable from *p*-isopropylbenzoic acid and its methyl ester by thin-layer and vapour-phase chromatographic techniques, respectively.

The formation of *p*-isopropylbenzoic acid from *p*-isopropyltoluene suggested that *p*-isopropylbenzyl alcohol and *p*-isopropylbenzaldehyde may be intermediates in a pathway analogous to that proposed by Dagley & Patel (1957) for the oxidation of *p*-hydroxytoluene. To investigate this possibility the organism was examined for simultaneous induction of the capacity to oxidize *p*-isopropyltoluene and the suspected intermediates. Bacteria growing in the presence of glucose plus *p*-isopropyltoluene exhibited a lag, corresponding to the exhaustion of glucose, after which growth was resumed (Fig. 1). Organisms sampled before the lag did not oxidize *p*-isopropylbenzaldehyde, *p*-isopropylbenzyl alcohol or *p*-isopropyltoluene (Table 1). After the resumption of growth the organisms oxidized the three substrates, and the increase in the specific activities of the enzymes was accompanied by the accumulation of *p*-isopropylbenzoic acid in the culture medium (Table 1). In addition, non-induced bacteria (time 2 hr) and induced bacteria (time 8 hr) were assayed manometrically for their ability to oxidize *p*-isopropyltoluene and the suspected aldehyde and alcohol derivatives (Fig. 2). These results indicate that the enzymes which were required for the oxidation of *p*-isopropyltoluene to *p*-isopropylbenzoic acid were simultaneously

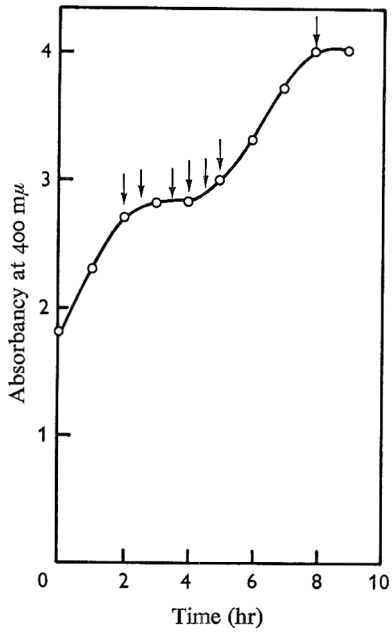

Fig. 1

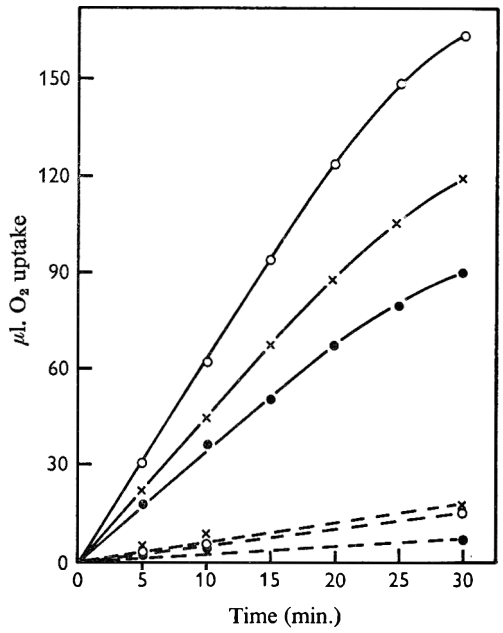

Fig. 2

Fig. 1. Growth of *Pseudomonas* sp. on a medium containing glucose plus *p*-isopropyltoluene. Glucose (0·1%) and *p*-isopropyltoluene (0·1%) were added at zero time to a culture that had been grown for 18 hr in the presence of glucose (0·1%). After 3 hr glucose was no longer detected in the medium. Samples (0·5 ml.) were removed at the points marked with arrows (see Table 1).

Fig. 2. Oxidation of *p*-isopropyltoluene (●), *p*-isopropylbenzyl alcohol (×) and *p*-isopropylbenzaldehyde (○) by glucose-grown (---) and *p*-isopropyltoluene-grown (—) bacteria. The organisms, both non-induced (time 2 hr, Table 1) and induced (time 8 hr, Table 1), were harvested and resuspended in distilled water. Oxygen uptake was measured manometrically in the presence of 20% (w/v) KOH; the incubation mixtures contained (2·0 ml. final volume): substrate, 33 μmoles; phosphate buffer pH 8·0, 100 μmoles; organisms, 0·5 mg.; and distilled water.

Table 1. *Induction of enzymic activities by p-isopropyltoluene*

Samples were removed during the incubation shown in Fig. 1 and tested for oxidative ability and *p*-isopropyl benzoic acid content. Reaction mixtures were as described in the Methods section.

Time (hr)	Substrate oxidation (μmole × 100/mg. organism/hr)			*p*-Isopropylbenzoic acid formed (μg./mg. of organisms)
	p-Isopropyl-toluene	*p*-Isopropyl-benzyl alcohol	*p*-Isopropyl-benzaldehyde	
2·0	0·0	0·0	0·0	0
2·5	0·0	21·0	11·9	64
3·5	8·1	66·5	23·8	142
4·0	14·3	76·5	57·2	156
4·5	11·9	81·0	95·0	102
5·0	8·6	67·0	66·6	49
8·0	8·6	57·0	62·0	44

induced by *p*-isopropyltoluene or a product of its oxidation, and that the enzymes were also repressed by glucose.

Neither *p*-isopropylbenzaldehyde nor *p*-isopropylbenzyl alcohol were effective either as inducers or as growth substrates when added to a liquid culture of organisms grown with a limiting concentration of glucose. However *p*-isopropylbenzoate proved to be a utilizable carbon source and its effect on the induction of the three enzymes was examined (Table 2). Bacteria grown in the presence of *p*-isopropylbenzoic acid were capable of oxidizing the three suspected intermediates at a rate which was slightly in excess of that of bacteria grown on *p*-isopropyltoluene. This observation suggests that *p*-isopropylbenzoic acid or its oxidation product induced the formation of the enzymes for the three reactions examined.

Table 2. *Oxidative capacities of p-isopropyltoluene-grown and p-isopropylbenzoate-grown organisms*

Reaction mixtures were as described in the Methods section.

	Oxidation of substrates (μmole × 100/mg. organisms/hr) by organisms grown on	
Substrate	*p*-Isopropyltoluene	*p*-Isopropylbenzoate
p-Isopropyltoluene	8·6	10·9
p-Isopropylbenzyl alcohol	81·6	114·0
p-Isopropylbenzaldehyde	43·5	50·0

Table 3. *Ability of various aromatic compounds to function as either inducers or growth substances*

Organisms were suspended to equal densities and assayed for their ability to oxidize *p*-isopropyltoluene as described in the Methods section.

Carbon source	Growth (17 hr)	Ability of organisms to oxidize *p*-isopropyl-toluene
p-Isopropyltoluene	+	+
p-Isoprpyolbenzyl alcohol	+	+
p-Isopropropylbenzaldehyde	+	+
p-Isopropylcatechol	+	−
Toluene	+	−
Benzaldehyde	+	−
Benzene	+	−
o-Cresol	−	−
m-Cresol	+	−
p-Cresol	−	−
Catechol	−	−
Isopropylbenzene	+	−

Since both *p*-isopropylbenzaldehyde and *p*-isopropylbenzyl alcohol were apparently toxic when added directly to the mineral salts medium, their effectiveness as inducers was re-examined by using the mineral medium solidified by 2 % (w/v) agar as culture medium and with the aromatic derivative furnished in the vapour phase. Other related aromatic compounds were tested for their ability to function as carbon sources or inducers (Table 3). Of all the compounds assayed, only *p*-isopropyltoluene, *p*-iso-

propylbenzaldehyde and *p*-isopropylbenzyl alcohol supported the growth of the organism and induced the formation of the enzymes necessary for the oxidation of *p*-isopropyltoluene.

Studies with cell-free preparations

The results with whole organisms suggested that the oxidation of *p*-isopropyltoluene proceeded by the oxidation of the methyl group via the alcohol to the aldehyde and then to the acid derivative. Additional evidence favouring this pathway was obtained with cell-free extracts prepared from glucose-grown and from *p*-isopropyltoluene-grown bacteria (Table 4). Extracts prepared from *p*-isopropyltoluene-grown bacteria oxidized *p*-isopropylbenzyl alcohol and *p*-isopropylbenzaldehyde but not *p*-isopropyltoluene. Extracts of glucose-grown organisms did not oxidize any of the substrates. The addition of NAD, NADP, or the reduced derivatives did not stimulate the oxidation of *p*-isopropyltoluene by either extract. Extracts prepared in the presence of 0·1 M mercaptoethanol oxidized *p*-isopropyltoluene. The mercaptoethanol was equally effective when added after extraction and could be replaced by the addition of cysteine plus ethanol to the reaction mixture (Table 5). Under the same conditions, extracts prepared from glucose-grown cells were completely inactive.

Table 4. *Oxidative capacities of extracts of glucose-grown and p-isopropyltoluene-grown bacteria*

Reaction mixture was as described in the Methods section.

	Oxidation of substrates (μmole × 100/mg. protein/hr) by extracts of organisms grown on	
Substrate	Glucose	*p*-Isopropyltoluene
p-Isopropyltoluene	0·00	0·00
p-Isopropylbenzyl alcohol	0·00	61·0
p-Isopropylbenzaldehyde	0·00	178·0

Table 5. *The effect of cysteine and ethyl alcohol on the oxidation of p-isopropyltoluene by extracts of p-isopropyltoluene-grown and glucose-grown bacteria*

Reaction mixture as described in the Methods section

	Oxidation of *p*-isopropyltoluene (μmole × 100/mg. protein/hr) by extracts of organisms grown on	
Additions	Glucose	*p*-Isopropyltoluene
None	0·00	0·00
Ethyl alcohol	0·00	0·95
Cysteine	0·00	3·8
Ethyl alcohol + cysteine	0·00	12·4

p-Isopropylbenzoic acid formation

After incubation for 3·5 hr in the supplemented reaction mixture, the formation of *p*-isopropylbenzoic acid from *p*-isopropyltoluene by cell-free extracts could not be

detected. However, after incubation for 18 hr an ultraviolet-absorbing acidic product was detected chromatographically. This acid was tentatively identified as *p*-isopropylbenzoic acid on the basis of its R_F value in the two solvent systems cited (solvent A, R_F value 0·66; solvent B, R_F value 0·96). A second acid was also detected chromatographically in the ethereal extract. It had a lower R_F value (solvent A, R_F value 0·09) and did not absorb in the ultraviolet region when observed under a lamp emitting ultraviolet radiation at 253 mμ; this may represent a product of ring fission, but further identification has not been attempted. A supplemented reaction mixture containing boiled extract and substrate did not accumulate any detectable products. Because of the prolonged incubation period (18 hr) an additional control was used to eliminate the possibility that the reaction mixture had supported the growth of bacteria which were themselves responsible for the formation of *p*-isopropylbenzoic acid. A supplemented reaction mixture was incubated for 18 hr with *p*-isopropyltoluene. The mixture was then centrifuged for 15 min. at 34,000*g* and the precipitated material resuspended in a solution containing tris buffer, ethanol, cysteine and *p*-isopropyltoluene. This mixture was incubated for 18 hr and then examined for *p*-isopropylbenzoic acid. No product was detected in this control reaction mixture. This observation substantiates the contention that despite the prolonged incubation period the formation of *p*-isopropylbenzoic acid from *p*-isopropyltoluene was catalyzed by the soluble extract.

When reaction mixtures, supplemented with NAD, and containing crude extract prepared from *p*-isopropyltoluene-grown bacteria were incubated with *p*-isopropylbenzyl alcohol or with *p*-isopropylbenzaldehyde for 3 hr under N_2. The formation of *p*-isopropylbenzoic acid was detected. Oxygen was excluded in order to eliminate autoxidation of the substrates and NAD was added because preliminary experiments indicated that this nucleotide cofactor was required for the oxidation of *p*-isopropylbenzaldehyde and of *p*-isopropylbenzyl alcohol. The acidic product was isolated from the reaction mixtures and identified chromatographically (solvent A, R_F, value 0·66; solvent B, R_F value 0·96; column A, retention time of methyl ester 4·5; column B, retention time of methyl ester 4·1). Reaction mixtures of boiled extract and either substrate did not contain any detectable reaction products after the period of incubation.

DISCUSSION

The pathway of *p*-isopropyltoluene degradation as suggested by these studies proceeds via the formation of *p*-isopropylbenzyl alcohol to *p*-isopropylbenzaldehyde and ultimately to *p*-isopropylbenzoic acid. By analogy to other aromatic systems (Evans, 1963) it is probable that *p*-isopropylbenzoic acid is hydroxylated before ring cleavage. Such products of ring hydroxylation are at the moment unknown, since they have not been shown to accumulate in the presence of either growing bacteria or cell-free extracts in the system studied.

The oxidation of *p*-isopropyltoluene by cell-free extracts is apparently dependent on the presence of both cysteine and ethanol although their precise role is at the present unknown. The possibility that the ethanol functions as a reductant for a cofactor appears unlikely, since neither reduced NAD nor NADP could be used to replace the alcohol. It is possible that the ethanol is in some way involved in the interaction between the water soluble enzyme and the water insoluble substrate. The whole organism pre-

sumably has a mechanism for bringing such an interaction about; a mechanism which is destroyed when the organism is ruptured. This explanation is at the moment under investigation.

The toxicity of *p*-isopropylbenzaldehyde and *p*-isopropylbenzyle alcohol is presumed to be a reflexion of the level of the unoxidized intermediate when added to the liquid medium. Supplying the intermediate in the vapour phase reduces this level to a minimum and permits growth to occur. Presumably the same result could be achieved by the slow, continuous addition of the hydrocarbon to the liquid medium, keeping the compound below a toxic level.

The observation of diauxic growth exhibited by bacteria grown in the presence of glucose plus *p*-isopropyltoluene and the comparative enzyme studies involving extracts prepared from bacteria grown on the respective carbon sources illustrates repression, by glucose, of the enzymes involved in the degradation of *p*-isopropyltoluene. It appears that these enzymes, presumably *p*-isopropyltoluene oxidase, *p*-isopropylbenzyl alcohol dehydrogenase and *p*-isopropylbenzaldehyde dehydrogenase, are inducible; but the compound responsible for induction has not been established. The fact that bacteria grown on *p*-isopropylbenzoic acid contain the enzymes necessary for the degradation of *p*-isopropyltoluene would implicate the acid as either an inducer or a source of the inducer. Moreover, a comparison of the formation of the acid during the metabolism of *p*-isopropyltoluene with the appearance and increase in specific activities of the three enzymes would suggest that the acid in addition to the hydrocarbon induces the formation of the first three catabolic enzymes. The control of mandelate degradation, involving both repression and induction, bears some similarities to the control of *p*-isopropyltoluene degradation; Hegeman (1966) showed that benzoyl formate induced the formation of the three early enzymes in the mandelate pathway. However, by using mutants blocked in the early reactions, he also showed that the D- and L-isomers of mandelate were equipotent inducers. Whether or not *p*-isopropyltoluene is itself as potent an inducer as *p*-isopropylbenzoic acid of the corresponding pathway remains to be decided. The advantage of induction by an intermediate of the early enzymes in each respective pathway is perhaps more easily understood in light of the nature of the substrates in the *p*-isopropyltoluene pathway. Because the starting compound is water-insoluble, its concentration at the site of induction may never be high enough to elicit the maximal degree of enzyme formation. *p*-Isopropylbenzoic acid, as its salt, does not suffer from this limitation and so an organism with an inductive apparatus capable of responding to the acid would have a selective advantage over one deficient in that ability. An extension of this explanation to include the mandelate and other pathways which are susceptible to such a control mechanism would then be a matter of evolution, proceeding naturally from the highly reduced hydrocarbon to the more oxidized substrates as carbon sources, but maintaining the initial, once selective, advantage of 'back induction'.

I am grateful to Mr F. X. Ryan for his excellent technical assistance.

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Studies on the Regulation of Extracellular Enzyme Formation by *Bacillus subtilis*

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SUMMARY

The progress of secretion of the extracellular enzymes ribonuclease, α-amylase and proteinase by *Bacillus subtilis*, growing in a defined and in a complex medium, containing maltose, starch, glycerol or glucose as carbon source, was followed. In either medium and in the presence of any of the carbon sources the general characteristics of secretion of all three enzymes were the same. There was a low but definite production of exoenzyme from the moment the cells started to grow until the end of the logarithmic phase after which, when the rate of increase in cell mass was decreased, the rate of enzyme secretion increased to a high linear value which was maintained even into the stationary phase.

The results are discussed in relation to a possible regulatory mechanism which might account for the observed characteristics of extracellular enzyme secretion. A mechanism is proposed whereby exoenzyme m-RNA formation and hence enzyme production is limited during growth by a limitation of nucleic acid precursors caused by the depletion of the precursor pool during rapid ribosomal RNA synthesis. When the growth rate is decreased, ribosome synthesis is also decreased; the nucleic acid precursor pool may then increase in size, thereby removing the limitation so that exoenzyme m-RNA and protein may be formed at the maximum rate.

INTRODUCTION

No detailed study has been made of the mechanism which controls the production of extracellular enzymes by *Bacillus subtilis*. There are, however, several reports describing the characteristics of formation of the most important of these enzymes. Thus α-amylase (Fukumoto, Yamamoto, Tsuru & Ichikawa, 1957; Coleman & Elliott, 1962) and ribonuclease (Nishimura & Nomura, 1959; Coleman & Elliott, 1964) have been shown to lag behind cell growth, maximum accumulation of these enzymes occurring after the end of the logarithmic phase. Nomura, Maruo & Akabori (1956), also studying α-amylase formation, reported a distinct separation of several hours between the end of the logarithmic growth of *B. subtilis*, during which no enzyme secretion was observed, and the phase during which enzyme was secreted in significant amounts. They concluded that α-amylase formation occurs only in old cells in which normal cellular multiplication no longer occurs. Fukumoto *et al.* (1957) studied the progress of proteinase secretion and reported that the production of this enzyme reached its peak before either maximum cell growth or α-amylase formation. Coleman & Grant (1966) studied the characteristics of α-amylase formation by *B. subtilis* in a defined medium containing different carbon sources. They showed that, irrespective

of the carbon source used, α-amylase was formed throughout the growth cycle, although the rate of production during the logarithmic phase was considerably less than after logarithmic growth has ceased. The present communication deals with an extension of the work on α-amylase reported by Coleman & Grant (1966) to ribonuclease and proteinase. The characteristics of formation of all three extracellular enzymes were studied in both defined and complex media, in the presence of different carbon sources. The results are discussed in relation to a possible regulatory mechanism which might account for the observed characteristics.

METHODS

Organism. The strain of *Bacillus subtilis* was supplied by Dr L. A. Underkofler (Takamine Laboratories Inc., Clifton, New Jersey, U.S.A.).

Media. The defined medium was as described by Coleman & Grant (1966). The complex medium was obtained by replacing the amino acids and vitamins of the defined medium with 0·5% casein hydrolysate (British Drug Houses Ltd)+0·05% yeast extract (Difco). The defined and the complex medium were both supplemented with either starch, maltose, glycerol or glucose at a concentration of 1%, as carbon source. Both media were adjusted to pH 7·2 before autoclaving and before the addition of carbon source, in the usual manner. However, at the beginning of the experimental period the pH of the complete medium was 6·9 in every case.

Growth conditions. The organism was grown as described by Coleman & Grant (1966).

Analytical methods. Culture tubes were taken at 2 hr intervals over a period of 14 hr, beginning early in the logarithmic phase. Each culture was centrifuged at 5000*g* for 5 min. and the supernatant fraction removed, this was retained and samples taken for enzyme assay. The dry weight of the bacterial pellet was determined (Coleman & Elliott, 1962) from which the bacterial concentration was calculated as dry wt bacteria/ml. It should be noted that in expressing bacterial concentrations in terms of dry wt bacteria/ml. the assumption is made that all the organisms are viable although there is no unequivocal proof of this. The reason why this assumption is necessary is that under the conditions of the experiments the bacteria readily form clumps, particularly in the post-logarithmic phase, which prevents the determination of true viable counts. However, counting individual aggregates of 5–20 organisms in a Petroff–Hausser counting chamber gives a figure which corresponds very closely to the 'viable count' of the preparation. Microscopic examination, both light and electron, together with heat-sensitivity tests showed that during the time course of these experiments the bacteria did not reach the stage of sporulation. Thus, as closely as can be determined, there is no evidence to suggest that all the bacteria in the preparations are not viable.

α-Amylase assays were done as described by Coleman & Elliott (1962).

Ribonuclease was assayed as described by Coleman & Elliott (1965).

Proteinase was assayed by what was essentially the method of Charney & Tomarelli (1947). The standard procedure was as follows: 1·0 ml. samples of suitably diluted enzyme (supernatant fluid) preparations contained in test tubes (1·5 cm. × 12·5 cm.) were equilibrated at 25°. To each tube was added 1·0 ml. azocasein solution (pH 8·3), also equilibrated at 25°, and the resulting reaction mixtures incubated at 25° for 15 min. The reaction was stopped by the addition of 8 ml. 5% (w/v) trichloroacetic

acid and the precipitated protein (substrate) was filtered off by using Whatman no. 54. filter papers. Five-ml. samples of the supernatant fractions were taken and 0·5 ml. 10 N-NaOH added to each to develop the colour of the 'proteinase-solubilized' material. Extinctions were measured at 440 mμ in a 1 cm. cuvette with a Unicam SP 500 spectrophotometer. All values were corrected for a zero time blank of $E_{440\,m\mu}$ approx. 0·040. The method was standardized with 'Novo' crystalline *Bacillus subtilis* proteinase; 1 μg. enzyme in the assay system produced a $\Delta E_{440\,m\mu}$ of 0·0025.

To bring this enzyme in line with α-amylase and ribonuclease—that is, so that a unit of enzyme activity in each case represents the same amount of protein—the unit of proteinase activity was defined as the amount of enzyme which, under the standard conditions of the assay procedure, produced an increase in extinction at 440 mμ of 0·0005. The assay gave a linear relationship between enzyme concentration and extinction up to $\Delta E_{440\,m\mu}$ of 0·200.

RESULTS

Characteristics of extracellular enzyme formation in a defined medium

The progress of bacterial growth and of proteinase, α-amylase and ribonuclease secretion in a defined medium with 1 % maltose as the carbon source is shown in Fig. 1. The organism grew logarithmically until a concentration equiv. of 0·3 mg. dry wt. bacteria/ml. was reached. During the logarithmic phase a low rate of secretion of all three exoenzymes was observed. The logarithmic phase was followed by a long lag during which the bacterial concentration increased about threefold. Concomitant with the decrease in growth rate the rate of extracellular enzyme synthesis increased in each case, reaching a maximum which was maintained during the remainder of the experimental period. The units of enzyme activity are related to the same amount of enzyme protein in each case, so, whilst at the end of the experimental period the amount of ribonuclease and α-amylase protein was about 1 % and 25 %, respectively, of the amount of proteinase-protein, all three enzymes were nevertheless formed parallel to each other. This is illustrated in Fig. 2, where the results plotted in Fig. 1 are expressed as percentages of the values reached at the end of the experimental period.

These same characteristics—namely, logarithmic growth to a bacterial concentration equiv. 0·3 mg. dry wt bacteria/ml., followed by a long lag, together with a low rate of exoenzyme secretion in the logarithmic phase, followed by an increase to a high linear rate in the post-logarithmic phase, and maximum concentrations of enzyme in the medium in the ratio 1:25:100 for ribonuclease:α-amylase:proteinase, respectively—were observed irrespective of whether maltose, starch, glycerol or glucose (at 1 %) was included in the culture medium.

Differences were, however, observed in the presence of the different carbon sources in the doubling times during the logarithimic phase which were 2·2 hr with starch and maltose and 1·6 and 1·7 hr, respectively, with glucose and glycerol. During the 14 hr experimental periods, in the case of starch, maltose and glycerol the pH value increased steadily by 0·2–0·3 pH unit. With glucose, where the fastest doubling time was recorded, the pH value decreased by 0·2 pH unit during the logarithmic phase and subsequently rose to the value of the other cultures by the end of the incubation.

Figure 3 shows the differential rates of ribonuclease formation in the presence of maltose, starch, glycerol and glucose as carbon sources. Similar sets of curves were obtained for α-amylase (Coleman & Grant, 1966) and proteinase. All showed a low

differential rate of exoenzyme formation during the logarithmic phase which increased after the end of the logarithmic phase to the same high level for each enzyme irrespective of the carbon source employed. The initial rates tended to an inverse relationship with the doubling times such that the greatest rates were observed with starch and maltose and the lowest with glucose. However, with glucose the lower rate may be connected in some way with the decrease of pH value due to acid formation. This may be a pH effect *per se*, or the decrease of pH value may simply reflect an accumulation of intermediates of glucose catabolism which lower the differential rate of exoenzyme production by catabolite repression.

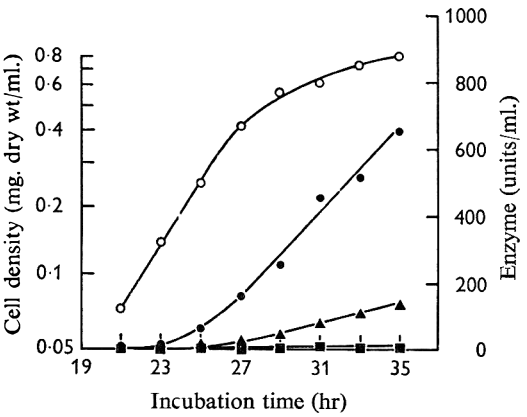

Fig. 1

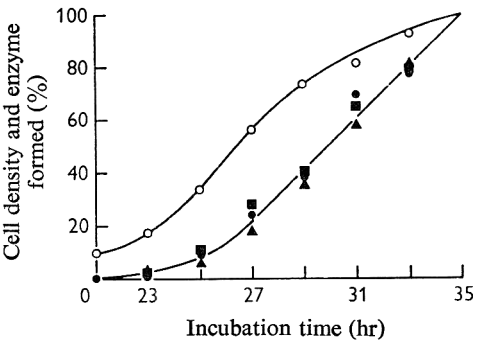

Fig. 2

Fig. 1. Time courses of growth (○) and of proteinase (●), α-amylase (▲) and ribonuclease (■) secretion by *Bacillus subtilis* growing in a defined medium with 1 % maltose as the carbon source.

Fig. 2. Progress of increase in cell density (○) and secretion of proteinase (●), α-amylase (▲) and ribonuclease (■), each expressed as a percentage of the values at the end of the experimental period.

Characteristics of extracellular enzyme formation in a complex medium

Experiments parallel to those already described were done in which the organism was grown in a complex medium containing 0·5 % casein hydrolysate + 0·05 % yeast extract in place of the amino acids and vitamins of the defined medium. Figure 4 shows the time courses of growth and extracellular enzyme secretion in a complex medium with 1 % maltose as the carbon source. It is apparent that the complex medium supported more growth and greater extracellular enzyme production than did the defined medium. With complex media logarithmic growth continued until a bacterial concentration equiv. 1·0 mg. dry wt bacteria/ml. was achieved, then after a lag during which the bacterial concentration doubled, a final stationary phase was quickly reached. During this stationary phase the exoenzymes continued to be secreted at the maximum rate and in a linear fashion. The ratios between the amounts of enzyme produced up to the end of the experimental period again, as in 1 % maltose defined medium, were 1:25:100 for ribonuclease:α-amylase:proteinase, respectively. The results plotted in Fig. 4, expressed as percentages of the final values, showed that the enzymes were again seen to be secreted in a parallel fashion (Fig. 5).

These same relationships were also observed when the organism was grown in media supplemented with starch, glycerol or glucose. Thus the end of the logarithmic phase was reached at a bacterial concentration equiv. 1·0 mg. dry wt bacteria/ml. and a final stationary phase at 2·0 mg. dry wt/ml. in each case. However, although the enzymes were secreted parallel to each other the ratio between the amounts present in the complex culture media at the end of each experimental period showed a deviation that was not observed when the organism was grown in defined media. The greatest variation was observed in the glucose-grown culture where the ratio proteinase:α-amylase:ribonuclease at the end of the experimental period was 100:10·7:0·8.

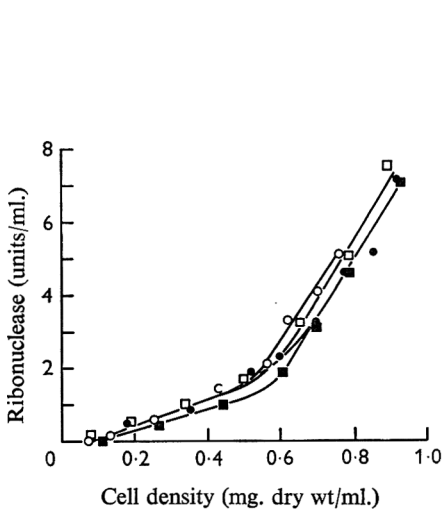

Fig. 3

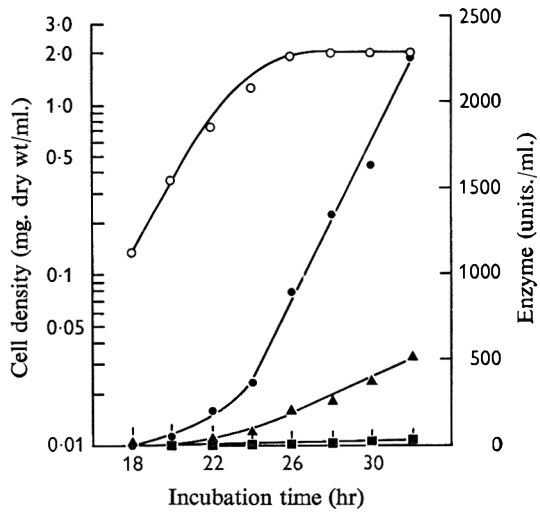

Fig. 4

Fig. 3. Differential rate of ribonuclease secretion by *Bacillus subtilis* growing in a defined medium with maltose (○), starch (●), glycerol (□) or glucose (■), at a level of 1% as carbon source.

Fig. 4. Time courses of growth (○) and of proteinase (●), α-amylase (▲) and ribonuclease (■) secretion by *Bacillus subtilis* growing in a complex medium with 1% maltose as the carbon source.

In the complex medium growth was faster than in the defined medium in all cases except in the presence of glycerol, where a doubling time of 2·0 hr was recorded. Doubling times of 1·3, 1·6 and 1·4 hr were observed when the organism was grown in the presence of starch, maltose or glucose, respectively.

In the maltose- and starch-containing media the pH value increased during the 14 hr experimental period by 0·1–0·2 pH unit, whilst with glucose there was a decrease of 0·4 pH unit during the logarithmic phase, followed by an increase of 0·6 pH unit to the end of the experimental period. With glycerol the pH decreased by 0·2 pH unit after 4 hr, and 6 hr later increased by 0·2 pH unit to the initial value, which was then maintained during the remainder of the experimental period.

The differential rates of synthesis of ribonuclease, α-amylase and proteinase in the presence of starch, maltose, glucose and glycerol, as carbon sources, showed the same general characteristics as were observed when the organism was grown in the defined medium; a representative set of curves is shown in Fig. 6. Thus there was a low initial

differential rate of formation in each case, which increased after the end of the logarithmic phase. However, in the complex medium final rates approaching infinity were achieved since the enzymes were secreted at the maximum rate even after the stationary phase had been reached and there was no further net increase in cell mass.

The initial differential rates of synthesis showed the same trend as in the defined medium with the highest values for maltose and starch and the lowest for glucose. However, in this case no simple relationship was observed between the initial differential rates and the doubling times. Again, the lower rate in the presence of glucose might be ascribed to a lowering of the pH value or to catabolite repression. Experiments were therefore made to test this.

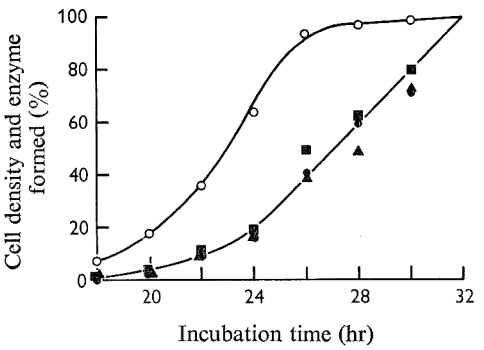

Fig. 5

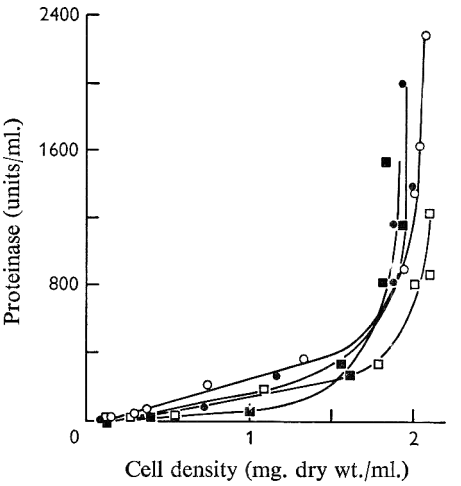

Fig. 6

Fig. 5. Progress of increase in cell density (○) and secretion of proteinase (●), α-amylase (▲) and ribonuclease (■), each expressed as a percentage of the values at the end of the experimental period.

Fig. 6. Differential rate of proteinase secretion by *Bacillus subtilis* growing in a complex medium with maltose (○), starch (●), glycerol (□) or glucose (■), at a level of 1% as carbon source.

Effect of maintaining constant pH value on the secretion of exoenzymes during logarithmic growth in a glucose-containing medium

The effect of the decrease of the pH value caused by acid formation, during growth in a glucose-containing complex medium, on extracellular enzyme secretion was examined by two experiments done in parallel. In the first experiment, extracellular enzyme secretion was followed through the logarithmic phase, the culture being allowed to maintain its own pH value, which was measured at hourly intervals. In the parallel experiment the pH value was adjusted hourly to the initial value by adding 10 N-NaOH, so that the pH value did not fluctuate by more than 0·1 pH unit. The progress of extracellular enzyme formation was again followed through the logarithmic phase. The results are shown in Table 1. It can be seen that 4 hr from the beginning of the experiment the pH value of the culture to which no additions of alkali were made had reached a minimum value, having decreased by 0·5 pH unit, and during the following 4 hr period the pH value increased by 0·3 pH unit.

In spite of the decrease in pH value of the unadjusted culture there was no significant difference between the amounts of enzyme secreted with and without pH adjustment during the first 4 hr. Subsequently, the amounts of extracellular enzyme secreted tended to be slightly higher in all cases where the pH value was kept constant. The results of the two experiments were not significantly different. Any gross effects on the initial differential rates of secretion of extracellular enzymes cannot be ascribed to the decrease in pH value during logarithmic growth in the presence of glucose.

Table 1. *Effect of maintaining constant pH on the secretion of exoenzymes by Bacillus subtilis growing on a 1 % glucose–complex medium*

The pH of both series was measured at hourly intervals and the 'constant pH' culture was maintained at pH 6·8–6·9 by the addition of 1ON-NaOH.

Incubation time (hr)	pH of control culture (no pH adjustment)	Ribonoculease (units/ml.)		α-Amylase (units/ml.)		Proteinase (units/ml.)	
		Control	pH 6·9	Control	pH 6·9	Control	pH 6·9
0*	6·9	0·0	0·0	0	0	0	0
2	6·6	0·4	0·6	1	1	20	32
4	6·4	1·6	1·9	11	13	76	76
6	6·5	3·5	4·2	30	34	216	228
8	6·7	5·6	7·2	69	74	436	476

* Zero time corresponds to a point in the logarithmic phase (14 hr) when growth is just apparent.

Effect of adding extra carbon source to a culture at the beginning of the stationary phase on the subsequent secretion of extracellular enzymes

The lower differential rates of exoenzyme formation during the logarithmic phase in the presence of glucose thus cannot be accounted for by the decrease of pH value which resulted from the accumulation of acids in the medium. A further possible explanation is that the accumulated intermediates of carbohydrate breakdown might have given rise to catabolite repression during the logarithmic phase, which was removed during the post-logarithmic phase as the repressing metabolites were further metabolized. Mandelstam (1961, 1962) made detailed studies on catabolite repression and concluded that any compound which an organism can use as a source of carbon and energy can cause catabolite repression of a sensitive system under the right conditions. Namely, when growth is prevented by the omission of an essential ingredient from the medium then in the presence of a carbon source intermediates can accumulate and conditions will favour the build-up of the repressing catabolite to a value at which it will exert an effect. To determine whether exoenzyme formation in *Bacillus subtilis* was subject to catabolite repression a culture in 1 % maltose complex medium was taken in the stationary phase at 28 hr which (as can be seen from Fig. 4) was expected to secrete exoenzymes at the maximum rate for at least 4 hr. An extra carbon source, maltose, glucose or glycerol, was dissolved in samples of culture and the progress of exoenzyme formation followed for a further 3 hr and the values compared with a control to which no extra carbon source had been added. The results (Table 2) showed that identical results were obtained with or without extra maltose, and in each case an insignificant increase in bacterial dry weight of 3–4 % was observed over the 3 hr period. If the formation of α-amylase, ribonuclease and proteinase, which are secreted parallel, were

subject to catabolite repression, then according to Mandelstam (1961, 1962) the enzyme concentrations achieved in the presence of excess maltose should have been decreased; clearly this was not so.

Table 2. *Effect of adding extra carbon source on exoenzyme secretion by a stationary phase culture of Bacillus subtilis in a 1 % maltose–complex medium*

	Increase in exoenzymes								
	ribonuclease (units/ml.)			α-amylase (units/ml.)			proteinase (units/ml.)		
Addition	1 hr	2 hr	3 hr	1 hr	2 hr	3 hr	1 hr	2 hr	3 hr
None (3%)	2·6	5·7	4·6	63	114	171	150	300	380
Maltose (4%)	2·6	5·6	4·6	56	104	158	150	280	380
Glycerol (15%)	2·6	5·4	6·1	51	83	114	110	240	310
Glucose (16%)	1·8	4·5	6·4	51	76	113	170	240	340
	(Zero time* level 10·3 units/ml.)			(Zero time level 239 units/ml.)			(Zero time level 1210 units/ml.)		

* Zero time corresponds to 28 hr incubation at which time the carbon supplements were added to a final concentration of 1%, in each case. The increases in cell mass observed over the 3 hr experimental period are shown in parentheses.

Table 3. *Effect of adding chloramphenicol on exoenzyme secretion by post-logarithmic phase culture of Bacillus subtilis in a 1 % maltose-complex medium*

	Increase in exoenzymes		
Addition	ribonuclease (units/ml.)	α-amylase (units/ml.)	proteinase (units/ml.)
None	4·45 (−)	124 (−)	380 (−)
Chloramphenicol (10 μg./ml.)	0·0 (100)	6 (95)	28 (93)
Chloramphenicol (100 μg./ml.)	0·0 (100)	0 (100)	0 (100)
	(Zero time* level 3·10 units/ml.)	(Zero time level 88 units/ml.)	(Zero time level 336 units/ml.)

Zero time corresponds to 24 hr incubation at which time 0·01 ml. and 0·1 ml. of an aqueous solution of chloramphenicol (5 mg./ml.) were added to 5 ml. samples of culture. Incubation was continued for a further 3 hr period. The figures in parentheses show the percentage inhibition of enzyme formation by chloramphenicol over the 3 hr period of the experiment.

When the added carbon source was glucose or glycerol greater deviation from the control values were observed, but this might be accountable to the accompanying significant increase in bacterial mass in the presence of these carbon sources. In relation to the actual amounts of enzyme in the cultures (the 28 hr zero time values were extremely high) these deviations amount to little more than experimental error. The results of this experiment therefore gave no support for the belief that catabolite repression played a part in controlling the extracellular enzyme formation.

Effect of adding chloramphenicol at the beginning of the post-logarithmic phase on the subsequent secretion of extracellular enzymes

The secretion of ribonuclease and α-amylase by washed suspensions of post-logarithmic *Bacillus subtilis* was shown to involve *de novo* synthesis and not liberation

or activation of preformed enzyme (Coleman & Elliott, 1962, 1965). However, this work was not extended to proteinase. It was desirable to ascertain that the apperance of all three exoenzymes in the post-logarithmic phase, under the conditions of the present experiments, represented the formation of new protein and so the sensitivity of their secretion to chloramphenicol, a specific inhibitor of protein synthesis, was examined.

Chloramphenicol was added to a 24 hr post-logarithmic culture of *Bacillus subtilis* grown in a 1 % maltose-complex medium which was then incubated for a further 3 hr. The changes in extracellular enzyme levels in the presence and absence of chloramphenicol were determined over the 3 hr experimental period. The results are shown in Table 3, where it can be seen that there was over 90 % inhibition of the further secretion of all three enzymes by 10 μg./ml. of chloramphenicol; the inhibition was complete when the chloramphenicol was increased to 100 μg./ml. This result is consistent with the appearance of all these enzymes involving *de novo* protein synthesis.

DISCUSSION

Before considering the implications of the results obtained in the present work it is expedient to consider relevant data obtained during earlier work. Coleman & Elliott (1965) compared the formation of α-amylase and ribonuclease in washed suspensions of post-logarithmic *Bacillus subtilis*. It was found that the appearance of both these enzymes in the medium had the characteristics of protein synthesized *de novo* during the period of secretion. In the earlier work the effect of actinomycin D on the formation of α-amylase and ribonuclease was examined and evidence was obtained that a stable messenger RNA was involved in ribonuclease synthesis, whilst α-amylase had an unstable messenger. These conclusions can be used to explain the different shapes of the progress curves of formation of the two enzymes in suspensions of washed bacteria.

Coleman & Elliott (1965) observed that when post-logarithmic phase bacteria, in a 1 % maltose casein hydrolysate medium and producing α-amylase and ribonuclease linearly, were suspended in new medium then ribonuclease was formed linearly, but at a rate 50 % higher than previously and for at least 3 hr. By contrast, the rate of α-amylase formation was drastically decreased, but after a 2 hr lag it was again secreted linearly at the same rate as in the post-logarithmic phase culture. Over the 3 hr experimental period there was an increase in bacterial mass of 90 % in the washed suspension as compared with the original culture in which there was a 30 % increase in bacterial mass over the same period.

It is obvious that as the bacteria are transferred from old medium, which is limiting in some nutrient, to new medium the culture will undergo a 'shift-up' transition with the concomitant formation of new ribosomes to enable growth to take place at a faster rate (Neidhardt & Fraenkel, 1961; Schaechter, 1961). During the transition the inevitable drain on the pools of RNA and protein precursors will occur as ribosomes are preferentially synthesized. However, during this same period ribonuclease is formed at a faster rate than previously, indicating that there is no shortage of protein precursors for the translation of the stable ribonuclease messenger. On the other hand, the rate of α-amylase formation is reduced and since there appears to be no shortage of protein precursors then the synthesis is probably limited by the reduction in nucleic acid precursor material, necessary for the formation of unstable m-RNA, due to its

increased use during the preferential ribosome formation in accelerating growth conditions.

The present study of the characteristics of the formation of the three extracellular enzymes, α-amylase, ribonuclease and proteinase, over the whole of the growth cycle, shows that irrespective of the medium used, defined or complex supplemented with either maltose, starch, glycerol or glucose, the enzymes are secreted parallel to each other, and although the amounts of each differ they approximate to a constant ratio to each other. A low differential rate of secretion is observed during the logarithmic phase of growth which undergoes a dramatic increase in the post-logarithmic phase.

Consideration of the whole of the data discussed above permits a regulatory mechanism to be described in terms of 'competition' at the level of the nucleic acid precursor pool.

Thus during logarithmic growth the nucleic acid precursor pool is depleted by the irreversible removal of material for ribosome synthesis (Stent, 1966) and to a lesser extent transfer RNA synthesis. The remaining nucleic acid precursors in the depleted pool are available for the formation of m-RNA involved in the synthesis of necessary enzymes and other proteins and will, in general, be removed and returned to the pool as described by Levinthal, Fan, Higa & Zimmermann (1963). During this phase the precursors available for exoenzyme m-RNA synthesis, which is not obligatory for growth, will be strictly limited.

After the end of the logarithmic phase a slowing down of ribosome synthesis results in less drain on the RNA precursor pool and, further, ribosome 'turnover' contributes towards replenishing the pool. The net result of slowing growth will be a greatly increased pool available for m-RNA synthesis permitting increased exoenzyme formation (Ben-Hamida & Schlessinger, 1966).

The observed characteristics can be explained in terms of such a mechanism provided that exoenzymes are produced in amounts sufficient to tax the organisms' anabolic resources. This is, in fact, the case and the increase in exoenzyme protein formation on passing from logarithmic to post-logarithmic growth conditions approximates to the reduction in cellular protein synthesis observed during the same transition.

Other mechanisms of control based on induction (Coleman & Grant, 1966), repression (Mandelstam, 1961, 1962; Jacob & Monod, 1961; Cline & Bock, 1966) and modulation (Stent, 1964) were considered but not one of them was as satisfactory in accounting for the experimental results as the simple model discussed. Thus if it is considered that the exoenzymes are formed constitutively with an overriding effect due to a limitation in nucleic acid precursors brought about by the potential for their synthesis on a massive scale then the characteristics of exoenzyme secretion are readily explained.

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Antigenic Relationships Between Oral Group D Streptococci, Some Haemophilus Species and *Mycoplasma hominis*

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SUMMARY

By gel-diffusion precipitin and absorption tests six antigens were identified in group D streptococci of human oral origin that were common to *Haemophilus influenzae*; four of these were common also to *Mycoplasma hominis*, and three to the L-form of a group A streptococcus. No cross-reactions were obtained with *Corynebacterium xerosis* or *C. hofmannii*. These antibodies occurred in commercial Streptococcus and Haemophilus typing sera, but not in enterobacterial agglutinating sera.

It should be concluded that the genus *Haemophilus* and *Mycoplasma hominis* are more closely related to the genus *Streptococcus* than to the genus *Corynebacterium*.

INTRODUCTION

Antigenic analysis of *Mycoplasma hominis* and of bacteria associated with, or believed to be derived from it, by the technique of gel-diffusion precipitin and cross absorption, has shown that they share common antigens (Pease, 1965*b*). Some of these bacteria have been classified with the genus *Haemophilus*, others with the genus *Corynebacterium* (Minck, 1953; Wittler, Cary & Lindberg, 1956; Smith, Peoples & Morton, 1957; Pease, 1962; Pease & Laughton, 1962, 1965) but the existence of Gram-positive variants, having a corynebacterial morphology, in bacteria attributed to the genus *Haemophilus*, or considered by some authorities to be related to it, is well documented (Deacon, Albritton, Edmundson & Olansky, 1954; McKay & Truscott, 1959; Lapage, 1961; Zinnemann & Turner, 1962, 1963), and Pease & Laughton (1965) showed that corynebacterial variants derived from the 'Haemophilus vaginalis' of Edmunds (1960, 1962), from *H. influenzae* and from mycoplasmas were antigenically related; but their classification has never been satisfactorily established in relation to true corynebacteria.

An antigenic relationship has also been determined, between *Mycoplasma pneumoniae* and the so-called Streptococcus MG (Pease, 1963; Marmion & Hers, 1963; Eaton, 1965), and the derivation of group D streptococci from PPLO has been claimed by Kelton, Gentry & Ludwig (1959). In addition, it is established that the physiology of PPLO markedly resembles that of group D streptococci (O'Kane, 1950; Niemark & Pickett, 1959; Tortellotte & Jacobs, 1959; Smith, 1964), and the possibility of a relationship has been emphasized. It was thus considered of interest to determine the antigenic structure of group D streptococci in relation to the analytical system of Pease & Laughton (1965) and Pease (1965*b*), previously applied to *Mycoplasma hominis* and its associated bacteria. Strains of *Corynebacterium xerosis* and of *C. hofmannii* were included for comparison.

METHODS

A group of strains was chosen as a basis for analysis which embodied a convenient spectrum of known antigens in the system of Pease & Laughton (1965) and Pease (1965*b*). These were two strains of 'Haemophilus vaginalis', Edmunds, (GP 2, GP 7) originally obtained from Dr P. N. Edmunds; one of a vaginal strain of *Mycoplasma hominis* (H 26), obtained from Dr D. G. ff. Edward, and two vaginal strains (GT. 2, GT. 3) and a derived, corynebacterial variant (Bact. OR. 9), isolated in this laboratory. Nine strains of group D streptococci (1–9) were isolated in this laboratory from human mouths and throats, and identified by the Lancefield grouping technique. One strain of group A *Streptococcus pyogenes* and its L-form were obtained from Dr W. R. Maxted. Five strains of *H. influenzae* (1–5) and L-forms of 1, 3 and 5 were isolated in this laboratory, six (NCTC 8465, 8467, 8469, 8470, 8472, 8473, originally types a–f, now uncapsulated), and one strain listed as *H. haemolyticus* (NCTC 8479) and one of *H. gallinarum* (NCTC 3438) were obtained from the National Collection of Type Cultures. Three strains isolated from pathological conditions in infants (KAIN 1, 2, 3) were obtained from Dr K. B. Rogers, by whom they were tentatively identified as species of *Haemophilus*; these were included in the analysis because of their strong serological reactions with the Pease & Laughton type antigenic strains already listed. Three strains of *Corynebacterium xerosis* and one of *C. hofmannii* were stock cultures maintained in this laboratory.

Ultrasonically disintegrated antigens were prepared from all strains; corresponding antisera against the group A streptococcus and L-form, four group D streptococci, four *Haemophilus influenzae* and one L-form, and the three type mycoplasmas, one bacterial mycoplasma-variant and two *H. vaginalis* strains were made, and gel-diffusion precipitin tests and antigen and antibody-absorption reactions were performed, all according to the methods described by Pease & Laughton (1965) and Pease (1965*b*), including all the same controls against false reactions.

The following Burroughs Wellcome typing and grouping sera were also used: Lancefield groups A, B, C, D (four batches), E, F, G, H, K, L, M, N; Haemophilus Pittman a, b, c, d, e, f; Pneumococcus I, II, III; *Shigella largei* (1–8), Flexner 6 (Newcastle 88), 'dispar', Boyd polyvalent 2 (7–11); Salmonella polyvalent (O and H sp. and non-sp.), *S. paratyphi A* (O and H), *S. paratyphi C* (O and H), *S. typhi* (O); *Vibrio cholerae* (Ogawa); Meningococcus polyvalent (A–D).

RESULTS

The results of precipitin reactions are shown in Table 1. There can be seen the imbalance that is customarily shown in reactions of this type, according to whether the antigenic component is represented in the antigen or in the antiserum (Taylor-Robinson, Canchola, Fox & Chanock, 1964; Pease & Laughton, 1965; Pease, 1965*b*).

The distribution in the newly examined strains of the *Haemophilus influenzae* group antigens, 17 and 39, in the scheme of Pease & Laughton (1965) and Pease (1965*b*) was then determined by coincident lines of precipitation between strains reacting with H 26 serum and with other sera. Antibodies occurring in the new sera, and corresponding to *H. influenzae* antigens not represented in the H 26 serum, appeared as spurs. Antigen 17 was identified from antibodies in streptococcus D 2 and D 3 sera and in the group A

Table 1.

Bacterial antigens	Sera: Strep. A	A L-form.	Strep D. 1	Strep D. 2	Strep D. 3	Strep D. 4	H. inf. 1	H. inf. 2	H. inf. 3	H. inf. 4	H. inf. L-fm 1	H. vag. GP 2	H. vag. GP 7	Myc. H. 26	Myc. Gt. 2	Myc. Gt. 3	Bact. Or. 9
Streptococcus																	
A	4	2	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—
A, L-form	2	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
D 1	—	—	4	2	2	2	—	—	—	—	—	—	—	1	—	—	—
D 2	—	—	3	3	2	2	—	—	2	—	—	—	—	—	—	—	—
D 3	—	—	3	2	2	2	—	—	—	—	—	—	—	2	—	—	—
D 4	—	—	3	3	2	3	—	—	—	—	—	—	—	—	—	—	—
D 5	2	2	3	2	3	4	—	—	2	—	—	—	—	1	—	2	1
D 6	2	2	2	2	2	4	—	—	2	—	—	—	—	1	—	2	1
D 7	2	—	4	1	2	3	—	—	1	—	—	—	—	1	—	2	—
D 8	—	—	1	1	2	3	—	—	1	—	—	—	—	1	—	2	—
D 9	—	1	2	2	2	1	—	—	1	—	—	—	—	1	—	2	—
H. influenzae																	
1	—	2	—	2	—	—	4	2	4	3	3	2	2	1	—	2	2
2	—	2	—	3	2	—	3	2	4	3	2	2	2	1	—	2	2
3	—	2	—	3	2	—	4	2	4	4	2	2	2	3	—	2	2
4	1	2	—	2	1	—	3	3	3	4	2	2	2	—	—	2	2
5	—	2	—	2	—	—	4	2	4	4	2	2	2	1	—	2	2
NCTC 8465(*a*) *	—	2	—	2	—	—	2	2	4	3	4	2	2	—	—	2	2
NCTC 8467(*b*) *	—	2	—	2	—	—	2	2	4	2	2	2	2	2	1	—	2
NCTC 8469(*c*) *	—	1	—	2	—	—	—	—	2	—	1	—	2	—	—	—	2
NCTC 8470(*d*) *	—	2	—	2	—	—	2	2	3	4	2	2	2	—	—	2	2
NCTC 8472(*e*) *	—	2	—	2	—	—	4	3	4	4	4	1	1	2	—	2	3
NCTC 8473(*f*) *	—	2	—	2	—	—	2	—	2	2	2	—	—	2	—	2	2
L-form 1	—	—	—	—	—	—	—	2	2	2	1	—	—	2	—	1	2
L-form 3	—	2	—	2	—	—	2	2	3	4	3	2	2	2	—	1	2
L-form 5	—	2	—	2	—	—	2	3	3	2	2	1	2	—	—	2	.
H. gallinarum	—	2	—	2	—	—	—	—	2	2	2	2	2	—	—	2	2
H. haemolyticus	—	2	—	2	—	—	2	—	2	2	2	—	—	—	—	2	.
'*H. vaginalis*' GP 2	—	—	—	—	1	—	—	—	—	—	—	3	—	2	—	2	—
'*H. vaginalis*' GP 7	—	—	—	—	—	—	—	—	—	—	—	—	3	2	—	—	—
Kain 1 (*Haemophilus* sp.)	—	3	—	2	—	2	—	—	2	—	—	—	—	1	—	2	3
Kain 2 (*Haemophilus* sp.)	1	1	—	—	—	—	—	—	—	2	—	2	2	—	1	—	2
Kain 3 (*Haemophilus* sp.)	2	2	—	2	—	2	—	—	2	2	—	2	2	1	2	—	.
Mycoplasma H 26	—	—	—	—	—	—	—	—	—	—	—	—	—	3	3	3	—
Mycoplasma GT. 2	—	—	—	—	—	—	—	—	—	—	—	—	—	3	3	3	—
Mycoplasma GT. 3	—	—	—	—	—	—	—	—	—	—	—	1	1	3	3	3	—
C. xerosis 1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C. xerosis 2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C. xerosis 3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C. hofmannii	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

1, Weak; *2*, moderate; *3*, strong, multiple lines; *4*, very strong, heavy and multiple lines; —, negative; ., not done

* Uncapsulated.

L-form serum. Antigen 39 was identified in Gt. 3 antigen also, and in smaller apparent quantity in the streptococcus group A L-form, by the ability of these antigens to absorb the precipitin from positive antisera. The mycoplasma common antigen *g* was identified by a spur between the two sera H26 and Bact. Or. 9, when these reacted against *H. influenzae* 8473 antigen.

Six further antigens, previously recognized only as specific, were now found to be group antigens when identified in newly examined strains of bacteria. The naming of these, with respect to the former specific numbers, is necessarily arbitrary, since they were originally numbered in arbitrary series (Pease & Laughton, 1965). This extension permits them to be defined more completely. Antigen 8 had previously been defined in 'Haemophilus vaginalis' of Edmunds (1960, 1962); it was now also detectable as a second line, distinct from that given by antigen 39 (described in the previous paragraph), between *Mycoplasma hominis* Gt. 3 serum and GP 2 antigen. The presence of antigen 9 was shown by the coincidence between the lines given by streptococcus D 2 serum with those given by both GP 2 and GP 7 sera against Kain 3 antigen. Antigen 10 appeared as a second line between Kain 3 and GP 7 serum, continuous with that produced by Kain 3 against Gt. 2 or H 26 sera. Antigens 36, 37 and 38 were originally defined in *Haemophilus influenzae*; 36 was now identified in Kain 1 because the lines produced by reaction of this bacterium against *Haemophilus influenzae* 3 serum were identical with those produced by Kain 1 reacting with *M. hominis* Gt. 3 serum. Antigen 36 was also identified in the remaining strains of *H. influenzae*, because of their ability to absorb these lines of precipitation. This antigen was subsequently found to be of such wide occurrence that its significance and validity as a group antigen in this system is accordingly suspect. The point is referred to in the Discussion. The presence of 37 in *H. influenzae* and in *H. gallinarum* was determined by absorption, and in sera of mycoplasma Gt. 3 and H 26, streptococcus group A L-form and Bact. Or. 9, by coincidence of lines. Antigen 38 was detectable in the reactions between D 2 antiserum and *H. influenzae* 2 and 3, by a further line, distinguishable by absorption from 17 and 39; 38 was similarly identified also in D 2 and in the antiserum of *H. influenzae* 3. A group antigen, not previously defined, was revealed by the phenomenon of dissociation recorded by Zwartouw, Westwood & Harris (1965) in relation to the antigens of vaccinia virus; this appeared as a new, heavy line of precipitation produced in the reactions between *H. influenzae* 3, GP 2, GP 7 and D 3 antigens on one side, and H 26 antiserum on the other, after the antigens had been stored at 4° for periods of *c.* 15 weeks; a coincident line was present in Gt. 3 and H 26 antigens without low-temperature storage, and accordingly this antigenic component was named *m*. A similar, but apparently specific, antigen was also revealed by low-temperature storage of GP 7, but was not further identified. A second, previously unrecognized group antigen was detected in the reaction of Kain 1 with D 4 antiserum, and was distinguishable from 9 by non-identity of lines between the same serum and Kain 3 in a contiguous cup; this was named 46.

A hitherto unreported phenomenon was observed in the process of antigen absorption by antisera: the addition of sera, containing homologous antibodies, to cups containing certain antigens, resulted not in the removal of the line of precipitation, but in an increase of visible density when the antigen–antibody complex reacted with a second positive antiserum; this must be attributable to the formation of a partial complex, that retains its diffusibility. Thus, for example, reaction between D 6 antigen and

Table 2. *Gel-diffusion precipitin reactions between antigens and Burroughs Wellcome typing and grouping sera*

1, Weak; *2*, moderate; *3*, strong, multiple lines; *4*, very strong, heavy and multiple lines; −, negative; ., not performed.

	Streptococcus Lancefield sera													Pneumococcus sera		Haemophilus sera						Salmonella, Shigella Vibrio, Neisseria sera
Bacterial antigens	A	B	C	D	D	D	D	E	F	G, H, N	K	L	M	I	II, III	a	b	c	d	e	f	
Streptococcus																						
A	*4*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
A, L-form	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D 1	−	−	−	*2*	*2*	*4*	*4*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D 2, 4, 5, 6	−	−	−	*2*	*2*	*3*	*3*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D 3	−	−	−	*2*	*2*	*3*	*3*	−	−	−	*1*	−	−	−	−	−	−	−	−	−	−	−
D 7	−	−	*2*	*2*	*3*	*4*	*4*	*2*	*2*	−	*1*	*2*	−	−	−	−	−	−	−	−	−	−
D 8	−	−	−	*2*	*3*	*3*	*3*	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D 9	−	−	*2*	*2*	*3*	*3*	*3*	*2*				*1*										−
*H. influenzae**																						
1, 4, 8467, 8469, L-forms	−	−	−	*1*	*2*	*1*	*2*	*1*	*2*	*2*	*2*	*2*	−	*1*	−	*2*	−	−	−	*1*	*2*	−
2	−	−	−	*1*	*2*	*1*	*2*	−	*2*	*2*	−	*2*	*2*	*1*	−	−	−	−	−	*2*	*2*	−
3	−	−	−	*1*	*2*	*1*	*2*	*1*	*2*	*2*	*2*	*2*	*1*	−	−	*2*	−	*2*	−	*2*	*2*	−
5, 8470, 8472	−	−	−	*1*	*2*	*1*	*2*	−	*2*	*2*	*2*	*2*	−	*1*	−	*2*	−	−	*1*	*2*	*2*	−
8465, 8473	−	−	−	*1*	*2*	*1*	*2*	*1*	*3*	*2*	*2*	*2*	−	−	−	*2*	−	*1*	−	*2*	*2*	−
H. gallinarum	−	−	−	*1*	*2*	*1*	*2*	−	*2*	*2*	*2*	*2*	*1*	*1*	−	.	.	.	.	.	.	−
H. haemolyticus	−	−	−	*1*	*2*	*1*	*2*	*1*	*2*	*2*	*2*	*2*	−	*1*	−	.	.	.	.	.	.	−
Corynebacterium xerosis, C. hofmannii	−	−	−	−	−	−	−	−	−	−	−	−	−	.	.	−	−	−	−	−	−	−

* Uncapsulated.

Haemophilus influenzae 3 serum was stronger when the former was absorbed with D4 antiserum.

Table 2 shows the results of precipitin reactions between the ultrasonically disintegrated antigens of the type genera and Burroughs Wellcome antisera: from which it can be seen that there was a marked degree of cross-reaction between Haemophilus antigens and streptococcus sera D–M, also with Pneumococcus I. Analysis showed that

Table 3. *Distribution of newly detected group antigens in strains examined*

+, Antigen detected; −, antigen not detected; ns, antigen detectable in other strains of group D streptococcus only by presence of antibody in serum, which was not available for strains D 5–9; nt, not tested for this antigen.

Strains	Group antigens										
	8	9	10	17	36	37	38	39	46	g	m
Streptococcus											
A	−	−	−	−	−	−	−	−	−	−	−
A, L-form	−	−	−	+	+	+	−	+	−	−	−
D 1	−	−	−	−	−	+	−	−	−	−	−
D 2	−	+	−	+	+	−	+	−	−	−	−
D 3	−	−	−	+	−	−	−	−	−	−	+
D 4	−	−	−	−	+	−	−	−	+	−	−
D, 5, 6, 7, 8, 9	−	−	−	ns	−	+	−	−	−	−	−
*H. influenzae**											
1, 5, 8467(*b*), 8472(*e*)	−	−	−	+	+	−	−	+	−	−	−
2	−	−	−	+	+	−	+	+	−	−	−
3	−	−	−	+	+	+	+	+	−	−	+
4	−	−	−	−	+	+	−	+	−	−	−
8465(*a*), 8469(*c*), 8470(*d*)	−	−	−	−	+	−	−	+	−	−	−
8473(f)	−	−	−	+	+	−	−	+	−	+	−
L-forms	−	−	−	−	nt	−	−	+	−	−	−
H. gallinarum	−	−	−	−	+	+	−	+	−	−	−
H. haemolyticus	−	−	−	−	+	−	−	+	−	−	−
'*H. vaginalis*' GP 2	+	+	−	−	−	−	−	+	−	−	+
'*H. vaginalis*' GP 7	−	+	+	−	−	−	−	−	−	−	+
Haemophilus sp. KAIN 1	−	−	−	−	+	−	−	−	+	−	−
Haemophilus sp. KAIN 2	−	−	+	−	+	−	−	−	−	−	−
Haemophilus sp. KAIN 3	−	+	+	−	+	−	−	−	−	−	−
Mycoplasma H. 26	−	−	+	−	+	+	−	−	−	+	+
Mycoplasma GT. 2	−	−	+	−	+	−	−	−	−	−	−
Mycoplasma GT. 3	+	−	−	−	+	+	−	+	−	+	+
Corynebacterium xerosis, *C. hofmannii*	−	−	−	−	−	−	−	−	−	−	−

* Uncapsulated.

Table 4. *Distribution of group antigens in Streptococcus, Haemophilus and Mycoplasma hominis*

Streptococcus Group D 9, 17, 37, 38, 39, 46, m
Streptococcus Group A L-form 17, 37, 39
Haemophilus spp. (excl. '*H. vaginalis*') 17, 37, 38, 39, 46, g, m
'*Haemophilus vaginalis*' 8, 9, 10, 17, 39
Mycoplasma hominis 8, 10, 17, 37, 39, g, m

most of the reactions between *Haemophilus influenzae* and these commercial sera were due to antigens 17 and 39, as were those between the streptococcus sera and the other Haemophilus species; the reaction with Pneumococcus I serum was provisionally

attributed to 17, but the antibody was too weak for reliable absorption reactions. The usual imbalance is seen in the absence of visible reaction between streptococcal antigens and *H. influenzae* typing sera.

The antisera against Enterobacteria, *Vibrio cholerae* and *Neisseria meningitidis* were negative throughout, and antigens from *Corynebacterium xerosis* and *C. hofmannii* were negative with all sera.

The results of these analyses are summarized in Tables 3 and 4.

DISCUSSION

The distribution of group antigens is shown in Table 4. *Haemophilus influenzae*, the other type species and the KAIN 1, 2, and 3 *Haemophilus* spp. form a group. 'Haemophilus vaginalis' Edmunds is distinct from, but nevertheless related to, this group.

The precipitin and cross-absorption reactions recorded and analysed reveal six antigens (17, 37, 38, 39, 46, *m*) that are common to group D streptococci of oral origin and to *Haemophilus influenzae*. Four of these (17, 37, 39, *m*) are common to *Mycoplasma hominis* also. Two (17, 39) are previously recorded antigens (Pease, 1965*b*), two (37, 38) were previously recorded as specific to *H. influenzae*, and two (46, *m*) are newly identified. The number of antigens recorded as common to *Mycoplasma hominis* and to *Haemophilus* species, including 'H. vaginalis', is now eight including the diffusible α (Pease, 1965*b*), and two others (17, 39) previously defined, two (8, 10) previously believed to be specific, and one (*g*) that is a group antigen previously known to occur in *M. hominis* and its associated bacterial strains. Another group antigen (9) was previously believed to be specific to 'Haemophilus vaginalis' Edmunds, and is now found to be shared by group D streptococci.

The group A streptococcus possessed no group antigens in this system, whereas three group antigens (17, 37, 39) were detected in its L-form. This observation is presumably of some significance in the problem of whether or not the mycoplasmas can be regarded as L-forms. Antigen 36 was widely present, and may not be significant in the present argument (Chapman & Osborne, 1942; Zepp & Hodes, 1943).

Since the antigenic relationships of *Mycoplasma hominis* and *Haemophilus influenzae* seem to be with streptococci rather than with the corynebacteria, which did not cross-react at all, the question remains of how the variably Gram-positive bacteria associated with mycoplasmas, and usually described either as *Haemophilus* or as *Corynebacterium*, should be classified.

Comparison of DNA base ratios, as listed by Hill (1966), is of considerable interest. *Streptococcus faecalis* (usually group D) and *Haemophilus influenzae* are similar if not identical at *c.* 38 moles % GC. Corynebacteria range from 48 to 58, which is in agreement with their lack of antigenic relationship, but among bacteria possessing a corynebacterial morphology, at least on occasions, *Listeria monocytogenes* has a ratio of 38 also, and shares several antigenic components with *S. faecalis* (Seeliger & Cherry, 1957). This may indicate a relationship closer than hitherto suspected with the genera under discussion.

The relationships of mycoplasma have been so little understood that Hayflick & Chanock (1965) recently concluded that they were protozoa. The present evidence reinforces the view that they are bacteria.

The occurrence of streptococcal antigens in *Mycoplasma hominis* is potentially

significant in the problem of the causation of auto-immune disease, in relation to the hypothesis of Pease (1965*a*).

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Changes in Sensitivity to Acriflavine of *Escherichia coli* Grown in Media of Different Glucose Contents

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SUMMARY

Organisms of *Escherichia coli* K-12 strains sensitive and resistant to acriflavine were plated on media with and without acriflavine after growth in media containing different concentrations of glucose. Proportionally more organisms produced colonies, in the presence of acriflavine, after growth in media containing a high concentration of glucose than in media with lower glucose contents. The final pH value of the growth medium was low with the high glucose media. With the resistant strain, the number of bacteria which survived acriflavine increased as the final pH value of the medium from which bacteria were harvested was decreased, but the initial glucose concentration rather than the final pH value of the culture was more influential in increasing survival with the sensitive strain. The acriflavine-binding capacity of the bacteria was affected by the initial glucose concentration of culture medium probably indirectly through a change of pH. Acriflavine sensitivity of the bacteria varied with the amount of acriflavine bound. The acriflavine-binding capacity of bacteria modified by the pH value of culture medium was stabilized in the course of several doublings of bacteria in that medium. The glucose concentration of the medium affected the acriflavine sensitivity of the sensitive strain through some mechanism other than the change of pH.

INTRODUCTION

In the studies on genetic control of acriflavine sensitivity of *Escherichia coli* K-12 it was found that the binding capacity of the bacteria for acriflavine and other basic dyes seemed to be controlled by a gene which controlled the acriflavine sensitivity and which lay in the vicinity of the marker for lactose utilization (Nakamura, 1966). A long period was needed for the acriflavine resistance to be expressed following introduction of the resistance gene into acriflavine-sensitive bacteria by mating (Nakamura, 1965). During these investigations it was observed that the acriflavine sensitivity of the bacteria was markedly affected by the initial glucose content of the medium in which the organisms had been grown. The present paper shows that this effect of glucose concentration was partly due to the change of pH value of the culture during growth, which in turn affected the acriflavine binding of the bacteria, and also that the pH shift was not the sole factor involved in the glucose concentration effect.

METHODS

Organisms. Two strains of *Escherichia coli* K-12 were used. One, 18/1042, is an acriflavine (AF)-sensitive strain, isolated as a spontaneous mutant from a wild-type AF-resistant female strain. The other, N90, is an AF-resistant female strain obtained

by a cross of 18/1042 with an AF-resistant Hfr strain of *Escherichia coli* K-12, strain W1895. The characters of these strains were described previously (Nakamura, 1965).

Media. The strains were maintained in brain–heart infusion (Difco). For experiments, bacteria were grown overnight at 37° in a liquid medium composed of (g./l.): Difco nutrient broth (solid), 8; NaCl, 5; glucose, 1; initially at pH 7·4. This medium will be referred to as standard broth. A stock solution of acriflavine was sterilized at 100° for 20 min. and stored in a refrigerator, for not more than 10 days.

For solid media, powdered agar (1·5 %) was added. AF-broth agar was prepared by adding AF solution to the broth agar after it had cooled to 60–70°.

Determination of acriflavine content of bacteria. As negative-charge density of bacteria is reported to decline remarkably during the active growth phase (Abramson, Moper & Gorin, 1942), bacteria in the stationary phase of a culture in broth were sampled and suspended in AF-media for the determination of AF-binding. The amount of bacteria to be suspended was turbidimetrically adjusted to be equivalent to about 700 μg. dry wt bacteria/ml. Samples taken from AF-media, after a varying period of time specified later, were centrifuged (14,500*g*) for 5 min. at 5°, and the dye concentration in the supernatant fluid determined spectrophotometrically at 450 mμ. The amount of AF lost from the supernatant fluid was taken as the AF-content of the bacteria and used as an index of their dye-binding capacity. Although the medium itself was coloured yellowish, the absorption spectrum of AF could be differentiated when the medium was diluted a few times; medium without dye used as reference.

Determination of pH value in culture media. A sample of the culture was centrifuged (14,500*g*) for 5 min. at 5°, and pH value of the supernatant fluid determined electrometrically.

Thus the experiments done took the following form: organisms were grown in various liquid media; after a given time the organisms were harvested and tested for their degree of survival in AF-media (by plating on AF-nutrient agar) and for AF-binding capacity; the pH value of the culture fluids at the time of harvesting was measured.

RESULTS

Glucose content of medium and AF-sensitivity of bacteria

Strains N90 (AF-resistant) and 18/1042 (AF-sensitive) were inoculated in broth media which contained glucose in concentrations ranging from 0·05 to 6·4 g./l. After overnight incubation at 37°, a part of each culture was diluted by a factor of 6×10^6 and plated on broth agar containing AF, 250 μg./ml., for resistant strain N90 and 1 μg./ml. for sensitive strain 18/1042, and on broth agar alone, as a control. Colony counts on AF-agar are expressed as % of those on the control plates; this gave a measure of the sensitivity (% survival) to acriflavine of the harvested organisms.

The rest of each culture was centrifuged and the pH value of the supernatant fluid determined

Figure 1 represents % survivals of the two strains on the AF-agar plates and the final pH values of the media from which the bacteria had been harvested. The % survival was high when culture medium originally contained high concentrations of glucose. The % survival on AF-agar of strain N90 was related to the pH value of the

culture at the time of harvesting, but with strain 18/1042 the initial glucose concentration rather than the final pH value of the culture showed a better correlation with % survival on AF-agar.

Effect of initial glucose concentration in buffered media

To examine the effect of initial glucose concentration in cultures kept at a relatively constant pH value, broth medium was supplemented with phosphate buffer (pH 7·05; M/15 final concentration). The initial glucose concentrations were from 0·05 to 3·2 g./l. After growing strains N90 and 18/1042 in the media overnight, samples from the cultures were diluted and plated on AF-broth agar containing 250 μg./ml. and 1 μg./ml. of AF, respectively. Figure 2 shows that the % survival of strain N90 in the presence of AF remained unchanged as long as the pH value of the culture did not change, namely with initial glucose concentrations up to 1·6 g./l. With strain 18/1042, in contrast, the % survival increased even when the pH value remained constant. Hence, the initial glucose concentration of the culture medium influenced the AF-sensitivity of bacteria through final pH value in the former case and not through pH value in the latter case.

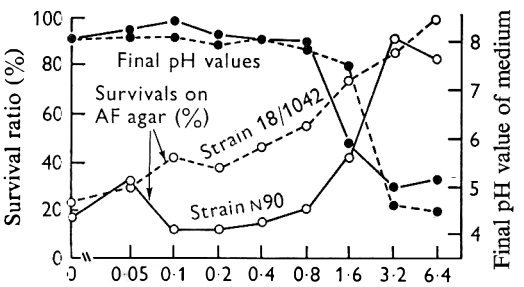

Fig. 1

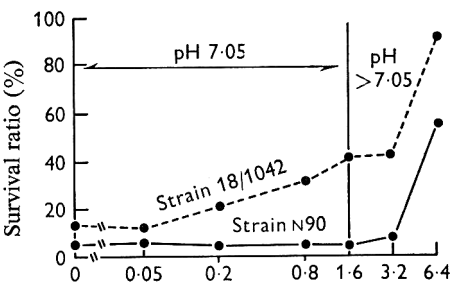

Fig. 2

Fig. 1. Effect of initial glucose concentration in culture medium on acriflavine (AF) sensitivity of *Escherichia coli* K12 AF-sensitive strain 18/1042 and AF-resistant strain N90. Bacteria were inoculated into broth media of graded glucose contents and, after overnight incubation at 37°, dilutions ($1/6 \times 10^6$) of cultures were plated on AF-containing broth agars. The pH values of the cultures at time of sampling were determined after centrifugation. – – –, Strain 18/1042; — strain N90; ○ survival ratio on broth agar containing 1 μg. AF/ml. for strain 18/1042 and 250 μg. AF/ml. for strain N90; ●, Final pH value of medium.

Fig. 2. Effect of initial glucose concentration of broth medium buffered at pH 7·05 by phosphates on the AF-sensitivity of *Escherichia coli* K12. AF-sensitive strain 18/1042 and AF-resistant strain N90. Organisms were inoculated into broth medium of graded glucose concentrations buffered at pH 7·05 by phosphates. After overnight incubation at 37°, dilutions ($1/6 \times 10^6$) of cultures were plated on AF-containing broth agars. – – –, % survival of strain 18/1042 on 1 μg. AF/ml. broth agar; —, % survival of strain N90 on 250 μg. AF/ml. broth agar.

Acriflavine binding capacity and pH value of medium

It is known that bacteria of the AF-sensitive strain 18/1042 bind more AF than do bacteria of the AF-resistant strain N90 (Nakamura, 1966). In connexion with the effects of initial glucose concentration and the pH value of the medium at harvest, on the AF-sensitivity of the bacteria, the effects of these factors on the AF-binding capacity of bacteria are interesting. Bacteria of the two strains were grown overnight

in broth media adjusted initially to pH 5·4, 6·2, 7·2 and 8·0 by M/15 phosphate buffer. The bacteria were then harvested, washed 3 times with 0·85% (w/v) NaCl solution, and suspended in a broth medium containing 5 μg AF/ml. After incubation for 60 min. at 37°, the AF-binding capacity of these bacteria was determined. The binding capacity of either strain increased with increase of initial pH value of the culture medium, as shown in Fig. 3A. When bacteria grown in the standard broth without phosphate buffer were directly transferred into AF-containing broth media of various pH values, the AF-binding of the bacteria increased with increase of pH value (Fig. 3B). Thus, pH value of the preceding culture medium and pH value of the AF-medium were both effective on the AF-binding of the bacteria. Since the AF-sensitivity was lower at low pH values (Fig. 1, 2), the effect of pH value on sensitivity was the inverse of the effect on AF-binding in both strains.

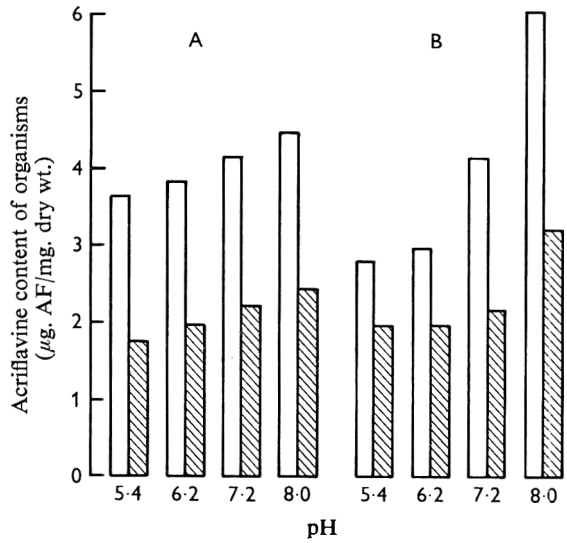

Fig. 3. Effect of pH value on AF-binding by *Escherichia coli* K 12 AF-sensitive strain 18/1042 and AF-resistant strain N90. A, strain N90 (shaded) and 18/1042 (unshaded) were grown overnight in broth media of initial pH values indicated, harvested, well washed and then incubated for 60 min. in the broth containing 5 μg. AF/ml. for measurement of AF-binding. B, Organisms of N90 (shaded) and 18/1042 (unshaded) grown in the standard broth were directly suspended in AF-broth (5 μg./ml.), buffered at the indicated pH values, and then incubated for 20 min. for determination of AF-binding.

Since the AF-sensitivity of 18/1042 organisms decreased as initial glucose concentration of the medium increased even at the same pH value (Fig. 1, 2), the effect of initial glucose concentration on AF-binding capacity was examined by using buffered culture media. Organisms of strains N90 and 18/1042 were grown in broth media containing different concentrations of glucose and adjusted to pH 7·4 by phosphate buffer. After overnight incubation, the bacteria were harvested and washed and their AF-binding capacities determined by suspension in AF-containing broth. Table 1 shows that for both strains the AF-binding varied with the pH value rather than by the initial glucose concentration.

Table 1. *Effect of glucose concentration of buffered medium on acriflavine(AF)-binding capacity of Escherichia coli K-12 AF-sensitive strain 18/1042 and AF-resistant strain N90*

Organisms were inoculated in media buffered at pH 7·4 and containing graded initial concentrations of glucose and incubated overnight. The organisms were then harvested, well washed, and then incubated for 60 min. in broth containing 5 μg AF/ml.

	Initial glucose concentration in culture medium (g./l.)						
Strain	0	0·05	0·20	0·80	1·60	3·20	6·40
	Final pH value of culture						
18/1042	7·31	7·32	7·29	7·19	7·18	6·91	5·80
N90	7·41	7·38	7·36	7·33	7·20	7·05	6·17
	AF-content of organisms after treatment (μg AF/mg. dry wt)						
18/1042	4·31	4·12	3·88	4·05	3·88	3·53	3·16
N90	2·44	2·44	2·53	2·60	2·55	2·38	1·90

Table 2. *The need for multiplication for modification of acriflavine(AF)-binding capacity by the pH value of the culture medium*

Strains 18/1042 and N90 were inoculated with the indicated inoculum sizes into broth media adjusted to pH 6·0 and pH 8·0 and, after overnight incubation at 37°, organisms were well washed and suspended for 60 min. in broth at pH 7·4 containing 5 μg. AF/ml.

Strain	pH of culture medium	Inoculum size (cells/ml.)	Doublings	AF-content (μg. AF/ mg. DW)
18/1042				
a	6·0	$9·1 \times 10^8$	2·2	3·44
	8·0	$9·0 \times 10^8$	1·2	3·48
b	6·0	$9·6 \times 10^7$	5·7	3·47
	8·0	$8·7 \times 10^7$	4·2	3·88
c	6·0	$9·3 \times 10^6$	8·6	3·44
	8·0	$8·1 \times 10^6$	7·6	3·80
d	6·0	$8·9 \times 10^5$	11·9	3·44
	8·0	$9·3 \times 10^5$	11·0	3·85
e	6·0	$9·8 \times 10^4$	15·0	3·44
	8·0	$9·8 \times 10^4$	14·2	3·85
f	6·0	$9·5 \times 10^3$	18·5	3·44
	8·0	$9·5 \times 10^3$	17·8	3·90
N90				
a	6·0	$9·9 \times 10^8$	1·4	1·81
	8·0	$1·1 \times 10^9$	1·0	1·73
b	6·0	$1·1 \times 10^8$	5·0	1·81
	8·0	$7·8 \times 10^7$	4·8	2·08
c	6·0	$9·6 \times 10^6$	8·2	1·78
	8·0	$1·1 \times 10^7$	7·5	2·08
d	6·0	$9·6 \times 10^5$	11·5	1·81
	8·0	$8·6 \times 10^5$	11·1	2·13
e	6·0	$1·0 \times 10^5$	14·7	1·81
	8·0	$9·9 \times 10^4$	14·1	2·12
f	6·0	$1·1 \times 10^4$	17·9	1·82
	8·0	$1·0 \times 10^4$	17·3	2·12

Modification of acriflavine-binding capacity and multiplication of the organisms

The AF-binding capacity of the bacteria was controlled not only by pH value of the AF-containing solution (Fig. 3B), but also by the pH of the medium in which they had been grown (Fig. 3A). The following experiment was designed to see whether growth in the medium was necessary for pH value of the medium to modify AF-binding capacity of the organisms.

Freshly grown organisms of strains N90 and 18/1042 were washed with saline and inoculated into broth media adjusted to pH 6·0 and 8·0, the inoculum sizes being graded as shown in Table 2. After overnight incubation at 37°, the bacteria were washed with saline and suspended in broth containing 5 μg. AF/ml. The AF-content of the bacteria after incubation for 60 min. is represented in Table 2. The pH value of the incubation did not affect the AF-binding when bacteria underwent only one or two doublings in that medium.

DISCUSSION

It is known that the surface of bacteria bears a negative charge under physiological conditions, mainly because of the Donnan equilibrium and the ionization of surface components. Albert (1951) showed that the positive ionization of acridine dyes is an important factor for their antibacterial action, and that an equilibrium between dye and bacteria is rapidly established. Hence, it may be considered that acriflavine (and other basic dyes) binds primarily with negatively charged sites on and near the cytoplasmic membrane, where acriflavine-sensitive metabolic machinery may be located.

It was shown by the experiments described above that the initial glucose concentration of the medium markedly affected the AF-sensitivity of the organisms grown in it, even after they had been well washed. And in the AF-resistant strain N90 a positive correlation was observed between the AF-sensitivity of organisms and the pH value of the medium from which they had been harvested. Three metabolic processes can operate to shift the pH value of culture medium used: (1) unbalanced uptake and output of anions and cations; (2) formation of basic substances from nitrogenous compounds; (3) formation of acids from neutral metabolites. The pH decrease observed with the high glucose media was probably due to the third factor.

The AF-binding capacity of organisms was markedly affected by pH value of the medium in which they had been grown, much acriflavine being bound by organisms from high pH media (Fig. 3A). This suggests a possible explanation for the observation that the organisms from high pH cultures were more sensitive to acriflavine than were those from low pH cultures. Thus, in the case of the AF-resistant strain (N90), and partly in the case of the AF-sensitive strain (18/1042), the effect of initial glucose concentration on the AF-sensitivity of the grown organisms might be accounted for by modification of the AF-binding capacity of organisms because of the pH shift of the culture to more acid values.

The AF-binding increased when the organisms were simply treated with acriflavine at an increased pH value (Fig. 3B) and thus it might appear that, in the experiments illustrated in Fig. 3A, growth at a given pH value might not have been necessary to produce the effect of pH on subsequent AF-binding after washing of the organisms. However, this effect of pH, prior to washing of the organisms and exposure to acriflavine, on the binding of acriflavine was lost when the organisms were not permitted

to multiply at all (unpublished data) or to undergo more than two doublings at a given pH (Table 2). When the organisms were allowed to multiply by a factor more than two or so in a buffered broth medium, the AF-binding capacity characteristic of the pH value of the buffered broth medium in which the organisms were grown was retained even after washing. It is assumed that, when the organisms grew in media of different pH values, irreversible modification corresponding to the pH value occurred in the nature and amount of AF-binding sites.

The present results also show that a large initial glucose concentration increased the AF-tolerance of the AF-sensitive strain (18/1042) even when the pH value of the medium did not change. In the buffered medium the AF-binding capacity of the organisms did not change according to the initial glucose concentration (Table 1). Hence the initial glucose concentration of the culture medium influenced the AF-tolerance of the AF-sensitive strain not through changes in the AF-binding sites. There may possibly be a second process connecting glucose concentration and modification of AF-sensitivity in the AF-sensitive strain. In both of the strains the degree of binding of acriflavine by cells is affected by the pH value of medium but not by the glucose concentration directly.

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ADDENDUM

After submission of this paper the author received a preprint from Dr S. Silver. They include the same experimental results as in this paper with respect to the effect of pH of acriflavine solution on the acriflavine uptake of *Escherichia coli.* SILVER, S., LEVINE, E., & SPIELMAN, P. M. Acridine binding by *Escherichia coli*: pH dependency and strain differences. Submitted to *J. Bact.*

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Nutritional Requirements of *Pasteurella tularensis* for Growth from Small Inocula

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SUMMARY

The ability of *Pasteurella tularensis* to grow from small inocula (less than 10^5 organisms) was found to be critically dependent upon the supply of materials produced by this organism (growth-initiating substance, GIS). In contrast, large inocula showed no requirement for added GIS. The growth-promoting activity of GIS was enhanced in the presence of blood. The evidence favours the conclusion that the inoculum-dependent growth characteristics of *P. tularensis* are attributable to a nutritional heterogeneity of the bacterial population. According to this interpretation, GIS is produced by a relatively small number of bacteria in the population and is excreted by them into the surrounding culture fluid. The excreted material enables growth of the GIS-requiring organisms which constitute a major proportion of the total population. This commensal relationship appears to account for the characteristic all-or-none type of growth response elicited by GIS.

INTRODUCTION

Previous studies elucidated the essential nutritional requirements of *Pasteurella tularensis* and led to the development of a chemically defined medium which supported luxuriant growth of various strains of this organism (Mager, Traub & Grossowicz, 1954; Traub, Mager & Grossowicz, 1955). These strains, however, required large inocula (in excess of 10^5 organisms) for growth initiation, both in the defined medium and in complex and specially enriched media, and did not produce colonies in the conventional viable (colony) count procedure. It was thought that the inoculum-dependent growth might reflect a demand of this organism for some endogenously produced metabolite. This assumption was borne out by finding that growth of *P. tularensis* from small inocula could be ensured by supplementing the culture medium with aqueous extracts of the same organism (Mager, 1964). Subsequent work showed that growing cultures of *P. tularensis* produced and accumulated in the medium material capable of initiating growth from single organisms. The present paper describes the conditions which govern the elaboration of the growth-initiating substance (GIS) by *P. tularensis* cultures and the characteristics of its growth-promoting action.

METHODS

Organisms. The various strains of *Pasteurella tularensis* used in this work (see Table 1) originated from the culture collections of Dr C. R. Owen (Rocky Mountain Laboratory, Hamilton, Montana, U.S.A.) and Dr L. Foshay (University of Cincinatti,

Ohio, U.S.A.). The cultures were maintained by weekly transfers on cysteine blood agar (see below).

Media and cultural procedures. The cysteine blood agar (CB) medium used for colony counts was composed of (%, w/v): 0·8, dehydrated nutrient broth (Difco); 1, glucose; 0·5, NaCl; 0·05, cysteine HCl; 2, agar; and 2 % (v/v) sterile defibrinated rabbit blood. The liquid medium (cysteine broth) was of the following composition (%, w/v): 2, Proteose peptone (Difco); 0·5, NaCl; 0·5, glucose; 0·05, cysteine HCl. The minimal medium used for cultivation of a Rhizopus strain was constituted as follows (%, w/v): 0·7, Na_2HPO_4; 0·3, KH_2PO_4; 0·1, NH_4Cl; 0·025, $MgSO_4.7H_2O$; 0·0015, $CaCl_2.2H_2O$; 1, glucose; with thiamine 5 μg./ml., $ZnCl_2$ 15 μg./ml. The media were prepared in distilled water, adjusted to pH 7 and autoclaved at 120° for 20 min. Heat-labile materials were sterilized by filtration through ultrafine sintered-glass filters or Millipore membranes (0·45 μ pore size; from Millipore Filter Corporation, Bedford, Mass., U.S.A.). The sterile solutions and defibrinated blood were added aseptically to the autoclaved basal medium.

Unless otherwise indicated, the following procedure was used to study the growth-initiation requirements of *Pasteurella tularensis*. Inocula of the various strains were prepared from 24 hr cysteine broth cultures. The organisms were washed once with fresh cysteine broth and suspended in the same medium to a final turbidity of 100 units on the scale of the Klett-Summerson photoelectric colorimeter fitted with a no. 42 filter. These suspensions, containing about 5×10^9 organisms/ml. (as estimated by colony counting on CB plates), served for preparing the final inocula by serial dilution in cysteine broth. Two-tenths ml. of bacterial suspension were added to 5 ml. cysteine broth dispensed in Erlenmeyer flasks (25 ml.) and the cultures incubated at 36–37° with continuous shaking in the New Brunswick gyrotory incubator-shaker. Growth was measured turbidimetrically in the Klett photometer (no. 42 filter) or in the Bausch and Lomb Spectronic-20 Colorimeter at 500 mμ. All the growth experiments were run in duplicate and the purity of the cultures routinely examined microscopically.

RESULTS

Colony counts made by a procedure described below established that the minimal size of inoculum required to secure growth in cysteine broth varied with different strains of *Pasteurella tularensis* from 10^5 to 10^8 organisms (Table 1). Confluent growth on the solid CB medium was consistently produced with massive inocula, whereas attempts to obtain colonial growth with appropriately diluted suspensions were unsuccessful. These observations suggested that the growth-determining role of the inoculum size might reflect a need of individual organisms for mutual 'feeding'. To test this, the surface of a CB agar plate was evenly inoculated by spreading about 1000 organisms of the Pasteurella S & D strain and a loopful of a 24 hr culture of the same strain in broth was streaked across the plate to produce a narrow zone of confluent growth. During incubation discrete colonies sprang up in successive crops at increasing distances from the streak of confluent growth, the sequential growth onset of the colonies being manifested by their distally diminishing size. It appeared reasonable to conclude that material produced by the organisms in the zone of confluent growth and excreted by them into the surrounding medium had enabled the more distant isolated organisms to produce colonies.

The presence of a growth-initiating factor was shown more directly in 24–48 hr broth cultures of *Pasteurella tularensis* sterilized by Millipore membrane filtration. Such sterile culture filtrates when added in small amounts to fresh cysteine broth gave growth of the various *P. tularensis* strains from as few as 10–100 organisms. The minimal quantity of culture-filtrate required for growth initiation was inversely related to the size of inoculum (Table 2). With a constant inoculum size the time lag

Table 1. *Inoculum-dependent growth of various strains of Pasteurella tularensis; effect of growth-initiating substance (GIS)*

Each strain was grown in 5 ml. cysteine broth. Where indicated, (GIS) was added in the form of 0·2 ml. sterile filtrate of a 70 hr culture of *P. tularensis* strain S & D. Growth was followed during 6 days of incubation at 37° with continuous shaking. The signs + and − denote maximal growth and no growth, respectively.

	Inoculum size (no. of organisms)									
	10^2		10^4		10^5		10^7		10^8	
	+GIS	−GIS	+GIS	−GIS	+GIS	−GIS	+GIS	−GIS	+GIS	−GIS
Strain	Growth response									
SCHU highly virulent	−	+	−	+	+	+	+	+	+	+
SCHU-M16 highly virulent	−	+	−	+	+	+	+	+	+	+
VAVENLY moderately virulent	−	+	−	+	−	+	+	+	+	+
425-F4G moderately virulent	−	+	−	+	−	+	+	+	+	+
30110 slightly virulent	−	+	−	+	−	+	−	+	+	+
JAP avirulent	−	+	−	+	−	+	−	+	+	+
VACCINE avirulent	−	+	−	+	+	+	+	+	+	+
S & D avirulent	−	+	−	+	−	+	−	+	+	+

Table 2. *Growth-promoting effect of Pasteurella tularensis strain S & D culture filtrate in relation to inoculum size*

The standard inoculum of *Pasteurella tularensis* strain S & D contained 2×10^9 organisms. The culture filtrate was derived from a 48 hr cysteine broth culture of *P. tularensis* strain S & D.

Dilution of the standard inoculum	Amounts of culture filtrate added to fresh cysteine broth (%, vv)				
	0	1	2·5	5	10
	Growth after incubation for 100 hr (Klett units)				
None	330	310	320	340	300
10^{-2}	0	320	320	310	320
10^{-3}	0	340	330	320	310
10^{-5}	0	0	0	310	310
10^{-7}	0	0	0	0	340
10^{-8}	0	0	0	0	0

for the appearance of visible growth decreased with increasing concentrations of filtrate (Fig. 1). Once initiated, however, growth proceeded in all instances at a nearly equal rate, attaining the same final yield irrespective of the duration of the lag (Table 2; Fig. 1). Essentially similar results were obtained on examining the ability of added culture filtrate to promote colonial growth on CB plates. Here again, the requisite

threshold dose varied within certain limits in roughly inverse proportion to the inoculum size (Fig. 2). The number of colonies, however, yielded by a given inoculum remained practically unaltered on increasing the amount of the filtrate added above the minimal effective dose. Thus, the all-or-none type of growth-promoting effect exhibited by the culture filtrates differed essentially from the graded dose response observed in conventional bioassays of vitamins and other essential metabolites.

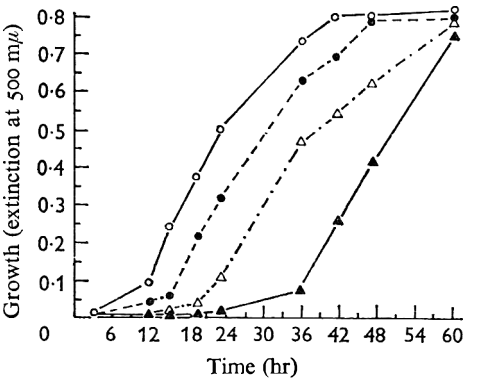

Fig. 1

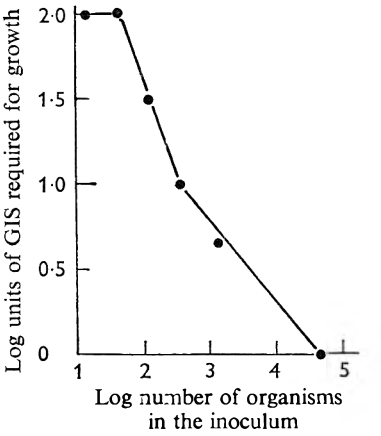

Fig. 2

Fig. 1. Growth curves of *Pasteurella tularensis* in cysteine broth supplemented with different amounts of culture filtrate as asource of growth-initiating substance (GIS). Sterile filtrate from a 48 hr culture of *P. tularensis* strain S & D was added to fresh double-strength cysteine broth and the final volume was made up with water to 15 ml. After inoculation with about 2×10^3 organisms of *P. tularensis* strain S & D, growth was determined turbidimetrically at intervals. Amounts of culture filtrate added: ○—○, 2 ml.; ●-·-●, 1 ml.; △—·—△ 0·5 ml.; ▲—▲, 0·1 ml.

Fig. 2. Dose response curve of *Pasteurella tularensis* strain S & D to growth-initiating substance (GIS) as a function of inoculum size. One unit of GIS is the amount required for growth of 200 organisms of *P. tularensis* strain S & D on blood-cysteine agar medium after incubation for 48 hr at 37°.

Requirement of different Pasteurella tularensis strains for growth-initiating substance

Apart from the avirulent Pasteurella S & D strain which was used throughout this study, a random assortment of 7 other strains *P. tularensis* of diverse origin and of widely different degrees of virulence was examined for their GIS requirements. All the strains tested showed essentially the same all-or-none pattern of response to GIS, although they differed considerably with regard to the minimal inoculum capable of growth without added GIS (Table 1). This quantity, which will be referred to as 'critical inoculum size', was fairly constant for each strain, provided that the conditions of subcultivation were rigidly standardized. It may be concluded, therefore, that the requirement for GIS constitutes a characteristic attribute of the *P. tularensis* species.

Occurrence of the Pasteurella growth-initiating substance in other micro-organisms

The production of GIS by organisms other than *Pasteurella tularensis* was not systematically studied, but its excretion by widely different species of bacteria and

moulds was frequently noted when contaminants appeared on the agar plates used for GIS assays. Of particular interest in this respect was an unidentified Rhizopus species which produced GIS in amounts largely exceeding those yielded by Pasteurella S & D. The formation of GIS by the Rhizopus in a minimal medium (see Methods) was markedly stimulated by trace amounts of Zn^{2+}. Further, as with *P. tularensis*, only a small portion of GIS elaborated by the Rhizopus was retained in the mycelium; the bulk of it was excreted into the culture medium.

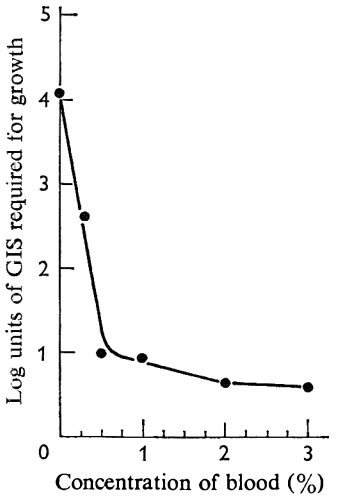

Fig. 3

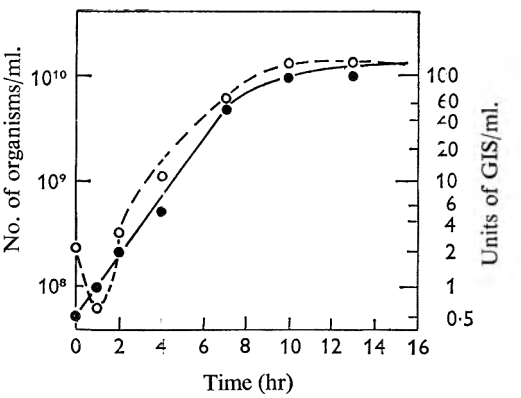

Fig. 4

Fig. 3. Sparing effect of blood on requirement of *Pasteurella tularensis* for growth-initiating substance (GIS). The numbers on the ordinate refer to threshold amounts of GIS required for growth of an inoculum of 200 organisms of *P. tularensis* strain S & D on CB agar in the presence of various amounts of blood.

Fig. 4. Time-course of growth and GIS production by cultures of *Pasteurella tularensis* in cysteine broth. Inoculum: $2{\cdot}5 \times 10^{8}$ organisms of *P. tularensis* strain S & D. Dashed line: growth; solid line: GIS produced.

Sparing effect of blood on requirement for growth-initiating substance

The quantitative requirement for GIS was substantially decreased by the addition of rabbit blood to the medium. The adjuvant effect of blood on GIS activity was nearly maximal at a 2 % (v/v), whereas without added GIS no growth resulted even when the amount of blood was increased to 20 % (v/v). An experiment on the sparing effect of blood on GIS requirement is shown in Fig. 3. The precise quantitative aspects of this relationship varied considerably in different experiments, for reasons not yet apparent. When plasma and washed red blood cells were tested separately, the adjuvant activity proved to be present mainly in the intact red cells. The activity of blood declined rapidly upon haemolysis induced by osmotic shock or by freezing and thawing.

Assay procedure

Based on the information derived from the above experiments, the following procedure for assaying GIS activity was adopted. Serial dilutions of culture fluid or other material to be tested for GIS activity were added to 30 ml. portions of the basal

CB medium (without blood) dispensed in suitable flasks. After autoclaving at 120° for 20 min., the medium was cooled in a water bath to about 50° and sterile defibrinated rabbit blood added aseptically to a final concentration of 2 % (v/v). After thorough mixing, the contents of each flask were poured into two sterile Petri dishes and allowed to solidify. The surface of each pair of duplicate plates was inoculated with about 20 and 200 organisms of Pasteurella S & D, respectively, by spreading 0·1 ml. of a culture suitably diluted in saline solution. The plates were examined after incubation for 48 hr at 37°. The value of 1 unit of GIS was assigned to the amount which enabled growth of the 200-organism inoculum but not of the 20-organism inoculum.

Kinetics of production of growth-initiating substance by Pasteurella tularensis

In an attempt to study the kinetics of GIS production in relation to the rate of growth proliferation, Pasteurella S & D was grown in cysteine broth and samples withdrawn at various intervals for colony counts and GIS titrations, which were run concurrently. As evident from Fig. 4, contrary to the typical lag-phase or even initial decline of the colony count displayed by the growth curve, there was no detectable delay in the start of GIS production. On the other hand, during the logarithmic and stationary phases of the growth cycle the time-course of GIS production closely paralleled the rate of division of the organisms.

Partition of growth-initiating substance between organisms and culture fluid

The distribution of GIS was determined in 100 ml. samples of 24 hr cultures of Pasteurella S & D. The organisms deposited by centrifugation for 20 min. at 20,000*g* at 5° were washed three times with 0·85 % NaCl solution to remove traces of GIS. The washed organisms were suspended in 20 ml. distilled water and after incubation for 30 min. at 37° were deposited by centrifugation as above. This treatment liberated into the suspending medium GIS activity which amounted to about 0·5–1 % of its overall titre in the culture (100 units out of about 10,000 units GIS/100 ml. culture fluid). A similar pattern of GIS distribution was found with other *P. tularensis* strains tested (SCHU, VAVENLY, 3001). The release of the cell-bound GIS activity appeared to be attributable to the injurious effect of the hypotonic environment (distilled water) on the permeability barrier of the cell (Mager, 1959), since similar treatment with 0·85 % NaCl solution did not release detectable amounts of GIS. The leakage of GIS coincided with the accumulation in the suspending fluid of considerable amounts of material with a maximum extinction at about 260 mμ. The yields of cell-bound GIS extracted by heating the organisms for 5 min. in a boiling water bath or by ultrasonic treatment for 5 min. in a 10 KC Raytheon oscillator were nearly identical to the amount of GIS liberated by osmotic shock.

Inoculum-dependent requirement for growth-initiating substance as a reflection of population heterogeneity

The available data did not permit a decision as to whether the requirement for added GIS was governed by the initial population density of the culture, or by the number of organisms in the inoculum. By varying the size of the inoculum and the volume of cysteine broth it was found that the ability of *Pasteurella tularensis* to multiply without added GIS was determined solely by the number of organisms pro-

vided in the inoculum (Table 3). Thus, an inoculum of *P. tularensis* strain S & D, which was within the minimal size range required for growth initiation in the absence of an exogenous source of GIS, exhibited an unimpaired capacity for growth, even when the initial population density was reduced up to 400-fold by increasing the volume of the culture medium. Furthermore, when 100 ml. broth were inoculated with the same 'minimal' number of organisms as above and dispensed in 5 ml. portions to 20 flasks, only 4 flasks showed growth without supplementation by GIS.

Table 3. *Effect of inoculum size and initial population density on growth of Pasteurella tularensis without added growth-initiating substance*

The various inocula, as defined by the number of colony-forming units of *P. tularensis* strain S & D, were added each to different amounts of cysteine broth, as indicated in the Table. Final growth was recorded after incubation for 5 days at 37° with continuous shaking.

Size of inoculum (colony count)	Volumes of culture medium (ml.)				
	2·5	5	25	100	1000
	Growth*				
5×10^7	+	+	+	+	+
$2{\cdot}5 \times 10^7$	+	+	+	+	+
10^7	−	−	−	−	−

* +, Maximal growth; −, no growth.

These results suggested that the growth-controlling effect of inoculum size reflected a heterogeneity of the bacterial population, the latter comprising a minor proportion of 'autotrophic' organisms and a majority of organisms which were 'auxotrophic' with respect to GIS. This conclusion agreed with the additional observation that the critical inoculum size was not appreciably affected by repeated washing with fresh broth or 0·85 % NaCl solution, thus arguing a possible role of carry-over of traces of GIS adherent to the organisms.

Corroborative evidence in favour of the above interpretation was afforded by an experiment to test the ability of Pasteurella S & D, prevented from multiplying by streptomycin, to serve as a source of GIS for a subminimal inoculum of a streptomycin-resistant Pasteurella mutant (Table 4). This experiment was similar in principle to that of Puck & Marcus (1955), in which non-proliferating animal cells obtained by X-ray radiation were shown to function as growth-supporting 'feeders' for the untreated cells. As evident from Table 4, however, an inoculum of the streptomycin-resistant Pasteurella mutant, which was about 5-fold lower than the critical size required for GIS-independent growth, was not induced to multiply in the presence of streptomycin, by adding streptomycin-sensitive pasteurellas in a 20-fold excess over the critical inoculum size. In a separate control experiment streptomycin did not interfere with the excretion of GIS by the streptomycin-resistant mutant strain.

Numerous unsuccessful attempts were made to arrive at segregation of a uniform population by rapid successive passages of large inocula, repeated subcultivation of growth produced with highly dilute inocula in the presence of GIS, and serial streaking of single colonies. The only positive result of these efforts was a transient shift in the ratio of the two 'nutritional' varieties as reflected in the difference of critical inoculum size.

Further insight into the nature of the population-dependent growth phenomenon was gained by depleting the organisms of their endogenous GIS content by osmotic shock treatment. The organisms so treated lost the ability to multiply without added GIS, even when the inoculum used was 1000-fold greater than that required for GIS-independent growth of the untreated organisms. When supplemented with GIS, however, the osmotically shocked culture proved to contain nearly the same number of colony-forming units as did the untreated control. The progeny of the osmotically shocked culture reverted after one to two passages to the original population-dependent growth pattern.

Table 4. *Inability of streptomycin-inhibited organisms of Pasteurella tularensis strain S & D to supply growth-initiating substance (GIS) to streptomycin-resistant mutants of the same strain*

The GIS-feeding capacity of non-growing organisms of *P. tularensis* strain S & D was tested in 5 ml. cysteine broth supplemented with streptomycin sulphate (200 μg./ml.), using an inoculum composed of a subminimal amount (10^7) streptomycin-resistant organisms of Pasteurella strain S & D (i.e. an amount incapable of growth without added GIS) and an amount (10^9) of streptomycin-sensitive mutant organisms representing about a 200-fold excess of the critical inoculum size of this strain. The culture was incubated for 70 hr at 37° with continuous shaking. Parallel controls were run with separate inocula of each mutant strain grown in the presence and in the absence of streptomycin. Growth of the parent strain was completely inhibited by streptomycin sulphate at 10 μg./ml. medium; the resistant mutant grew normally with amounts of streptomycin sulphate as high as 2 mg./ml. medium. In the absence of streptomycin both mutant strains showed equal growth response to GIS supplied in the form of a sterile filtrate from a 48 hr culture of *P. tularensis* strain S & D.

Composition of inoculum (no. of organisms)		Growth*	
Streptomycin-sensitive	Streptomycin-resistant	Without streptomycin	With streptomycin 200 μg./ml.
10^9	0	+	−
5×10^7	0	+	−
10^7	0	−	−
0	5×10^7	+	+
0	10^7	−	−
10^9	10^7	+	−

* + Maximal growth; − no growth.

DISCUSSION

The requirement of *Pasteurella tularensis* for an endogenously produced metabolite (GIS), as a factor essential for initiating division, accounts for the inoculum-dependent growth characteristics displayed by virulent and avirulent strains of this organism in artificial culture media. Such a requirement is patently inconsistent with the notoriously high virulence of the tularaemia agent, which implies a virtual capacity of a single organism to multiply *in vivo* and to produce a fatal infection in mice. The possibility that a substance similar to GIS or a functionally equivalent compound may be present in the host tissues appears rather unlikely, in view of the negative outcome of an extensive search for such a growth factor in a variety of tissue extracts and other natural materials (Traub *et al.* 1955; Halmann & Mager, 1967). Another possible explanation is that the requirement for GIS exhibited by small inocula *in vitro* stems

from a metabolic derangement due to the inadequacy of the artificial environment in imitating the requisite physico-chemical conditions prevailing *in vivo* (see Traub *et al.* 1955). It is not unlikely that such a mechanism may underlie also the sparing effect exerted by blood on GIS requirement, which appears to be mediated by some labile and as yet elusive factor apparently associated with intact red cells. The currently available evidence, however, does not warrant a definite choice between these alternative conceptions.

The seeming paradox implicit in the demand of *Pasturella tularensis* for the exogenous supply of a factor which is abundantly synthesized by the same organism in the course of its growth appears to be resolved by the evidence for a genetically determined nutritional heterogeneity of the cell population prevailing in the *P. tularensis* cultures. The coexistence of a minority of autotrophic cells and a vast majority of auxotrophic mutants is based on the ability of the autotrophs to manufacture and excrete a large excess of GIS, largely sufficient to cater to their auxotrophic offsprings. This commensal relationship offers an explanation for the apparent autocatalytic kinetics of GIS implied by its all-or-none action pattern. The considerable stability observed in the population dynamics of the different strains of *P. tularensis* seems to be determined on the one hand by their high mutation rates and on the other hand by the lack of selection pressure inherent in the 'feeding' phenomenon which cancels the advantage of autrophy for the survival of the species.

The association of an enhanced requirement for added GIS with the release of the cell-bound GIS activity induced by osmotic shocking of the cells suggests that the autotrophic growth capability is critically dependent upon a certain minimum level of endogenous GIS. The as yet unexplored question of the chemical identity of the cell-associated and diffusible forms of GIS may be relevant to the understanding of the factors governing the retention and excretion of GIS by *Pasteurella tularensis* cells.

The inoculum-dependent nature of the GIS requirement and some general characteristics of its growth-promoting action bear a distinct resemblance to those of the 'lag-reducing factors' described by Lankford and his associates in certain Bacillus species and designated by them as 'schizokinens' (Sergeant, Lankford & Traxler, 1957; Lankford, Walker, Reeves, Nabbut, Byers & Jones, 1966). On the other hand, the mechanism underlying the population-dependent growth of *Pasturella tularensis* differs essentially from that observed in mammalian cell cultures, in which the requirement for diffusible endogenous factors appears to be governed by the density of the cell population (see Eagle, 1965).

The authors are greatly indebted to Dr C. R. Owen, custodian of the stock cultures at the Rocky Mountain Laboratory in Hamilton (Montana, U.S.A.), for her generous help in providing us with the *Pasteurella tularensis* strains and detailed information regarding their origin, cultural characteristics and virulence.

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An Endogenously Produced Substance Essential for Growth Initiation of *Pasteurella tularensis*

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SUMMARY

Material produced by growing *Pasteurella tularensis* and required for the initiation of growth of *P. tularensis* from small inocula (growth-initiating substance, GIS) was purified by Sephadex gel filtration and ion-exchange resin chromatography. The purified material was characterized as an anionic compound of low molecular weight, heat-stable at neutral pH and in dilute alkali but destroyed by heating in dilute acid. Out of large variety of known nutrients tested, only iron salts and some iron-chelating compounds (siderамines) replaced partially GIS in supporting growth of different *P. tularensis* strains from small inocula. GIS formed complexes with iron and copper ions. The production of GIS by *P. tularensis* in a chemically defined medium was enhanced by added ornithine. Isotopic evidence established the role of ornithine as a biosynthetic precursor of GIS.

INTRODUCTION

Halmann, Benedict & Mager (1967) showed that the growth of *Pasteurella tularensis* from small inocula was governed by a specific requirement for endogenously produced material termed 'growth-initiating substance' (GIS). The present paper is concerned with the purification of GIS, the determination of its characteristic properties and a study of its biosynthesis in a chemically defined medium.

METHODS

The various biological compounds used were acquired from the Sigma Chemical Co. (St Louis, Miss., U.S.A.). The Sephadex preparations were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The other resins used were purchased from the Bio-Rad Laboratories (Richmond, California, U.S.A.). Radioactive compounds were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Spectra were determined with a Beckman model DU spectrophotometer (Fullerton, California, U.S.A.) or with the Unicam SP 800 double-beam recording spectrophotometer (Cambridge, England), with silica cells of 1 cm. light path. Radioactivity was measured in a Tri-Carb model 314 F liquid scintillation counter (Packard Instrument Co., Downers Grove, Ill., U.S.A.), with Bray's scintillator solution (Bray, 1960). Sephadex gel filtration was done according to the procedure described by Flodin (1961). Paper electrophoresis was done in a conventional apparatus under the conditions outlined below. The location of the GIS-active material on the electropherograms was

facilitated by using ^{14}C-labelled preparations of GIS (see below). The radioactive spots were detached with the aid of a Packard radiochromatogram scanner (model 7201) and eluted with distilled water. The eluted material was tested for GIS activity by determining the highest dilution capable of supporting growth on cysteine-blood agar of an inoculum containing 200 organisms of *P. tularensis* strain S & D (see Halmann *et al.* 1967). The cultural procedures and other experimental details were as described by Halmann *et al.* (1967).

RESULTS

Purification of growth initiating substance

A 48 hr cysteine broth culture of the *Pasteurella tularensis* strain S & D was centrifuged for 20 min. at 20,000 *g*. The resulting bacterial deposit was discarded and the supernatant fluid concentrated in a flash evaporator at 40° and then passed through a 14 × 200 mm column Sephadex G-25. The column was then washed with distilled water and the effluent collected in 15-drop portions, each fraction being assayed for GIS activity. Under these conditions GIS emerged from the column as a rather sharp peak, preceded by proteins and nucleic acids and followed by the bulk of amino acids, sugars and salts. The GIS-active samples were pooled and applied to a 9 × 160 mm column of the anion-exchange resin Dowex 1 chloride. After rinsing the column with distilled water, the adsorbed material was eluted with stepwise increasing concentrations of NaCl, successive 10 ml. samples of effluent being collected and tested for GIS activity. The elution profile of GIS is illustrated in Fig. 1.

The combined GIS-active fractions were de-salted by filtration through a Sephadex G-10 column, treated batchwise with the cation-exchange resin Dowex 50 (hydrogen form) and then freeze-dried. The overall recovery of GIS in the final material amounted to 70–90 % of the original GIS activity of the culture. In some instances, the fractionation on the Dowex 1 column was repeated with a NaCl-gradient elution system. The specific activities of the purest GIS preparations obtained were in the range of 5 units/μg. dry weight (a unit is an amount of GIS enabling growth of a 200-organism inoculum of *Pasturella tularensis* strain S & D on blood-cysteine agar; see Halmann *et al.* 1967). However, the degree of purity and homogeneity of these preparations could not be precisely evaluated by the criteria available.

From a brief survey of other microbial species, culture filtrates of a Rhizopus (see Halmann *et al.* 1967) were found to offer a rich and convenient source of GIS. The substance was isolated in high yields by the procedure described above; its properties, including biological potency, proved to be identical to those of the material produced by the *Pasteurella tularensis* strains.

Properties of growth-initiating substance

The material isolated by the above procedure was readily soluble in water but insoluble in a variety of organic solvents (e.g. absolute ethanol, *n*-propanol, acetone, diethyl ether, chloroform, benzene). The activity of GIS was stable to autoclaving for 90 min. at 120° in neutral solution and in 0·1 N-NaOH. It withstood treatment for 2 hr at about 25° with 0·5 M-H_2O_2, 0·3 M-nitrous acid and 0·05 M-2,4-dinitrofluorobenzene solution. Boiling GIS in 0·1 N-HCl resulted in a rapid destruction of its activity. Occasionally, the unpredictable occurrence of a drastic loss of activity was noted in neutral aqueous solutions of GIS. Further work showed that this 'spon-

taneous' inactivation of GIS was induced by its exposure to visible light. Moreover the photo-inactivation was found to be critically dependent upon the presence of traces of iron in the GIS solution and was completely prevented by treating the solution with the metal-chelating resin Chelex 100.

The low molecular weight of GIS was indicated by its diffusibility through cellophan membranes and its retention by Sephadex G-10. The anionic properties of the substance were reflected in its adsorption on anion exchange resins (Dowex 1, Amberlite IR-120). In addition, a ^{14}C-labelled preparation of GIS obtained by growing *Pasteurella tularensis* in the presence of [^{14}C]ornithine (see below) exhibited anodic migration as a discrete radioactive spot, when subjected to electrophoresis on Whatman no. 3 MM paper in 0·025 M-barbital buffer (pH 8·6).

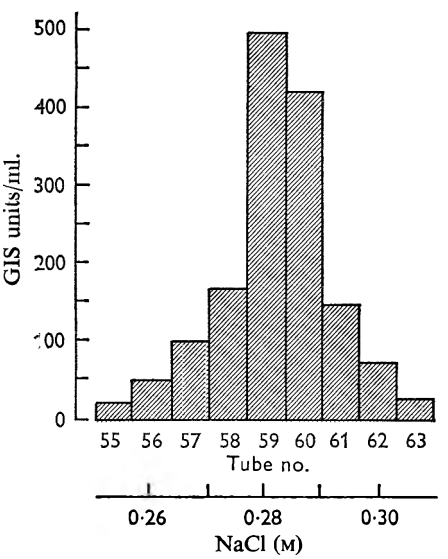

Fig. 1

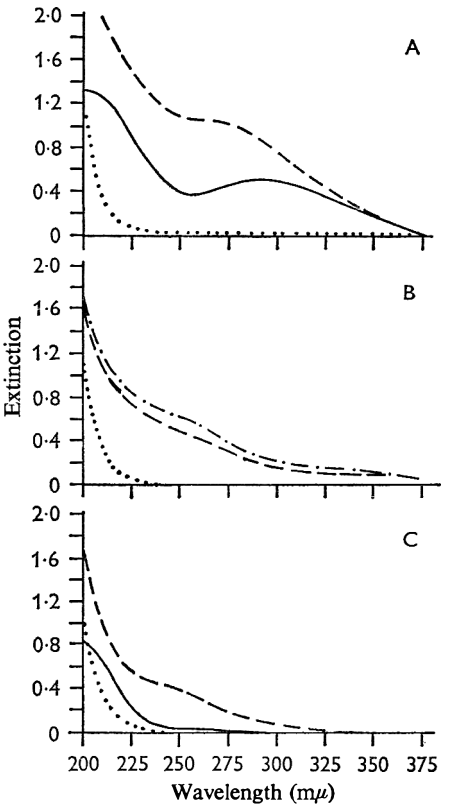

Fig. 2

Fig. 1. Chromatography of growth-initiating substance GIS on a 9 × 160 mm column of Dowex 1-X8 (100–200 mesh, chloride form); elution profile. Each fraction was assayed for GIS activity as described by Halmann, Benedict & Mager (1967). For unit definition see legend to Table 1.

Fig. 2. Spectral changes attending formation of complexes between growth-initiating substance GIS and metals. The metals and GIS were dissolved in 1 ml. distilled water in the amounts specified below and the spectra were recorded with a Unicam SP 800 spectrophotometer, with distilled water in the reference cell. A: ···, GIS alone, 50 μg.; ——, $FeCl_3$ alone, 0·25 μmoles; - - -, GIS, 50 μg. + $FeCl_3$, 0·25 μmoles. B: ···, GIS alone, 50 μg.; ——, $FeSO_4$ alone, 0·25 μmoles; - - -, GIS, 50 μg. + $FeSO_4$, 0·25 μmoles, 5 min after mixing; - · -, GIS 50 μg. + $FeSO_4$, 0·25 μmoles, 60 min. after mixing. C: ···, GIS alone, 50 μg.; ——, $CuSO_4$ alone, 0·5 μmoles; - - -, GIS, 50 μg. + $CuSO_4$, 0·5 μmoles.

GIS treated with the Dowex 50 cation exchanger and then titrated with 0·1 N-KOH with the aid of an automatic titrator (from Radiometer, Copenhagen, Denmark) yielded a pK_a value of 5·1 and a neutralization equivalent of about 280. The latter value was reasonably near the maximum molecular weight of 274–276, as determined with an Atlas PH-4 (Bremen, Germany) mass spectrometer. Details of these results will be presented elsewhere.

Qualitative tests for phosphate and sulphur were negative. Negative results were also obtained with ninhydrin, the Folin phenol reagent (Lowry, Rosebrough, Farr & Randall, 1951) and the anthrone sugar reagent (Seifter, Dayton, Novic & Montwyler, 1950). Elemental analysis gave the values (corrected for 2 % ash): C, 35; H, 5·4; N, 4·8.

The most highly purified preparations of GIS showed no significant extinction in the range of about 250–800 mμ and a progressively increasing end absorption in the shorter wavelengths (see Fig. 2).

Metal-binding capacity of growth-initiating substance

The iron-dependent photo-inactivation of GIS suggested a possible chemical interaction between these substances. The formation of different GIS complexes with both ferrous and ferric iron salts was revealed by their distinctive ultraviolet (u.v.) spectra (Fig. 2A, B). The spectrum of the GIS complex with $FeSO_4$ exhibited a progressive change towards the stable ferric complex. This change was prevented by bubbling nitrogen through the GIS + $FeSO_4$ solution.

GIS also produced a complex with Cu^{2+}, with a characteristic absorption in the ultraviolet region (Fig. 2C). The rapid inactivation of GIS which occurred on heating it at pH 1–2 manifested itself in the ultraviolet spectrum of the copper complex by a shift of extinction towards shorter wavelengths. The biologically inactive compound was further differentiated from native ^{14}C-labelled GIS (see below) by the faster anodic migration of the inactive compound on paper electrophoresis in 0·025 M-acetate (pH 4·7).

Production of growth-initiating substance in a chemically defined medium

Although the chemically defined medium of Traub, Mager & Grossowicz (1955) supported profuse growth of different strains of *Pasteurella tularensis*, the amount of GIS produced in it amounted to about 1–2 % only of the average GIS yield obtained in the complex cysteine broth. Addition of Difco Proteose peptone (0·5 %, w/v) or yeast extract (0·25 %, w/v) to defined medium and then growing the organism permitted a 20 to 100-fold increase in GIS titre. About a fourfold purification of the material which increased GIS production (in terms of activity/dry weight) was achieved by filtering a 20 % (w/v) solution of the Difco yeast extract through a Sephadex G-25 column and treating the combined GIS-stimulatory fractions with Norit A charcoal. The purified material exhibited no appreciable extinction over the spectral range 220–700 mμ, and yielded with the ninhydrin reagent (Yemm & Cocking, 1955) approximately 30 % of the colour obtained with the equivalent amount of untreated yeast extract. The activity of the preparation in promoting GIS production in the defined medium was not appreciably decreased by autoclaving at 15 lb./sq.in. in the presence of N-NaOH or 6N-HCl for as long as 6 hr.

Subsequent work showed that the active component of the yeast extract could be replaced by ornithine. Addition of glutamate resulted in a further enhancement of

GIS production, but the magnitude of this effect varied considerably in different experiments. Citrulline was less active than ornithine; arginine produced only a very limited degree of enhancement of GIS production (Table 1).

Table 1. *Effect of added glutamate and basic amino acids on production of growth-initiating substance by Pasteurella tularensis strain S & D*

The composition of the chemically defined medium was as described by Traub, Mager & Grossowicz (1955). The GIS titres were determined after 48 hr incubation at 37° by adding serial dilutions of the sterile culture filtrate to cysteine blood agar (CB). One unit of GIS is defined as the quantity required for growth of a 200-organism inoculum of *P. tularensis* strain S & D on CB agar after 48 hr incubation at 37° (see Halmann, *et. al*, 1967).

Additions to the defined medium (μg./ml.)	GIS (units/ml.)
None	3
L-Glutamate, 1000	5
Yeast extract, 2500	60
Purified fraction of yeast extract, 600	60
L-Ornithine, 30 + L-glutamate, 1000	30
L-Ornithine, 100 + L-glutamate, 1000	90
L-Ornithine 100	20
L-Citrulline, 30	30
L-Citrulline, 100 + L-glutamate, 1000	30
L-Arginine, 100 + glutamate, 1000	8
L-Arginine, 3000 + L-glutamate, 1000	8

Table 2. *Incorporation of various ^{14}C-compounds into growth-initiating substances GIS*

The ^{14}C-labelled compounds were added to 3 ml. of defined medium of Traub, Mager & Grossowicz (1955) at a final specific activity of about 5 μc/μmole. After incubation at 37° for 48 hr the organisms were removed by centrifugation. To the supernatant fluid 2000 units GIS were added as carrier and re-isolated by chromatography on a Dowex 1 column. Under these conditions all the compounds tested separated well from GIS. The corresponding fractions were tested for GIS activity and for radioactivity. The values for GIS-bound radioactivity were corrected for quenching and for % recovery of GIS which varied in different experiments from 80 to 100%. In calculating the radioactivities of DL-amino acids, the L-isomer was assumed to be the only biologically active form. Other conditions were as in Fig. 1.

Compound tested	Amount of radioactivity supplied in the medium (counts/min.) (*A*)	Amount of radioactivity recovered in the GIS fraction (counts/min.) (*B*)	% radioactivity incorporated into GIS ($B \times 100/A$)
[2-^{14}C]DL-Ornithine	20,000	13,890	69
[5-^{14}C]DL-Ornithine	36,000	23,500	65
[^{14}C]L-Lysine, uniformly-labelled	130,000	0	0
[^{14}C]L-Valine, uniformly labelled	79,000	0	0
[^{14}C]L-Glutamate, uniformly-labelled	57,000	0	0
[^{14}C]D-Glucose, uniformly-labelled	680,000	2,100	0·31
[^{14}C]Acetate	650,000	1,450	0·22

Incorporation of labelled precursors into growth-initiating substance

The above data pointed to a possible role of ornithine as a biosynthetic precursor of GIS. This possibility was tested by growing *Pasteurella tularensis* strain S & D in the defined medium supplemented with ^{14}C-labelled ornithine and isolating GIS by

the chromatographic procedure described above (see legend to Table 2). The distribution of the radioactivity closely paralleled the fractionation profile of GIS. When the isolated ^{14}C-labelled GIS was subjected to paper electrophoresis in barbital buffer (pH 8·6), the radioactivity and the biological activity migrated together. Regardless of whether the isotopic label was in the α or δ position of ornithine, the percentage of radioactivity incorporated into GIS was practically the same (Table 2). It appeared likely, therefore, that ornithine was incorporated as a whole into the molecule of GIS.

Several other ^{14}C-labelled amino acids (e.g. glutamate, lysine, valine) showed no significant incorporation into GIS. Substantial incorporation was obtained with [1-^{14}C]acetate and uniformly labelled [^{14}C]glucose (Table 2). However, the data recorded in Table 2 do not represent true incorporation yields of the various compounds tested (in terms of relative specific activities), because of the disparity in the extent of dilution of their isotope contents by the respective endogenous pools.

Specificity of growth-initiating substance; relationship to sideramines

A large number of growth factors, including vitamins, coenzymes and other known essential metabolites, did not replace GIS in promoting growth from small inocula of *Pasteurella tularensis* on blood cysteine agar. The substances tested and found inert (at mg./100 ml. blood-cysteine agar) were: acetic acid, 10; *N*-acetyl glucosamine, 10; $NaHCO_3$, 20; catalase (beef liver, crystalline), 5; citric acid (sodium salt), 10; cobamide coenzyme (dimethyl benzimidazole derivative), 0·01; coenzyme A (70 % pure), 2; cyanocobalamine, 0·01; *m*-diaminopimelic acid, 5; D-galactosamine, 10; D-glucosamine, 10; L-glutamine, 10; flavin-adenine dinucleotide, 1·5; flavin-adenine mononucleotide, 1; haemin, 0·1; *m*-inositol, 10; mixture of nucleosides (adenosine + guanosine + uridine + cytidine + thymidine), 2 mg. each; mixture of nucleoside-5′-monophosphates (AMP + GMP + UMP + CMP + TMP), 2 mg. each; nicotinamide-adenine dinucleotide, 1; nicotinamide-adenine dinucleotide phosphate, 1; α-oxoglutaric acid (sodium salt), 10; pyridoxal, 2; pyridoxal phosphate, 2; thioctic acid (sodium salt), 1; thioctic acid amide, 1; thiamine pyrophosphate, 1; ubiquinone, 0·5.

The iron-complexing ability of GIS and the precursor role of ornithine in its biosynthesis suggested a possible relation of GIS to a group of compounds of microbial origin known as sideramines, which are characterized by their potent iron-chelating capacity inherent in the repeating hydroxylamino groups, the latter being essential components of their structural pattern, most commonly derived from δ-*N*-hydroxyornithine (Neilands, 1957; Zaehner *et al.* 1963; Emery, 1965, 1966).

The chemical dissimilarity between GIS and the sideramines was shown by the negative reaction of GIS for hydroxamic acid when tested by the method of Csáky (1948), either directly or following heating with N-HCl. A certain degree of overlapping in their biological activities was indicated by the finding that some of the sideramines examined (ferrichrome, ferrioxamine B, ferrichrysin) were capable of decreasing by a factor of 100–1000 the critical size of inoculum for growth of several strains of *Pasteurella tularensis* in the absence of added GIS (Table 3). However, the minimal inocula required for growth initiation were still about 10^2–10^6 times larger with the sideramines than with GIS. Furthermore, the effective dose of the sideramines was in the range of 10–20 μg./ml. culture medium, i.e. nearly 500–1000 times higher than that of GIS. On the other hand, GIS in amounts as high as 100 μg/ml. showed no growth-factor activity for Arthrobacter JG-9, whose growth has been shown by

Burnham & Neilands (1961) to be dependent upon ferrichrome or other sideramines. With the majority of strains of *P. tularensis* tested the effect of sideramines was duplicated by inorganic iron salts (ferrous and ferric); $FeSO_4$ was reported by Tresselt & Ward (1964) to support under certain conditions colonial growth of the 'Schu' strain of *P. tularensis*.

Table 3. *Effect of inorganic iron and some sideramines on the critical inoculum size of some strains of Pasteurella tularensis*

The various substances were added each to 5 ml. cysteine broth, as indicated. The final medium was inoculated with various numbers of organisms of each strain and incubated for 70 hr with continuous shaking.

Substance added (μg./ml. medium)	Strain of *P. tularensis*: Minimal inoculum required for growth (log. no organisms)				
	S & D	SCHU	JAP	30110	VACCINE
None	7	6	7	7	5
GIS, 0·02	1	1	2	1	1
$FeCl_3$, 0·4*	6	2	6	4	3
$FeSO_4$, 0·4*	6	2	7	4	3
Ferrichrome, 20	6	2	5	4	3
Ferrioxamine B, 20	7	2	5	5	2
Ferrichrysin, 20	6	3	7	6	2
Fusarinine, 20	5	3	7	5	4

* Refers to iron content.

DISCUSSION

A variety of conventional peptone-containing or chemically defined media have been reported as capable of supporting colonial growth of different strains of *Pasteurella tularensis* (Snyder, Engley, Penfield & Creasey, 1964; Downs, Corriell, Chapman & Klauber, 1947; Won, 1958; Nagle, Anderson & Gary, 1960; Hood, 1961; Gaspar, Tresselt & Ward, 1961). These claims are at variance with our findings about the apparently novel and specific nature of the endogenously produced growth initiating substance GIS required for initiating growth of *P. tularensis* from small inocula. The discrepancy cannot be accounted for solely by strain differences, i.e. disparities in the degree of dependence of different strains on the exogenous supply of GIS, since some of the strains used in the studies quoted above and in the present investigation were of identical origin. It appears that some subtle differences in the cultural conditions used, perhaps of a physical or physico-chemical nature, may be of greater importance than hitherto recognized in determining the inoculum-dependent growth characteristics and the requirement for GIS. The metal-binding property of GIS, as well as its partial replacement by iron salts and some sideramines point to a possible role of GIS in the trace-metal metabolism of *P. tularensis*.

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The Molecular Basis of an Inhibition by Tetracyclines

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SUMMARY

The pyruvate-consuming system of *Aerobacter aerogenes*, which is inhibited by bacteriostatic concentrations of tetracyclines in an unaerated mineral salt medium, was isolated as a cell-free extract. The isolated system was inhibited by bacteriostatic concentrations of eight tetracyclines. The inhibitory molecular form of each of these tetracyclines, all of which were acids releasing either two or three protons successively in aqueous solution, was the first dissociation product. Quantitatively the inhibition was directly dependent on the concentration of the inhibitory form of the antibiotic present. The tetracyclines tested were derived structurally from tetracycline by changes of the group attached at position 5, 6 or 7 (type *a*), a de-(dimethylamino) tetracycline (type *b*) and anhydrotetracycline. None of the tetracyclines was inhibitory in the absence of free magnesium ions. In the presence of micromolar concentrations of these ions, the inhibitory form of the tetracycline competed with flavine mononucleotide: the effectiveness of different tetracyclines as inhibitors of unaerated cultures correlated with the stability constants of postulated tetracycline–magnesium–enzyme complexes. The intracellular concentrations of tetracyclines in organisms of sensitive and resistant strains were determined by measurements of inhibition of the pyruvate-consuming system. In medium containing bacteriostatic concentrations of types *a* and *b* tetracyclines, the intracellular concentrations of the sensitive organisms were the same as the extracellular concentrations. Anhydrotetracycline did not penetrate these organisms so readily. One of the resistant strains was insensitive because the tetracyclines did not penetrate into the organisms. There were no intracellular accumulations of tetracyclines from medium containing bacteriostatic concentrations. The bearing of these results on current theories to account for the bacteriostatic actions of tetracyclines is discussed.

INTRODUCTION

Tetracyclines inhibit a considerable number of metabolic reactions (Eagle & Saz, 1955; Snell & Cheng, 1962; Franklin, 1966) and three main theories have been advanced to account for their bacteriostatic actions. (1) It has been suggested that the ability of tetracyclines to combine with essential metallic cations is the cause of their biological effects (Albert, 1953; Albert & Rees, 1956). Although some tetracyclines, which have the ability to form co-ordination compounds with cations, have no antibacterial activity (Weinberg, 1957), some of the effective tetracyclines combine with fused ring compounds of biological importance such as riboflavin, adenylic acid and deoxyribonucleic acid (Higuchi & Bolton, 1959) and there is a cationic bridge between the macromolecules of deoxyribonucleic acid and the tetracyclines. Serum albumin combines similarly with tetracyclines (Kohn, 1961). (2) Saz & Martinez (1958) suggested that the antibiotic action of chlortetracycline on *Escherichia coli* is due to

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an inhibition of the electron transport system. Decreases of the rates of growth due to the presence of tetracyclines have been associated with interferences with oxidation-reduction processes in *Aerobacter aerogenes* (Jones & Morrison, 1962; Benbough & Morrison, 1965) and Staphylococcus 209E (Plakunov, 1963*b*; 1964). (3) Franklin (1963) attributed the antibiotic action on *E. coli* to inhibition of the incorporation of amino acids into ribosomal protein, and the relative efficiencies of five tetracyclines as inhibitors of polyphenylalanine synthesis can be correlated with their efficiencies as antibiotics (Laskin & Chan, 1964).

Snell & Cheng (1962) considered that tetracyclines can inhibit many reactions and that the problem is to determine the order of importance as a function of increasing concentration. If this be so then the reaction which is most sensitive may not be the same for all microbial species or for a given species under different experimental conditions. Many of the arguments for and against particular theories have been based on comparisons of the concentration of the tetracycline that was effective against the isolated system and the growing organism. However, the tetracyclines are acids which not only ionize in aqueous solution, releasing successively either two or three protons, but also form complexes with divalent cations such as magnesium. Hence the total concentration of tetracycline present is distributed between a number of chemically distinct molecular forms in equilibrium with each other. From the data normally available in reports it is not possible to identify the particular molecular form which is inhibitory, nor to calculate the actual concentrations of the various forms. It has also been suggested that sensitive organisms possess an active transport mechanism for tetracyclines which produces an intracellular accumulation of these antibiotics (Franklin & Godfrey, 1965). Clearly arguments based on total concentrations may therefore be misleading.

The decrease of the rate of growth of a strain of *Aerobacter aerogenes* in a glucose mineral salt medium with tetracycline has been studied quantitatively (Jones & Morrison, 1962; Benbough & Morrison, 1965). Inhibitions of rate of growth were expressed as the 'index ratios' (Harris & Morrison, 1961)—that is, the mean generation time of the inhibited culture when in its logarithmic phase divided by the mean generation time of the corresponding uninhibited culture. This is more useful mathematically than the '% inhibition'. At concentrations of inhibitor greater than the threshold concentration, the index ratio became linearly dependent on the concentration of inhibitor provided that the retardation of the rate of growth stemmed from interference with the same metabolic reaction throughout the range of concentration considered. The rate of change of the index ratio with respect to change of concentration of the inhibitory tetracycline was characteristic of the particular tetracycline and of the inhibited reaction. When the primary cause of the retardation of growth changed from interference with one metabolic reaction to another, within the studied range of concentration of a particular tetracycline, there was an abrupt alteration of the rate of change of the index ratio from the value characteristic for the one reaction to that characteristic of the new rate-controlling reaction. For convenience each vulnerable metabolic reaction was referred to as a 'mode' and the modes were distinguished from each other by assigning numerals.

Oxytetracycline had two different modes of action: mode 1 was attributed to interference with the utilization of amino acids by aerated cultures, and mode 2 to interference with a hydrogen transfer reaction or the production of a hydrogen acceptor

required for unaerated growth (Jones & Morrison, 1962). The rate of consumption of pyruvate by non-multiplying organisms in the absence of aeration, but not when the medium was aerated, was inhibited (Jones & Morrison, 1963). Korotyaev (1962) reported similar results for a strain of *Escherichia coli*. The effectivenesses of six different tetracyclines as inhibitors of pyruvate consumption by *Aerobacter aerogenes* were directly and quantitatively related to their effectivenesses as inhibitors of growth by mode 2, and consequently these retardations of pyruvate consumption were considered to arise from inhibition by mode 2 (Benbough & Morrison, 1965). The 7-chlor substituted tetracyclines inhibited aerated growth more severely (mode 3) but the presence of D-glutamate in the medium eliminated this mode whilst permitting inhibition by mode 1. The 7-chlor substituted tetracyclines and tetracycline itself, at the lowest bacteriostatic concentrations, inhibited the rate of growth of unaerated cultures by mode 1, but at higher concentrations by mode 2. 6-demethyl-6-deoxytetracycline inhibited the rates of growth of both aerated and unaerated cultures by mode 1, but weakly inhibited the consumption of pyruvate by non-multiplying organisms in unaerated medium by mode 2. Since the presence in the medium of 0·2 % (w/v) brilliant cresyl blue or methylene blue eliminated all three modes of action, each mode had to be attributed to interference with an electron transfer reaction.

Inhibition by each mode was related quantitatively, and linearly, to the concentration in the medium of the product of the first dissociation of the tetracycline, whether this was altered by varying the total concentration of the tetracycline, varying the pH value of the medium or by varying the concentration of magnesium ions. The inhibition could not be related directly to the concentration of any other of the molecular forms of a tetracycline. Thus either the first dissociation products are the inhibitors of the primary sites or are the only molecular forms able to penetrate into the organisms. The rate of change of index ratio with respect to change of concentration of the inhibitory molecular form of a tetracycline, 'the intrinsic activity' (Benbough & Morrison, 1965), was used as a measure of the maximal effectiveness of the tetracycline as an inhibitor by a particular mode.

The present work examines the inhibition by tetracyclines of the isolated pyruvate-consuming system of *Aerobacter aerogenes* NCTC418 in order to establish which molecular form of a tetracycline inhibits the primary site responsible for mode 2, to identify more closely the affected site and to investigate the mechanism of the inhibition. Comparisons of the effectivenesses of tetracyclines as inhibitors of the isolated system and of the system whilst in its normal place in the organisms, are used to provide direct evidence of the concentrations of the inhibitory form within the organisms. The eight tetracyclines studied include seven variations in the side groupings and anhydrotetracycline.

METHODS

Organism, media and cultivation techniques. The organism used was *Aerobacter aerogenes*, strain NCTC418, which had been maintained for twelve years by regular subcultivation at 37°, with and without aeration, in a basal medium containing (g./l.): 5·4, KH_2PO_4; 12, glucose; 1·8, $(NH_4)_2SO_4$; 0·0203, $MgSO_4.7H_2O$ (all Analar) in demineralized water of resistance greater than 2 MΩ/cm., adjusted to pH 7·00 (Radiometer type PHM4C, V. A. Howe and Co. Ltd., 46, Pembridge Road, London, W.11) with NaOH. Other media were prepared by making the appropriate additions or

omissions of materials. The preparation of inocula, measurements of rate of growth, preparation of solutions of pyruvic acid from pure lithium pyruvate and measurements of concentration of pyruvate were as described previously (Jones & Morrison, 1962; Jones & Morrison, 1963; Benbough & Morrison, 1965).

Resistant substrains of the organism. Six subcultures in unaerated basal medium initially at pH 7·00 containing (*a*) 0·2 μM tetracycline and (*b*) brilliant cresyl blue 20 mg./ml. (Benbough & Morrison, 1965) produced substrains A and B respectively. The rates of growth of both substrains were unaffected by concentrations of tetracycline sufficient to treble the mean generation time in cultures of the parent strain (index ratio of 3·0).

Preparation of suspensions of bacteria and of cell-free extracts. Bacteria were obtained from fully grown unaerated cultures in medium containing (g./l.): 1·08, glucose; 0·15, $(NH_4)_2SO_4$; with the usual concentrations of KH_2PO_4 and $MgSO_4.7H_2O$, adjusted to pH 7·00. When the growth amounted to two-thirds of the maximum obtainable in normal medium, the glucose had been consumed completely and only about 0·14 mM-ammonium ion remained. Suspensions of unwashed bacteria were obtained by suspending them in enough of the culture liquid to give equiv. 0·5 mg. dry wt bacteria/ml. Suspensions of washed bacteria were obtained by suspending an equivalent amount of bacteria washed in KH_2PO_4 9·0 g./l. buffer (pH 7·00) in the equivalent volume of appropriately buffered solution. Freshly prepared suspensions were used for each set of experiments after incubation at 37° for 1 hr.

Suspensions prepared as above were treated at 0° in a 60 W M.S.E. ultrasonic disintegrator to bursts of 15 sec. duration in each of 20 successive minutes. This killed almost all the bacteria but there was no serious loss of ability to consume pyruvate. The debris was removed by centrifugation at 3000 *g* and the extract incubated for 1 hr at 37° before use, to consume metabolities in it.

Measurements of inhibition and inhibitory power. Inhibitions of rates of growth of culture are expressed as the index ratios, and the effectiveness of a tetracycline as an inhibitor by a particular mode by the intrinsic activity. As the equilibria between the different molecular forms of anhydrotetracycline are greatly affected by small changes of pH value, and growing cultures changed their pH values progressively, the growth curves (log equiv. mg. dry wt bacteria/ml. plotted against time) of cultures inhibited by anhydrotetracycline were markedly sigmoid in shape. The index ratios for cultures inhibited by anhydrotetracycline were obtained from the maximum rates of growth, and the intrinsic activities from these values and the concentrations of the inhibitory molecular form (calculated from the total concentrations of anhydrotetracycline and the hydrogen ion concentration prevailing at the times of maximum rates of growth).

Inhibitions of the rate of consumption of pyruvate are expressed as the reciprocal of the 'fractional activity' (Hunter & Downs, 1945), which corresponds to the index ratio. The experimental data were obtained by analysis of 3 ml. samples withdrawn at suitable intervals from 20 ml. of appropriately buffered solution containing 5 ml. of freshly prepared suspension of bacteria or of cell-free extract, 0·8–1 mM pyruvate, and test substances. After 5–10 min. the progress curves were linear and the ratios were calculated from the slopes of the linear portions.

Determination of dissociation constants of tetracyclines and the stability constants of their complexes with Mg^{2+}. These constants were calculated from the results of potentiometric titrations (Monk, 1961).

Antibiotics used. Six of the tetracyclines were given by Charles Pfizer Ltd. and the 7-chlorotetracyclines by Lederle Laboratories Ltd. The nomenclature used is essentially that of Benbough & Morrison (1965): $TH_3^+Cl^-$ denotes a tetracycline hydrochloride and TH_2 the 4-de(dimethylamino)-6-demethyl-6-deoxytetracycline which does not form a hydrochloride. The dissociation constants, the stability constants of the complexes with Mg^{2+} and the intrinsic activities as inhibitors of growth have been listed previously for six of the tetracyclines (Jones & Morrison, 1962; Benbough & Morrison, 1965). The corresponding data for anhydrotetracycline and the 4-de(dimethylamino)-tetracycline are given in Table 1. The first dissociation products, TH_2 and TH^-, are the molecular forms inhibitory to growing cultures and to the pyruvate consumption by unaerated suspensions, and the complexes with Mg^{2+} are $THMg^+$ and TMg^+.

The concentration of the first dissociation product of a tetracycline is given by C_t/Σ, where C_t is the total concentration of the tetracycline (all molecular forms taken together) and

$$\Sigma = [H^+]/K_1 + 1 + K_2/[H^+] + K_2K_3/[H^+]^2 + K_2K_{stab}[Mg^{2+}]/[H^+]$$

for tetracyclines with three dissociation constants, and

$$\Sigma = [H^+]/K_1 + 1 + K_2/[H^+] + K_{stab}[Mg^{2+}]$$

for the de(dimethylamino)tetracycline. K_{stab} is the stability constant of the magnesium-tetracycline complex. $[Mg^{2+}]$, the concentration of free magnesium ions, was calculated from the total concentration by allowing for association with phosphate and sulphate ions in the medium (Jones & Morrison, 1962). The importance of these calculations is illustrated by the facts that whereas 88 % of a concentration of tetracycline is in the form TH_2 at pH 7·00 and 54 % at pH 7·80, the corresponding figures for anhydrotetracycline are 15 % and 2·6 % respectively.

Table 1. *Anhydrotetracycline and 4-de(dimethylamino)-6-demethyl-6-deoxytetracycline* (TH_2)

The intrinsic activity is the change of index ratio per mμM of the first dissociation product of the tetracycline (which is the inhibitory molecular form) measured in the basal growth medium. The index ratio is the mean generation time of the inhibited culture divided by the mean generation time of the corresponding uninhibited culture. The intrinsic activities of other tetracyclines are given in Benbough & Morrison (1965; Table 5).

Tetracycline	Dissociation constants (*p*K)	Stability constant of of Mg complex (log K_{stab})	Intrinsic activity as inhibitors of growth	
			Mode 1 (aerated)	Mode 2 (unaerated)
Anhydro-	3·4 6·3 8·7	4·0	0·0018	0·0044
TH_2	5·8 8·5	2·8	0·00015	0·00035

RESULTS

Inhibition of pyruvate consumption by cell-free extracts

For each tetracycline in turn, the inhibitions of pyruvate consumption by cell-free extracts (made from suspensions of washed *Aerobacter aerogenes* organisms) were determined over a range of concentration of the antibiotic in media containing (g./l.):

4·45, KH_2PO_4; 0·0203, $MgSO_4.7H_2O$; adjusted to pH 6·20–7·80 with NaOH; and in media containing (g./l.): 4·45, KH_2PO_4; 0·0203–0·203, $MgSO_4.7H_2O$; adjusted to pH 7·00. The concentrations of Mg^{2+} calculated by the method of Jones & Morrison (1962) ranged from 16·4 to 164 μM. For each tetracycline above its threshold concentration, the ratio of the uninhibited rate to the inhibited rate was related linearly to the concentration of the first dissociation product of the tetracycline, TH_2 or TH^-, irrespective of whether this was being varied by changing the total concentration of antibiotic, changing the pH value of the medium or changing the concentration of Mg^{2+}. Thus the molecular forms TH_2 and TH^- were the inhibitors of the isolated system.

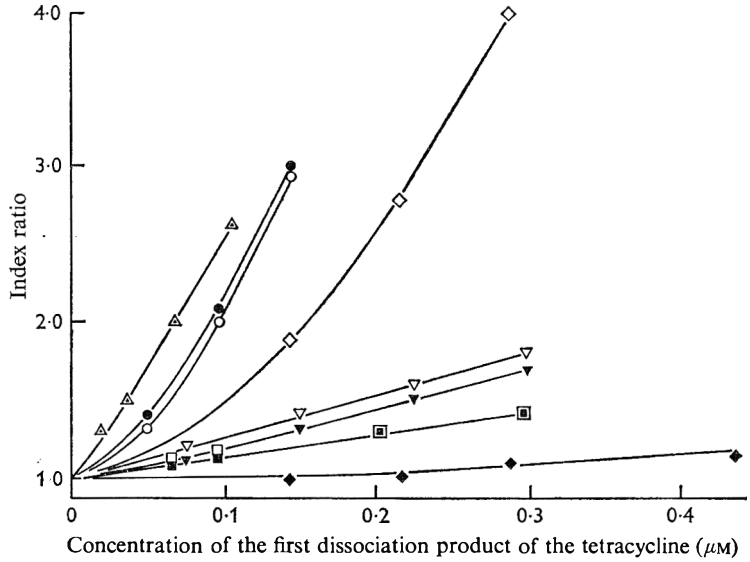

Fig. 1. Inhibition by tetracyclines of the consumption of pyruvate by suspensions and cell-free extracts of *Aerobacter aerogenes* NCTC418 in medium containing: (g./l.) 4·45, KH_2PO_4; 0·0203, $MgSO_4.7H_2O$; 0·0704, pyruvic acid initially; adjusted to pH 7·00 with NaOH. Measurements of rates of reaction described under Methods. The solid points show the results with suspensions of bacteria (equiv. 0·125 mg dry wt/ml.) and the open points the results with cell-free extracts prepared from the same suspensions. ▲, △, Chlortetracycline; ●, ○, methylene-oxytetracycline; ▼, ▽, tetracycline; ■, □, demethyl-deoxytetracycline; ◆, ◇, anhydrotetracycline. Index ratio is the rate of the uninhibited reaction divided by the rate f the inhibited reaction.

Comparisons of inhibitions of whole organism suspensions and of cell-free extracts

Part of a suspension of unwashed organisms was reserved whilst the rest was treated to produce a cell-free extract. The inhibitions of pyruvate utilization by various concentrations of one of the tetracyclines were measured for each preparation. The results for five tetracyclines are shown in Fig. 1. The slopes of the linear portions of these graphs (change of index ratio per μM change of concentration of the first dissociation product of the antibiotic; results for suspensions given first) were: 7-chlortetracycline, 16·0, 16·0; 6-methylene-oxytetracycline, 16·9, 17·1; tetracycline, 3·7, 4·0; 6-demethyl-6-deoxytetracycline, 1·45, 1·45; anhydrotetracycline, 0·95, 16·6; (not illustrated) 6-demethyl-7-chlortetracycline, 20·4, 22·0; oxytetracycline, 2·3, 2·3;

4-de(dimethylamino)-6-demethyl-6-deoxytetracycline, 0·27, 0·30. Thus in seven cases the tetracycline was 1·0–1·1 times as effective against the isolated system as it was against whole organisms under the same conditions. Anhydrotetracycline, however, was 17·5 times as effective against the isolated system. The system, when part of whole organisms, was protected from anhydrotetracycline but not from the other compounds. This suggests that the system was inside the organisms and that, of the eight tetracyclines examined, all but anhydrotetracycline penetrated readily so that the intracellular concentration of the inhibitory form of the antibiotic was essentially that of the medium. This finding could help to explain why anhydrotetracycline is as effective as other tetracyclines against actinomycetes but not against most other micro-organisms (Goodman, Matrishin & Backus, 1955): actinomycetes will grow in slightly acid media which would increase the proportion of the anhydrotetracycline in the form of the first dissociation product to more nearly that of other tetracyclines, and it may penetrate the actinomycetes more readily than it does other micro-organisms.

Table 2. *Lipids of Aerobacter aerogenes* NCTC *418 and substrains* A *and* B

Data from Dr A. M. James, Department of Chemistry, Queen Elizabeth College, London. The bacteria were grown in aerated medium containing (g./l.): 5·4, KH_2PO_4; 1·8 $(NH_4)_2SO_4$; 1·08, glucose; harvested and washed with distilled water; dried and extracted with $CHCl_3$+ CH_3OH. The extract was washed free from water-soluble material, dried and methylated. The analysis was by gas chromatography. For details see Hill, James & Maxted (1963).

		mg. methyl ester obtained from 1 g. dry intact cells		
Chromatogram peak	Ester	NCTC418	Substrain A	Substrain B
A	Laurate	0·014	0·036	trace
B	Myristate	0·088	0·089	0·017
C	?	0·018	0·018	0·017
D	Palmitate	0·810	0·930	0·279
E	Oleate	0·510	0·466	0·161
F	?	0·037	0·018	—
G	?	Trace	—	—
H	?	0·386	0·613	0·100
I	?	0·159	0·219	0·034
J	Stearate	Trace	0·018	Trace
K	?	0·306	0·358	0·034
L	?	0·194	0·174	0·017
M	?	0·124	0·210	—
N	?	0·107	0·141	—
	Total	2·76	3·32	0·66

Examination of the two resistant substrains of *Aerobacter aerogenes* confirmed this suggestion. None of the tetracyclines at concentrations that inhibited severely the parent strain *A. aerogenes* NCTC418, affected the consumption of pyruvate by intact organisms of either substrain. The system isolated from substrain A (obtained by subcultivation in presence of tetracycline) was exactly as sensitive to the antibiotics as the system isolated from the parent strain; but that isolated from the substrain B (obtained by subcultivation in presence of brilliant cresyl blue) was not sensitive. The resistance of substrain A was due to the development of impermeability to the tetracycline whereas that of substrain B arose from the development of independence from any sensitive reaction. Franklin & Godfrey (1965) and Laskin & Chan (1964) reported cases in which systems isolated from resistant *Escherichia coli* organisms were just as

sensitive as the systems isolated from sensitive organisms, but these systems could not be tested directly whilst in their normal positions in the organisms. A nitroreductase from an aureomycin-resistant *E. coli* was also resistant, although that from aureomycin-sensitive organisms was not (Saz, Brownell & Slie, 1956). The resistant nitroreductase bound flavine mononucleotide firmly, whereas the corresponding complex with the enzyme from sensitive organisms was readily dissociable (Saz & Martinez, 1956). Plakunov (1963*a*) reported that the major change in metabolism of a tetracycline resistant strain of *Staphylococcus aureus* was an increase of flavine mononucleotide to 4·5 times the normal value.

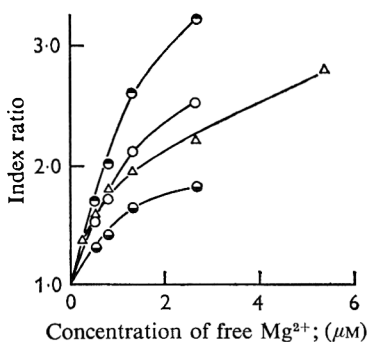

Fig. 2

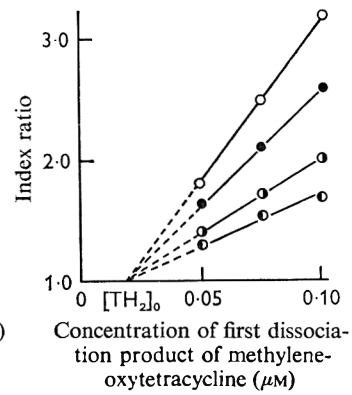

Fig. 3

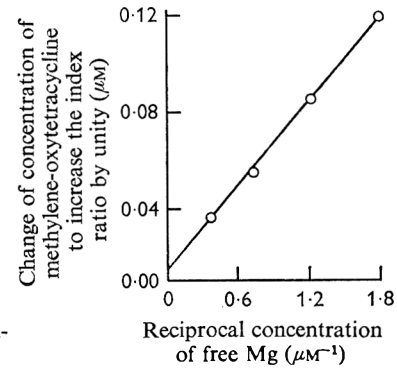

Fig. 4

Figs. 2, 3, 4. Magnesium and inhibition by tetracyclines of the consumption of pyruvate by dialysed cell-free extracts of the *Aerobacter aerogenes* NCTC 418 (equivalent to suspensions of 0·125 mg. dry wt bacteria/ml.) in medium containing: (g./l.) 2·25, KH_2PO_4; 0·0704, pyruvic acid initially; adjusted to pH 7·00 with NaOH. Measurement of rates as under Methods; concentrations of free magnesium ion by method of Jones & Morrison (1962). Index ratio is the rate of the uninhibited reaction divided by the rate of the inhibited reaction. Fig. 2. Dependence of inhibition on concentration of free magnesium ion. Concentration of the first dissociation product of the tetracycline (μM): demethyl-chlortetracycline, 0·084, △; methylene-oxytetracycline, 0·0506, ◒; 0·0760, ○; 0·1013, ◓. Fig. 3. Dependence of inhibition on concentration of the first dissociation product of methylene-oxytetracycline. Concentration of free magnesium ion (μM): 0·56, ◐; 0·82, ◑; 1·36, ●; 2·72, ○. Fig. 4. Relation between slopes of linear portions of graphs in Fig. 3 and the concentration of free magnesium ion for inhibitions by methylene-oxytetracycline.

The possible differences of permeability of the parent strain and substrain A may be connected with the increased content of lipid of the cell walls of some tetracycline-resistant bacteria obtained from clinical sources noticed by Hill, James & Maxted (1963). The analyses of the lipids extracted from *Aerobacter aerogenes* NCTC 418 and from the substrains A and B are given in Table 2 (Dr A. M. James, personal communication).

Magnesium and inhibition of cell-free extracts

Dialysis of cell-free extracts of *Aerobacter aerogenes* NCTC 418 against demineralized water (specific conductance $0·5 \times 10^{-6}$ reciprocal Ω) slowly and progressively decreased their abilities to consume pyruvate, and decreased more rapidly their sensitivities to tetracyclines. After 18 hr the residual rates of consumption of pyruvate were un-

affected by usually inhibitory concentrations of a tetracycline, but the sensitivity was restored by 1–10 μM free magnesium ion. Such low concentrations of Mg^{2+} have a negligible effect on the proportion of a tetracycline which is in the inhibitory form. This general dependence of inhibition by tetracyclines on the presence of Mg^{2+} is illustrated in Fig. 2 by results for 6-demethyl-7-chlorotetracycline and 6-methylene-oxytetracycline. Figure 3 shows that for each given concentration of Mg^{2+} the inhibition by the methylene-oxytetracycline became directly proportional to the concentration of the first dissociation product of the tetracycline, i.e. index ratio $= 1 + B\{[TH_2] - [TH_2]_0\}$, where $[TH_2]_0$ is a constant, but the proportionality constant B was a function of the concentration of available Mg^{2+} ions. Figure 4 shows that experimentally $1/B = C + D/[Mg^{2+}]$, where C and D are constants and hence

$$B = [Mg^{2+}]/(C[Mg^{2+}] + D).$$

At a relatively high concentration of Mg^{2+} the effectiveness of a tetracycline becomes independent of the concentration of Mg^{2+}, i.e. the function of $[Mg^{2+}]$ becomes equal to unity and hence the equation for B must be rewritten as $B = \alpha[Mg^{2+}]/([Mg^{2+}] + K_\gamma)$, where α and K_γ are constants, to give the overall equation

$$\text{index ratio} = 1 + \frac{\alpha[Mg^{2+}]}{([Mg^{2+}] + K_\gamma)} \{[TH_2] - [TH_2]_0\}. \qquad (1)$$

$[TH_2]_0$ is the threshold concentration.

From the slope and the intercept with the vertical axis of the graph in Fig. 4 $K_\gamma = 4{\cdot}3$ μM. The shape of the function of concentration of magnesium suggests that either Mg^{2+} forms a complex with an enzyme (stability constant $1/K_\gamma$), which then combines further with the tetracycline to form an inactive enzyme–magnesium–tetracycline complex, or the magnesium combines primarily with the tetracycline and then with the enzyme. The latter theory can be discarded since potentiometric titration of the tetracycline in the presence of Mg^{2+} detected only one such complex and its stability constant was only 1/280 of $1/K_\gamma$.

Antagonists of the inhibition

As it is known that yeast extract contains an antagonist to this inhibition by tetracyclines (or some substance from which an antagonist is produced) and that certain redox dyes also annul this inhibition (Jones & Morrison, 1962; Benbough & Morrison, 1965), substances of suitable redox potential which occur in yeast extract were tested for antagonism. Riboflavin in the range of concentration 0–4c μM progressively and markedly decreased the inhibition of cell-free extracts due to a normally bacteriostatic concentration of one of the tetracyclines, but flavine adenine dinucleotide (FAD) at higher concentrations had no effect. Incubation of the dialysed cell-free extract with riboflavin before the addition of pyruvate and tetracycline increased the effect. Foster & Pittillo (1953) found that riboflavin annulled an inhibition by chlorotetracycline. A sample of flavine mononucleotide (FMN), prepared by hydrolysis of FAD, decreased the inhibition over a lower range of concentration than did riboflavin, and incubation of the system with FMN did not increase the effect. As an illustration of a general finding, Table 3 presents the results for inhibition by 6-demethyl-6-deoxytetracycline.

Table 3. *Effects of riboflavin and flavine mononucleotide on inhibition by 6-demethyl-6-deoxytetracycline of pyruvate consumption by dialysed cell-free extracts of Aerobacter aerogenes* NCTC *418.*

The index ratio is the rate of the uninhibited reaction divided by the rate of the inhibited reaction. The extracts were equivalent to suspensions containing equiv. 0·125 mg. dry wt bacteria/ml. in medium containing KH_2PO_4 2·25 g./l.; 0·56 μM free magnesium ion; adjusted to pH 7·00 with NaOH. Measurements of rate described under Methods.

Concentration of co-factor (μM)	Index ratio: Antibiotic and co-factor added together	Index ratio: Extract and co-factor preincubated together
Riboflavin		
0·00	2·0	2·0
6·67	1·95	1·4
13·33	1·75	1·15
26·67	1·5	1·1
40·00	1·3	1·0
Flavine mononucleotide		
0·00	2·0	2·0
0·90	1·8	1·8
1·80	1·4	1·4
2·70	1·4	1·4
3·60	1·3	1·3
4·50	1·2	1·2
5·40	1·15	1·15

DISCUSSION

Tetracyclines inhibit the consumption of pyruvate by *Aerobacter aerogenes* in nearly anaerobic conditions and the primary site of the inhibition is either an electron transfer or a step in the provision of an essential hydrogen acceptor (Jones & Morrison, 1963). The present work indicates that the primary site of inhibition is a reaction which requires flavine mononucleotide (FMN) and that the tetracycline competes with FMN provided the enzyme is already combined with Mg^{2+}. Combination between enzyme and Mg^{2+} alone does not affect the activity of the enzyme. As the concentration of the tetracycline is increased the fraction of the total enzyme complexed with it increases, the rate of utilization of the normal substrate decreases and hence the concentration of this substrate increases. This must compensate partially for the loss of active enzyme by causing a more efficient use of the remaining active enzyme. Eventually this ability to compensate must be exhausted and at greater concentrations of antibiotic all available enzyme molecules are saturated with substrate. The concentration of a co-factor such as FMN does not increase as readily since other reactions, in particular those that replenish it from its reduced form tend to maintain its normal concentration. The reaction becomes the rate-controlling reaction of the system and experimental kinetic data for the system apply to it. A kinetic model (Fig. 5) for this situation, therefore, may be proposed.

Equations for the rates of formation of all complexes containing enzyme can be written and equated to zero when the enzyme level steady state pertains (Briggs & Haldane, 1925). The expression for the rate, v, of the reaction in the presence of excess

substrate in terms of the reaction velocity constants and the concentrations of Co, TH_2 and Mg^{2+}, obtained by algebraical manipulation of the equations, is

$$v = \frac{K_\delta}{\left\{K_\delta + \frac{[Mg^{2+}]}{[Mg^{2+}]+K_\gamma}\frac{K_M}{([Co]+K_M)}[TH_2]\right\}}\left\{\frac{k}{[Co]+K_M}\right\}[En], \qquad (2)$$

where

$$K_\gamma = j_1/j_{-1},\ K_\delta = j_{-2}/j_2,\ k = \frac{k_2k_3}{k_2+k_{-2}+k_3}$$

and

$$K_M = \frac{k_{-1}k_{-2}+k_{-1}k_3+k_2k_3}{k_1(k_2+k_{-2}+k_3)}.$$

The details of the derivation of equation (2), and of the subsequent equations (3) and (4), are available in the library of the Edward Davies Chemical Laboratory.

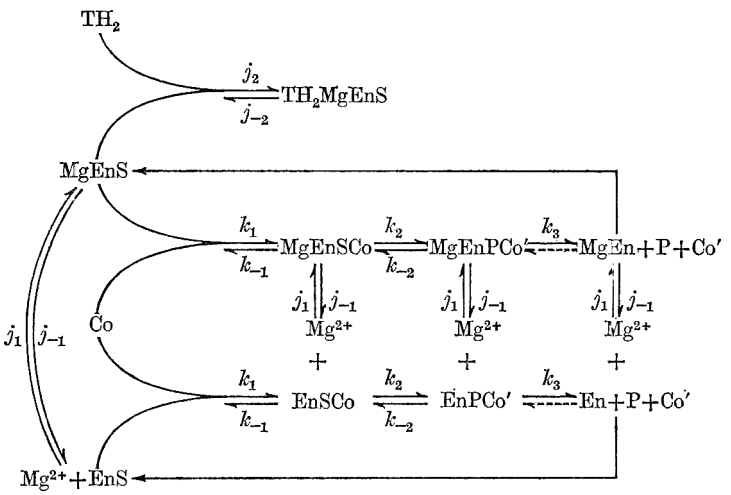

Fig. 5. Kinetic model of the inhibition of the electron transport mechanism of *Aerobacter aerogenes*. En represents free enzyme; S, the substrate; Co, flavine mononucleotide; Co′, reduced flavine mononucleotide; P, the product. The reaction velocity constants $j_1 \rightarrow j_2$ and $k_1 \rightarrow k_3$ are as shown. TH_2MgEnS is the inactive enzyme complex, EnS and MgEnS are active enzyme complexes.

A simpler model in which MgEnSCO and EnSCO are considered to break down directly to MgEn, P and Co′, and En, P and Co′, respectively, gives the same formal result, except that the composite constants k and K_M are equal to k_2 and $(k_{-1}+k_2)/k_1$ respectively.

It follows that if v_0 is the rate of the uninhibited reaction in the presence of excess substrate that

$$v_0/v = 1 + \frac{[Mg^{2+}]K_M}{K_\delta([Mg^{2+}]+K_\gamma)([Co]+K_M)}[TH_2]. \qquad (3)$$

v_0 cannot be determined directly as in the absence of inhibitor the reaction may not be rate controlling for the system, and hence, until the concentration of the inhibitor is considerable, the substrate may not saturate the enzyme. However, in a particular experiment the rate of the uninhibited system, v_u is directly related to v_0, i.e. $v_u/v_0 = \beta$.

Thus the index ratio, v_u/v, should give a linear plot against $[TH_2]$ when $[TH_2]$ is sufficiently large to cause an excess concentration of substrate. Fig. 3 shows that this was realised experimentally. β can be calculated from the intercept with the index ratio axis, of the extrapolation of the linear portion of the plot. When $[Mg^{2+}]$ is large compared with K_γ, as for the measurements in Table 1 and Fig. 1, the function of $[Mg^{2+}]$ is unity and the slopes of the linear plots are equal to

$$\frac{K_M}{K_\delta}\frac{\beta}{[Co]+K_M}.$$

The concentration of flavine mononucleotide, Co, is unknown and therefore it is not possible to calculate the individual values of K_M/K_δ for each tetracycline. However, provided the conditions are constant, the relative values of $1/K_\delta$ can be determined from a comparison of the slopes of the individual plots. In Fig. 6 these relative values obtained from the results for cell-free extracts are compared with the relative intrinsic activities of the tetracyclines as inhibitors of growth by mode 2 (Benbough & Morrison, 1965, table 5; present report Table 1). The effectivenesses of tetracyclines as inhibitors of growth of *Aerobacter aerogenes* NCTC418 by mode 2 reflect the calculated stability constants ($1/K_\delta$) of the postulated tetracycline–magnesium–enzyme complexes.

It follows further from equation (3) that

$$\frac{v/v_0}{1-(v/v_0)}\quad\frac{[Mg^{2+}]}{[Mg^{2+}]+K_\gamma}\quad[TH_2] = K_\delta+\frac{K_\delta}{K_M}[Co], \tag{4}$$

which is equivalent to the Hunter & Downs (1945) test for competitive inhibition. Figure 7 shows that the results for the annullment by flavine mononucleotide (FMN) of inhibition of cell-free extracts by 6-demethyl-6-deoxytetracycline (Table 3) fits this equation. v_0 was calculated from v_u and the relationship between inhibition of the same preparation and concentration of inhibitor (preceding paragraph); K_γ was derived earlier from Fig. 4. The concentrations of FMN and its reduced form are unknown and hence it is not possible to calculate K_δ and K_M. K_M/K_δ is approximately 0·01.

The three main variations of structure of tetracyclines are illustrated by 6-demethyl-6-deoxytetracycline, anhydrotetracycline and 4-de(dimethylamino)-6-demethyl-6-deoxytetracycline in aqueous acidic solution (Fig. 8). Stephens, Murai, Brunings & Woodward (1956) assigned the dissociations of the 4-dimethylamino-tetracyclines to the systems A, B and C in that order, but the results of experiments by Leeson, Krueger & Nash (1963) and Kalnis & Belenski (1964) require the order to be A, C and B. The present results are in accord with the latter. It is likely that the forms of the three types which are inhibitory have a close structural resemblance, i.e. the products of the first dissociations of all three types should resemble each other. The common loss of a first proton from part A must increase the electron density of the oxygens at C1 and C3 and thus may render one of these oxygen atoms capable of forming a magnesium bridge with the affected enzyme. Though not essential, the substituted amino group at C4 (probably with a positive charge) clearly increases the ability to combine with the enzyme and this is supported by the fact that the epi-tetracyclines are even less effective than the 4-de(dimethylamine) compounds (McCormick, Jensen, Miller & Doersch, 1960). The products of the second dissociation of anhydrotetracycline and of the demethyl-deoxytetracycline are not inhibitory. If this were due merely to the loss of a second proton from part B, then the first dissociation product of the

de(dimethylamino)-tetracycline, in which the whole group is missing, would also be expected to be non-inhibitory; but this was not so. However, the loss of a second proton might be from part C in all three cases. Conover (1956) concluded that in the potentiometrically detectable oxytetracycline-magnesium complex the magnesium is attached to the oxygens at C11 and C12. The loss of a second proton from part C (probably from the hydroxyl at C10) would increase the electron density of the oxygen at C11, and thus combination of magnesium and the C11 and C12 oxygens is likely for the second dissociation products: in the case of anhydrotetracycline the hydrogen of the hydroxyl at C11 bonding with the negatively charged oxygen at C10, and in the ordinary tetracyclines the hydrogen of the hydroxyl at C12 bonding with the oxygen

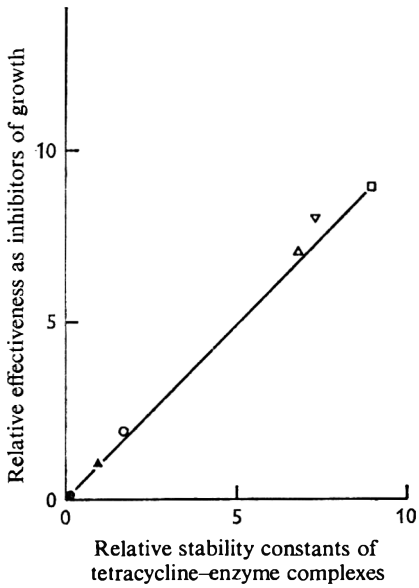

Fig. 6

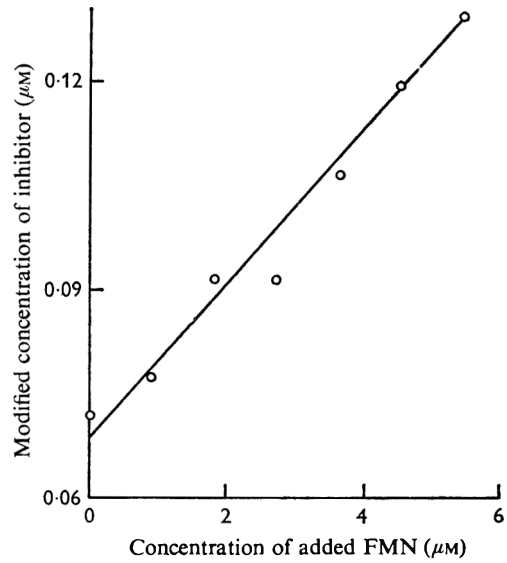

Fig. 7

Fig. 6. Comparisons of the relative effectivenesses of different tetracyclines as inhibitors of unaerated growth of *Aerobacter aerogenes* (in medium containing, g./l.: 5·4, KH_2PO_4; 1·8, $(NH_4)_2SO_4$; 12, glucose; 0·0203 $MgSO_4.7H_2O$; adjusted to pH 7·00 with NaOH) and the relative stability constants of complexes between the tetracyclines and the enzyme controlling the rate of pyruvate consumption by cell-free extracts of *A. aerogenes* (in medium containing (g./l.): 5·4, KH_2PO_4; 0·0203 $MgSO_4.7H_2O$; adjusted to pH 7·00 with NaOH). Measurements of rates described under Methods. Growth data from Table 1 and Benbough & Morrison (1965, Table 5). Values for oxytetracycline taken as unity. ●, 4-de(dimethylamino)-6-demethyl-6-deoxytetracycline; ▲, oxytetracycline; ○, tetracycline; △, chlortetracycline; ▽, methylene-oxytetracycline; □, 6-demethyl-chlortetracycline.

Fig. 7. Competition between the first dissociation product of demethyl-deoxytetracycline and flavine mononucleotide (FMN) for the enzyme controlling the rate of consumption of pyruvate by dialysed cell-free extracts of *Aerobacter aerogenes* NCTC 418 in medium containing (g./l.): 2·25, KH_2PO_4; 0·000772, $MgSO_4.7H_2O$; 0·0704, pyruvic acid; adjusted to pH 7·00 with NaOH. $[TH_2]$ represents the concentration of the first dissociation product of the antibiotic, $[Mg^{2+}]$ the concentration of free magnesium ion (0·56 μM), K_y the dissociation constant of the magnesium–enzyme complex (4·3 μM), v the rate of the inhibited reaction and v_0 the maximum rate of the uninhibited reaction. The modified concentration of inhibitor is

$$\frac{v/v_0}{1-(v/v_0)} \quad \frac{[Mg^{2+}]}{[Mg^{2+}]+K_y} \quad [TH_2].$$

See equation (4) in text.

at C1. The first dissociation product of the de-(dimethylamino)-tetracycline has an overall negative charge and hence a greater electron density on the oxygen at C11 than have the first dissociation products of the other tetracyclines. Apparently this is sufficient for bonding to magnesium ions. It is noteworthy that the stability constants for the complexes with the other tetracyclines are from 14–140 times that of the de-(dimethylamino)-tetracycline complex, and that potentiometric titrations indicated a continuation of complex formation at high pH values when the form T^{2-} forms a considerable proportion of the total concentration of the de-dimethylamino tetracycline.

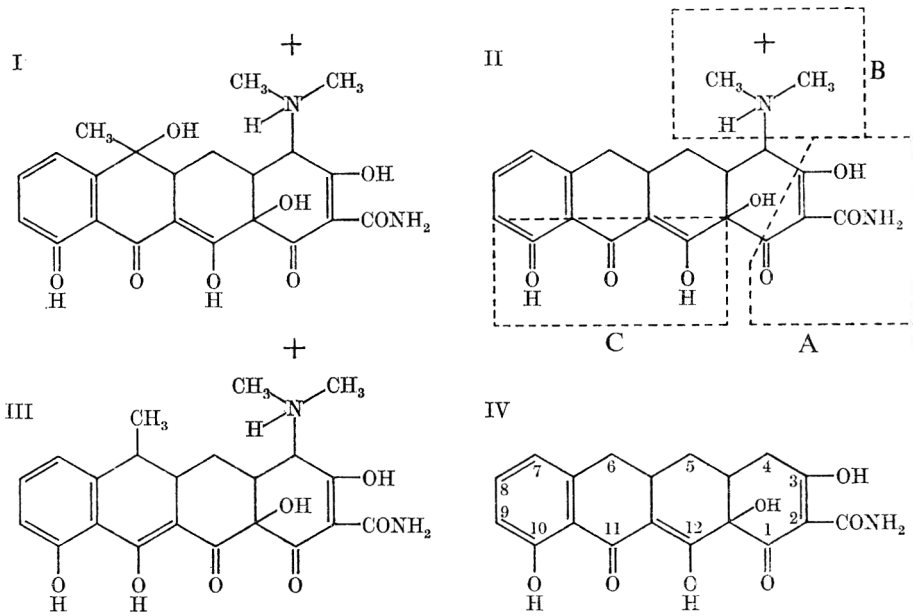

Fig. 8. Structures of some tetracyclines in aqueous acidic solution. I, Tetracycline; II, 6-demethyl-6-deoxytetracycline; III, anhydrotetracycline; IV, 4-de(dimethylamino)-6-demethyl-6-deoxytetracycline.

A similar mechanism to that suggested for mode 2 may be concerned with mode 1 (Jones & Morrison, 1962) and mode 3 (Benbough & Morrison, 1965) inhibitions of the growth of *Aerobacter aerogenes* which are also eliminated when the medium contains an appropriate redox dye (Benbough & Morrison, 1965). Each mode may arise from inhibition of a different FMN-requiring enzyme. The values of K_S and K_M would be likely to differ from enzyme to enzyme, but the different tetracyclines would be expected to show the same general trend of effectiveness as inhibitors by the three modes as was found by Benbough & Morrison (1965). Similarly, since enzymes performing the same function in different organisms may not be identical in all chemical properties, and the relative importance of a function may also vary from organism to organism, the effectiveness of a mode may vary from organism to organism. One resistant strain of the *A. aerogenes*, substrain B, was resistant because the tetracyclines were ineffective at the enzyme level. Another cause of variation between organisms is a difference in abilities of a tetracycline to penetrate into the bacteria. The other

resistant substrain A, resisted penetration by the antibiotics and its organisms had greater lipid contents. The importance of this was shown by anhydrotetracycline which inhibited *A. aerogenes* relatively poorly because it does not penetrate the bacteria readily. The effect of this inability was enhanced by the fact that only a small fraction of the total concentration of anhydrotetracycline was in the inhibitory form in the range of pH values at which the organism grew. The mechanism may also be applicable in principle to other inhibitions that have been reported. Catalytic surfaces containing bound magnesium ions, or on which magnesium ions are absorbed, could complex with one or other of the anionic forms of a tetracycline that are present in solution. Detailed examination of the possibility depends upon knowledge of which form of the tetracycline is the inhibitor of the affected reaction; the data required for determining this are not yet available. There is clear evidence that multiplying organisms of sensitive strains, e.g. *Escherichia coli* (Franklin & Godfrey, 1965), bind tetracyclines to their cellular material whereas organisms of resistant strains do so less readily. Only that part of the bound tetracycline which is attached to the active site of the catalyst for the rate-controlling reaction can be expected to inhibit the system. The results of the present experiments with *A. aerogenes* require that the intracellular concentration of the inhibitory form of the tetracycline is essentially the same as its concentration in the medium, and in this instance at least, there is no enhanced intracellular concentration of the actual inhibitor. The impermeability to tetracyclines of organisms of one resistant substrain would prevent both inhibition of the crucial reaction and binding of a tetracycline to other cellular material.

The concentrations of inhibitors (first dissociation products) needed to decrease the rate of growth of sensitive *Aerobacter aerogenes* NCTC418 by 90% (by mode 2) are (μM): tetracycline, 0·6; oxytetracycline, 1·1; methylene-oxytetracycline, 0·14; and demethyl-chlortetracycline, 0·13. The total concentrations of these tetracyclines in the blood of patients during treatment vary between (μM) 1·8 and 9; 1 and 5; 0 8 and 6; and 1 and 6, respectively (Dr G. M. Williamson, Charles Pfizer Ltd., personal communication). Even though a high proportion of the tetracyclines in the blood may be in non-inhibitory forms because of dispersion between the different dissociation products and binding to divalent cations and serum constituents, bacterial strains as sensitive to mode 2 as is *A. aerogenes* NCTC418 would be vulnerable under clinical conditions. Mode 3 is rather more severe than mode 2 which is somewhat more severe than mode 1. Cultures of *Escherichia coli* ATCC 12299/198 also were just as sensitive to tetracycline as were those of the *A. aerogenes* (G. A. Morrison, unpublished). It is noteworthy that 400 μM-chlortetracycline was needed to inhibit by 89% the incorporation of leucine by ribosomes from *E. coli* (Franklin, 1963), and that the anaerobic utilization of pyruvate by a strain of *E. coli* was sensitive to tetracyclines (Korotyaev, 1962).

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The Saccharolytic Activity of *Acinetobacter lwoffii* and *A. anitratus*

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(*Accepted for publication 26 June 1967*)

SUMMARY

The activity of 75 strains of *Acinetobacter* (16 strains of *A. lwoffii* and 59 strains of *A. anitratus*) against carbohydrates is described. It is concluded that no firm distinction can be made between these species which represent extreme ends of a group of organisms possessing widespread but variable activity.

INTRODUCTION

This paper deals with the saccharolytic properties of *Acinetobacter lwoffii* and *A. anitratus*.

METHODS

Bacteria used. These were 75 strains of the genus *Acinetobacter*, 15 strains from type culture collections and 60 wild strains. Their sources and designations were as follows.

A. lwoffii: *Mima polymorpha* ATCC9957; *Moraxella lwoffi* Inst. Pasteur 5382, 53116, A162; *A. lwoffii* 12 wild strains.

A. anitratus: *Acinetobacter anitratus* NCTC7250, 7412, 7422, 7461, 7844, 9427, 10292; *Moraxella glucidolytica* Inst. Pasteur 5367, 5497; *Neisseria winogradskyi* Inst. Pasteur Paris; *Acinetobacter anitratus* 49 wild strains.

The wild strains were alloted to the species *A. anitratus* or *A. lwoffii* on the basis of acid production or failure to produce acid from any carbohydrate in peptone water.

Acid production in carbohydrates was detected by two methods. Method A, the carbohydrates were used at 1 % (w/v) in peptone water; the indicator was acid fuchsin. Inoculation was from an 18 hr peptone water culture. Method B, the carbohydrates were used at 1 % (w/v) in the medium of Smith, Gordon & Clark (1952) without agar; the indicator was bromocresol purple. Inoculation was from an 18 hr culture on nutrient agar.

The utilization of carbohydrates as sole carbon source was detected in the mineral salt medium of Chan (1964). The carbohydrates were used at 0·1 % (w/v); the nitrogen requirements were satisfied by the potassium nitrate. Inoculation was from a nutrient agar colony emulsified in autoclaved tap water (method C).

Reduction of nitrate to nitrite was tested in the medium of Jyssum & Joner (1965), by using the reagents of McLean & Henderson (1966).

In all cases, cultures were incubated at 37° for 7 days.

RESULTS

The bacteria received as *Mima polymorpha*, *Moraxella lwoffi* or the wild strains regarded as *Acinetobacter lwoffii* (16 all told) did not produce acid from any carbohydrate when tested by method A (6 of 6 strains) or B (5 of 5 strains) and did not utilize carbohydrate by method C (9 of 9 strains).

The bacteria (59 strains) received as *Acinetobacter anitratus*, *Moraxella glucidolytica*, *Neisseria winogradskyi* or the wild strains regarded as *A. anitratus* produced acid readily from pentoses and hexoses, but not from higher carbohydrates, alcohols or glycosides when tested by method A (59 strains). When tested by method B (29 strains) a wider spectrum of activity was noted, extending through the disaccharides to the trisaccharides which were split by 2 strains. When tested by method C (28 strains), 8 strains utilized arabinose and xylose, 4 utilized arabinose but not xylose, and the remainder did not utilize any carbohydrate tested. These results are summarized in Table 1.

Table 1. *Acinetobacter strains: acid production from carbohydrates (methods A and B) or utilization of carbohydrates (method C)*

	Method A		Method B		Method C	
	A. lwoffii	*A. anitratus*	*A. lowffii*	*A. anitratus*	*A. lwoffii*	*A. anitratus*
Number tested ...	16	59	5	29	9	28
Acid production from or utilization of						
Pentoses						
Arabinose	0	45	0	27	0	14
Rhamnose	0	3	0	2	0	0
Xylose	0	49	0	28	0	10
Hexoses						
Fructose	0	3	0	17	0	0
Galactose	0	21	0	28	0	0
Glucose	0	45	0	28	0	0
Mannose	0	19	0	28	0	0
Sorbose	0	0	0	11	0	0
Disaccharides						
Cellobiose	0	0	0	23	0	0
Lactose	0	0	0	14	0	0
Maltose	0	0	0	0	0	0
Melibiose	0	0	0	10	0	0
Sucrose	0	0	0	2	0	0
Trehalose	0	0	0	0	0	0
Trisaccharides						
Melizitose	0	0	0	0	0	0
Raffinose	0	0	0	2	0	0

All the 34 strains tested, including 5 of *Acinetobacter lwoffii* and 29 of *A. anitratus*, produced urease. Of the same 34 strains grown in the medium of Jyssum & Joner (1965), 5 strains of *A. anitratus* but no strain of *A. lwoffii* reduced nitrate to nitrite.

DISCUSSION

Acinetobacter lwoffii is usually recognized by its inactivity towards carbohydrates in contrast to *A. anitratus* which attacks them. At the outset of this work it was thought

that the apparent inactivity of *A. lwoffii* might be due to neutralization of acid produced from the carbohydrate by ammonia produced from the peptone when the organisms were grown in peptone water sugars. Elimination of the peptone failed in my hands to reveal saccharolysis by *A. lwoffii* but allowed the *A. anitratus* group to show a wider range of activity. On the other hand, 14 of 28 strains of *A. anitratus* did not utilize carbohydrate as sole carbon source for growth, so resembling the *A. lwoffii* strains. Also both groups readily utilized 2,3-butanediol as carbon source for their isolation from earth, and it is now known that both species produce urease uniformly (Henderson, 1967) when tested by the method of Elek (1946).

A difficulty in dividing the species has been the hitherto apparent inactivity of the organisms on the substrates usually employed; however, Fewson (1967) recorded the activities of one strain on 150 of 450 compounds. Mitchell & Burrell (1964) suggested that although the species within the 'Mima-Herellea' group may be distinguishable, they are closely linked serologically. The investigations of other workers have led to the conclusion that the large number of species hitherto recognized can be reduced to two (review by Henderson, 1965). Pintér & Bende (1967) made an Adansonian analysis of 31 strains of *Acinetobacter lwoffii* and 8 of *A. anitratus*, and while affirming the validity of both species did not find a sharp distinction between them. The results of the present work lead the author to the conclusion that no firm distinction can be made between the two species and that they represent only extreme ends of a group exhibiting widespread but variable activity.

The author thanks Dr H. Girard (Institut Pasteur, Paris) for the strain of *Neisseria winogradskyi*.

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Stability of Cephaloridine and Cephalothin to Staphylococcal Penicillinase

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(*Accepted for publication 28 June 1967*)

SUMMARY

The rates of destruction of benzylpenicillin, cephaloridine, cephalothin and (in some cases) cloxacillin by cocci from induced cultures of 17 strains of *Staphylococcus aureus* were measured. Rates of destruction of cephaloridine, relative in each case to those of benzylpenicillin, by the 10 methicillin-resistant strains were not significantly different from those by the 7 methicillin-sensitive strains. V_{max} for cephaloridine and cephalothin were about 0·2 % and 0·009 %, respectively, that for benzylpenicillin; cloxacillin was destroyed still more slowly. The inactivation of the cephalosporins is almost certainly brought about by the action of penicillinase. Notwithstanding its greater stability to staphylococcal penicillinase, cephalothin is less effective than cephaloridine at suppressing the growth of the methicillin-resistant strains, although there is cross-resistance between methicillin, cloxacillin, cephalothin and cephaloridine.

INTRODUCTION

Cephaloridine, a semisynthetic cephalosporin introduced for therapeutic use in 1964 (Muggleton, O'Callaghan & Stevens, 1964), has been shown in many laboratory trials to be highly effective *in vitro* against penicillinase-producing strains of *Staphylococcus aureus*. The most comprehensive of these studies to date seems to be that made by Vymola & Hejzlar (1966), who studied the sensitivities of 800 strains of clinically isolated *S. aureus* which were benzylpenicillin-resistant. The general conclusion reached by the workers who did laboratory trials is that cephaloridine is more active against staphylococci than is cloxacillin, especially in the presence of serum (Barber & Waterworth, 1964), and that it is at least as effective as cephalothin, *in vivo* and *in vitro* (Thornton & Andriole, 1966). Three recent papers (Ridley & Phillips, 1965; Benner, Bennett, Brodie & Kirby, 1965; Hye-Knudsen, 1966) have indicated that cephaloridine is inactivated relatively rapidly by staphylococcal penicillinase, and from the first two of these papers it might be concluded that methicillin-resistant strains possess some special ability to destroy cephaloridine; however, all three reports give only qualitative results. The present work was done to determine whether methicillin-resistant *S. aureus* strains inactivate cephaloridine and cephalothin more rapidly, either relatively or absolutely, than do methicillin-sensitive strains. Quantitative measurements were made of the breakdown of benzylpenicillin, cephaloridine and cephalothin, by ten naturally occurring methicillin-resistant and seven methicillin-sensitive *S. aureus* strains. Cross-resistance between methicillin, cephaloridine and cephalothin was also examined.

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METHODS

Bacterial strains. The 17 strains of *Staphylococcus aureus* used in this investigation are listed in Table 1, with place of origin and phage-type (we are grateful to Mr J. Gibb for phage-typing strains 1735, 1736, 6467, 33, 63, 69). The division into 'methicillin-resistant' and 'methicillin-sensitive' was originally made on the basis of the reported methicillin sensitivities of the strains, later substantiated by disc and plate methods as described below.

Table 1. *Strains of Staphylococcus aureus used*

Strain	Source	Reference	Pattern of resistance*	Phage type
1735, 1736, 6467, 6637	Carshalton	Stewart & Holt, 1963	PN, S, T, CB, OB, CR	75/77
5974	Copenhagen	Eriksen & Erichsen, 1963	PN, S, T, CB, OB, CR	6/75/77
10395, 10396, 13136, 13137, 14668	Colindale	Jevons, 1961	PN, S, T, CB, OB, CR	7/47/53/54/75/77
E3	Guy's Hospital	Knox, 1960	PN, S, T	77
GALT			PN, T	52/80/+
33			PN, S	54/75/77/83a
63	Mancheste	Fairbrother, Parker & Eaton, 1954	PN, S, T	Not typable
69				
8511	NCTC	—	PN	53
8534			PN, S	75/77

* Strains were resistant (disc technique) to the drugs specified: PN = benzylpenicillin; S = streptomycin; T = tetracycline; CB = methicillin; OB = cloxacillin; CR = cephaloridine. See Methods.

Sensitivity testing. Organisms were incubated at 37° overnight in infusion broth (Southern Group Laboratories, Hither Green, London, S.E. 13); 0·1 ml. of each culture was spread on Oxoid sensitivity agar to give a uniform lawn. An Oxoid Multodisk (code 11–14D), or an appropriate individual sensitivity disc, was then laid on the surface of the agar, and the plate incubated at 37° overnight. The compounds tested were (amount in each disc shown in parentheses): chloramphenicol (50 μg.), erythromycin (50 μg.), novobiocin (30 μg.), oleandomycin (10 μg.), streptomycin (25 μg.), tetracycline (50 μg.), lincomycin (2 μg.), methicillin (10 μg.), cloxacillin (5 μg.), cephaloridine (5 μg.). Strains which produced confluent growth up to the edge of a disc were taken to be (qualitatively) resistant to the drug contained in that disc.

Penicillins and cephalosporins. The following compounds were used as substrates, antibacterial agents or inducing agents: benzylpenicillin (Crystapen; from Glaxo Ltd., Greenford, Middlesex); cephaloridine (Ceporin) and cephalothin (both generously given by Glaxo Laboratories Ltd.); methicillin (Celbenin) and cloxacillin (Orbenin)—both generously given by Beecham Research Laboratories, Betchworth, Surrey.

Culture methods and penicillinase preparations. Each strain was grown overnight statically at 37° in several 20 ml. amounts of infusion broth (containing methicillin 0·5 μg./ml. as inducing agent), each 20 ml. sample being in a separate 100 ml. bottle.

Cultures of each strain were pooled, and organisms were harvested by centrifugation (3000 *g* for 20 min.). The pellet of cocci was then resuspended in a volume of sterile sodium phosphate buffer (25 mM, pH 7·4) equal to half that of the original pooled culture. Such a preparation is called 2 × 'normal', meaning that the cell concentration of organisms was double that of the original overnight culture; a 'normal' suspension usually contained 4–7 × 10^8 cocci/ml. The resulting suspension was counted (0·1 ml. samples of a 10^{-6} dilution were spread in duplicate or triplicate on blood agar plates, incubated at 37° overnight, and the colonies counted), and separate samples were assayed against benzylpenicillin, cephaloridine, cephalothin and (in some cases) cloxacillin, as described below.

Assay methods. Activity against benzylpenicillin was assayed at 37°, against samples of cocci which had been inactivated by treatment with mM-sodium *p*-chloromercuribenzoate, by the hydroxylamine method as described by Knox & Smith (1962); the coccal concentration was 0·22 × normal, and the substrate concentration was initially 4·6 mM (1·7 mg./ml.). Activities against cephaloridine, cephalothin and (where applicable) cloxacillin were assayed by a microbiological method: appropriate amounts of coccal suspension, substrate solution and sterile buffer were placed in bottles, to a final volume of 20 ml., and incubated at 37°. At intervals (2 and 4 hr after the start of the assay for cephaloridine, 3 and 6 hr for cephalothin and cloxacillin), 5 ml. samples were withdrawn, the cocci removed by centrifugation (3000 *g* for 20 min.) and the supernatant fraction placed in a sterile bottle in a deep-freeze cabinet (−20°). These fractions did not contain detectable penicillinase activity (see below). When all the samples were to hand (after 6 hr) the concentration of antibiotic in each one was assayed by the cup-plate method, with *Sarcina lutea* NCTC 8340 (ATCC 9341) as test organism (Knox & Smith, 1963). The following controls were run in parallel with the assays: compound in absence of staphylococci, and compound in presence of a suitable dilution of *Staphylococcus aureus* OXFORD. (These two controls always gave identical results.) Coccal concentrations and initial substrate concentrations were as follows: cephaloridine 5 μg./ml., cocci at 0·05 × normal; cephalothin 5 μg./ml., cocci at 1 × normal; cloxacillin 10 μg./ml., cocci at 1 × normal.

Graphs were constructed of drug concentration remaining against time, and the nett value of the rate of destruction (as mμmoles/hr/ml.) of each drug for every strain was calculated from these; all rates were then corrected for coccal concentration, to an arbitrary figure of 10^9 cocci/ml.

In preliminary experiments, sample solutions of benzylpenicillin (containing between 400 and 1000 μg./ml.) were assayed in parallel by the hydroxylamine and microbiological techniques. As the results obtained by the two methods showed satisfactory numerical agreement, it was considered legitimate to compare the results obtained with benzylpenicillin using the chemical assay with those obtained with the other compounds using the biological assay.

Control experiments verified that all detectable traces of penicillinase were removed by the centrifugation procedure used during the bioassay technique; 5 ml. samples were taken from mixtures of cephaloridine or cephalothin and induced cocci which had been incubated at 37° for 2½ hr. Each sample was centrifuged and the supernatant fraction removed; a portion of each fraction was assayed immediately, while the remainder was incubated at 37°; after 18 hr and 24 hr portions of each sample were again removed and assayed. Control samples (put up in the absence of cocci) were

treated in a similar way. In no case did the samples which had been originally incubated in the presence of cocci contain less intact cephalosporin than the corresponding control samples: it was hence deduced that enzymatic breakdown of the cephalosporins ceased when the cocci were removed.

Chromatography. (*a*) Cephaloridine and cephalothin were applied by a calibrated loop in 100 μg. amounts to Whatman no. 1 paper, and the chromatogram run overnight, descending, in an acid solvent (*n*-butanol + acetic acid + water, 12 + 3 + 5 by vol.); spots were visualised by inspection under ultraviolet radiation, when the cephalosporins were distinguishable by their yellow fluorescence.

(*b*) Chromatograms to be developed by bio-autography were run on specially cut Whatman no. 1 papers (9 in. wide × 13 in. long): 3 in. strips were left intact at the top and bottom of the paper, and strips were cut out of the middle part to leave 4 strips 0·5 in. wide. Samples (containing approximately 1·5 μg. of intact drug, for optimal results) were applied to the top of each strip, and the chromatogram was then run (2–4 hr) in either the acid solvent described above, an alkaline solvent (*n*-butanol + pyridine + water, equal vols.), or a neutral solvent (organic phase of *n*-butanol + ethanol + water, 4 + 1 + 5 by vol.). Papers were dried until solvent smell was not noticeable, the strips were cut out and laid on nutrient agar containing 2 % by volume of an overnight shaken broth culture (37°, 80 cyc./min.) of *Sarcina lutea* NCTC 8340, in Perspex plates (12 × 10 in., containing 250 ml. medium). The plates were incubated at 37° overnight, and R_F values calculated from the distance of the middle of each zone of inhibition from the origin.

RESULTS

Sensitivity tests

Disc tests established that no strain was resistant to chloramphenicol, erythromycin, novobiocin, oleandomycin or lincomycin; all strains except 8511, 8534, GALT and 33 showed multiple resistance (i.e. resistance to at least benzylpenicillin, streptomycin and tetracycline). Methicillin, cloxacillin and cephaloridine discs gave no zones of inhibition when tested against methicillin-resistant strains, while all the methicillin-sensitive strains showed large zones of inhibition (greater than 15 mm. in diameter) around these discs (Table 1).

Methicillin-resistant strains contain a proportion of individual bacteria with increased intrinsic resistance to the drug (Sutherland & Rolinson, 1964). To determine to what extent this phenomenon also applied to the strains used here, 0·1 ml. samples of suitably diluted preparations from overnight broth cultures of the methicillin-resistant *Staphylococcus aureus* strains were plated on nutrient agar (a 10^{-6} dilution was used), and nutrient agar containing 10 μg./ml. methicillin (10^{-5} and 10^{-6}), 10 μg./ml. cephalothin (10^{-5}), or 10 μg./ml. cephaloridine (10^{-4} and 10^{-5}). Plates were incubated for 15 hr, the colonies counted, and those plates on which few (or no) colonies were observed were incubated for a further 24 hr and again counted. From these counts, the proportions of cocci able to grow at each concentration of the three drugs were calculated; the results are shown in Table 2. In most cases more than half the cocci were able to grow in the presence of methicillin 10 μg./ml., and a significant proportion also grew in the presence of cephalothin 10 μg./ml.; far fewer cocci possessed intrinsic resistance to cephaloridine. However, only in the case of strain 6467

were no cephaloridine-resistant clones observed. In three cases, with strains 5974, 6637, 13137, semiconfluent growth was seen on the cephaloridine-containing plates inoculated with the 10^{-4} dilution, but no colonies, or at best very few, were observed at the 10^{-5} dilution. This phenomenon was interpreted as being due to an inoculum-size effect, caused by the destruction of the drug by the larger inoculum. On plates containing methicillin or cephalothin, colonies were usually countable after incubation for 15 hr, while on the plates containing cephaloridine the further 24 hr period of incubation was necessary before counts could be made. In common with the findings of earlier workers (Barber, 1964; Sutherland & Rolinson, 1964), there was a considerable degree of colonial variation on plates containing methicillin, cephalothin or cephaloridine.

Table 2. *Intrinsic cross-resistance between methicillin, cephaloridine and cephalothin*

Dilutions of overnight broth cultures ($3{\cdot}9 \times 10^8$ to $8{\cdot}6 \times 10^8$ bacteria/ml.) were plated out on solid medium alone and in the presence of the 3 compounds shown, at 10 μg./ml.

Staphylococcus aureus strains	Compound		
	Methicillin	Cephaloridine	Cephalothin
	Proportion (%) of bacteria capable of growth		
1735	45	0·6	46
1736	44	0·5	25
6467	71	0·1	40
6637	77	*	31
5974	45	*	44
10395	100	0·2	46
10396	26	0·7	100
13136	80	11	38
13137	98	*	100
14668	56	0·3	40

* In these cases, heavy growth occurred with 10^{-4} dilution, no growth with 10^{-5} dilution.

Samples (0·1 ml.) of overnight cultures of the methicillin-sensitive strains were also spread, undiluted, on plates containing methicillin, cephalothin or cephaloridine at 10 μg./ml.; no growth was observed in any case (the inoculum size was at least 3×10^7 bacteria/plate). Thus, the results obtained by the disc technique were confirmed by the results of the plate tests. There was cross-resistance between methicillin and the two semi-synthetic cephalosporins, although it appeared that cephalothin was less active than cephaloridine against methicillin-resistant strains.

Destruction of β-lactam drugs

The rates of destruction of benzylpenicillin, cephaloridine and cephalothin by the 17 staphylococcal strains used here are given in Table 3. Cephaloridine was inactivated at about the same rate, relative to benzylpenicillin, by the methicillin-resistant and methicillin-sensitive strains alike (mean values 0·18 %, S.D. 0·044 and 0·18 %, S.D. 0·059 respectively; difference between means not significant). Cephalothin was 10–20 times less labile than cephaloridine, and there was no significant difference between methicillin-resistant and methicillin-sensitive strains (mean values 0·010 %, S.D. 0·0023, and 0·0087 %, S.D. 0·0017, respectively). Similarly, there was no significant

difference between the 13 multiple-resistant strains and the 4 which did not show multiple-resistance, in respect of their relative abilities to inactivate the cephalosporins. In the 6 cases where the destruction of cloxacillin was measured at the same time as that of the cephalosporins, cloxacillin was found to be even more stable than cephalothin.

Table 3. *Rates of destruction of various compounds by induced preparations of 17 Staphylococcus aureus strains*

Figures are given as mμmoles substrate destroyed/ml./hr at 37° in phosphate buffer (pH 7·4); all values are corrected to a coccal concentration of 10^9 cocci/ml. Figures in parentheses are rates relative to benzylpenicillin (rate for benzylpenicillin put at 100 for each strain).

Strain	Benzyl-penicillin	Cephaloridine	Cephalothin	Cloxacillin
	Rates of destruction (mμmoles/hr)			
1735	33,200	72 (0·217)	3·7 (0·011)	1·4 (0·00422)
1736	40,500	79 (0·195)	3·4 (0·00835)	2·1 (0·005)
6467	52,300	106 (0·203)	6·2 (0·0119)	—
6637	41·200	84 (0·204)	3·8 (0·00923)	1·2 (0·00292)
5974	28,400	33 (0·116)	2·9 (0·0102)	1·2 (0·00422)
10395	26,800	61 (0·228)	3·8 (0·0140)	1·2 (0·0045)
10396	36,400	63 (0·173)	3·0 (0·00825)	—
13136	32,500	67 (0·203)	4·0 (0·0124)	1·3 (0·004)
13137	47,500	70 (0·148)	4·8 (0·0101)	—
14668	31,800	35 (0·110)	1·95 (0·0061)	—
E5	33,400	61 (0·183)	2·3 (0·007)	—
GALT	94,100	94 (0·099)	8·8 (0·0094)	—
33	36,200	74 (0·204)	3·7 (0·0102)	—
63	46,800	120 (0·256)	4·1 (0·0088)	—
69	49,800	115 (0·231)	5·1 (0·0102)	—
8511	68,100	78 (0·11)	6·5 (0·0092)	—
8534	15,600	25 (0·163)	0·9 (0·0059)	—

It seemed unlikely that induction of penicillinase would take place during the incubation period (up to 6 hr), since the cocci were suspended in a non-nutrient medium (in fact washed suspensions in buffer were used rather than whole broth cultures deliberately to avoid induction occurring during this incubation period). To test this point, the penicillinase activities of coccal suspensions were measured against benzylpenicillin before and after incubation for 6 hr with cloxacillin 10 μg./ml.; in every case the activities of the two suspensions were precisely the same. It was confirmed that cloxacillin 10 μg./ml. did not inhibit staphylococcal penicillinase, so that it may be concluded that induction of penicillinase did not occur during the assay procedure adopted.

Since cephaloridine and cephalothin have extremely high affinities for staphylococcal penicillinase (of the order of 0·05 μM; Hamilton-Miller, 1966, and unpublished results), the rates of destruction given in Table 3 are indistinguishable from the values of V_{max}; the same argument applies for benzylpenicillin (K_m approximately 15 μM), but not for cloxacillin, which is known to have a very low affinity for staphylococcal penicillinase (Nayler *et al.* 1962). The rates of cloxacillin destruction given in Table 3 thus bear no relation to the V_{max} values for this compound. The argument in this paragraph presupposes that the inactivation of all these drugs was due to penicillinase (see next section).

Nature of destruction of cephalosporins

It can be seen from Table 3 that there seems to be a correlation between the rates of hydrolysis of benzylpenicillin and of the two cephalosporins. If this could be established, it would be excellent circumstantial evidence for the proposition that penicillinase is responsible for the destruction of the cephalosporins. The correlation was investigated by regression analysis, and was found to be highly significant (for benzylpenicillin against cephaloridine, $0{\cdot}01 > P > 0{\cdot}001$; for benzylpenicillin against cephalothin, $P < 0{\cdot}001$).

Removal or modification of the functional group in the 3-sidechain of cephalosporins can cause significant changes in biological activity (Chauvette *et al.* 1963). It was therefore considered necessary to examine the possibility that such a reaction might be responsible for some or all of the loss of activity observed in these experiments. Samples of cephalothin and cephaloridine which had been incubated in the presence and in the absence of the various staphylococcal strains were consequently subjected to chromatography. In no case was more than one spot apparent when the chromatograms were developed (whether by bioautography or by ultraviolet irradiation). Desacetylcephalosporins run more slowly in the commonly used chromatography

Table 4. *R_F values of cephaloridine and cephalothin in different solvent systems*

Values given are means of at least two determinations (number of determinations in parentheses).

Acid solvent: *n*-butanol + acetic acid + water, 12 + 3 + 5 by vol.
Neutral solvent: *n*-butanol + ethanol + water, 4 + 1 + 5 by vol.
Alkaline solvent: *n*-butanol + pyridine + water, equal vols.

Substance	Method of development	Acid Solvent	Neutral solvent	Alkaline solvent
			R_F values	
Cephaloridine	Bioautography	0·54 (6)	0·34 (4)	0·73 (2)
	Ultraviolet radiation	0·52 (4)		
Cephalothin	Bioautography	0·78 (2)	0·53 (4)	0·80 (2)
	Ultraviolet radiation	0·82 (4)		

solvents than do their parent compounds (see the figures given for cephalosporin C/desacetylcephalosporin C by Jeffery, Abraham & Newton, 1961, and for cephaloram/desacetylcephaloram by O'Callaghan & Muggleton, 1963). This also applies to cephalothin/desacetylcephalothin (C. H. O'Callaghan, personal communication: using paper buffered at pH 6·0, the R_F value of desacetylcephalothin in butanol + ethanol + water, 4 + 1 + 5 by vol., is 90 % that of cephalothin, and in propanol + water, 7 + 3, by vol. the corresponding figure is 85 %). Special attention was hence made to seek a spot with an R_F value less than that of cephalothin (see Table 4). No such spot was ever found, even in overloaded chromatograms (10 μg. nominal spot of cephalothin or cephaloridine). Assuming desacetylcephalothin to have about one-quarter of the activity of cephalothin (Wick, 1966), any amount of the former greater than about 1 μg. would be detectable by using the system which was employed in these experiments Since no trace was found on any occasion, it may be assumed that hydrolysis of the 3-sidechain did not, by itself, contribute significantly to the decrease in biological

activity which was observed under the assay conditions. Furthermore, on chemical grounds (Cocker *et al.* 1965) it is extremely unlikely that the pyridinium group of cephaloridine would be removed (or exchanged for some other nucleophile) in aqueous solutions such as used in these experiments, unless the β-lactam ring were first broken, in which case the product would be biologically inactive. Thus, the findings reported in this paragraph, taken in conjunction with the correlation between activity against benzylpenicillin and that against cephalosporins, suggest that the cephalosporins are inactivated by hydrolysis of the β-lactam ring mediated by staphylococcal penicillinase.

DISCUSSION

The finding that cephaloridine was more rapidly hydrolysed by staphylococcal penicillinase than was cephalothin is in line with the experience of Crompton *et al.* (1962), who reported that replacement of the acetoxy group of a cephalosporin by a pyridinium group increased the lability of the β-lactam ring. The evidence presented in the present paper shows that the methicillin-resistant staphylococci used did not have a special ability to hydrolyse cephaloridine, except in as much that such strains tend to produce larger amounts of the enzyme than do methicillin-sensitive strains (Richmond, Parker, Jevons & John, 1964). With the staphylococcal strains used here there was no significant difference between rates of benzylpenicillin hydrolysis by the two groups. Although these experiments showed that cephaloridine was about 15 times more labile than cephalothin, it must be stressed that the V_{max} for cephaloridine of staphylococcal penicillinase is only about a five-hundredth that of benzylpenicillin. Furthermore, cephaloridine is stable enough to staphylococcal penicillinase to act as a competitive inhibitor of the enzyme (Hamilton-Miller, 1966). The papers of Benner *et al.* (1965), Ridley & Phillips (1965) and Hye-Knudsen (1966), although their findings are not expressed in quantitative terms, might be interpreted as implying that cephaloridine is very labile to staphylococcal penicillinase; such a finding cannot be reconciled with the clinical and laboratory results (as regards penicillinase-producing *Staphylococcus aureus*) of, for example, Apicella, Perkins & Saslaw (1966), Vymola & Hejzlar (1966), Thornton & Andriole (1966). In the first published report on cephaloridine, Barber & Waterworth (1964) found that it was more effective than either cephalothin or methicillin against both large and small inocula of 5 methicillin-resistant strains of *S. aureus*. Seligman & Hewitt (1966) reported that cephaloridine was more effective *in vitro* than cephalothin against intrinsically resistant *S. aureus* strains; this finding has been confirmed in the present work (Table 2), although the degrees of intrinsic resistance were still too high for these strains to be regarded as fully sensitive to cephaloridine.

When cephaloridine solutions were incubated at 37° for 4 hr in buffer (a procedure carried out each time rates of breakdown of this compound were determined by biological assay), about 10 % of the original biological activity was lost, and the solutions had an odour of pyridine. Such incubation mixtures contained neither a detectable amount of any biologically active metabolite (e.g. a hypothetical 'despyridinium' derivative), nor any new compound detectable by ultraviolet irradiation. These findings suggest that pyridine is expelled from the cephaloridine molecule only as a consequence of fission of the β-lactam ring (under which circumstances expulsion of pyridine is known to be spontaneous; Sabath, Jago & Abraham, 1965). Thus, the

loss in activity of the control solutions of cephaloridine can be explained by spontaneous rupture of the β-lactam ring as the primary reaction. Control solutions of cephalothin did not lose any biological activity after incubation at 37° for 6 hr; this may reflect the greater intrinsic stability of the β-lactam ring in the cephalothin molecule.

Care must be taken in the interpretation of data about the stability of 'penicillinase-stable' β-lactam antibiotics to staphylococcal penicillinase, especially when comparing results obtained with high substrate concentrations (when, for instance, relative stabilities are measured by a chemical method) with those obtained at low concentrations (e.g. when measurements are made by a biological method). To take a specific example: Smith, Hamilton-Miller & Knox (1962) reported that the rate of hydrolysis

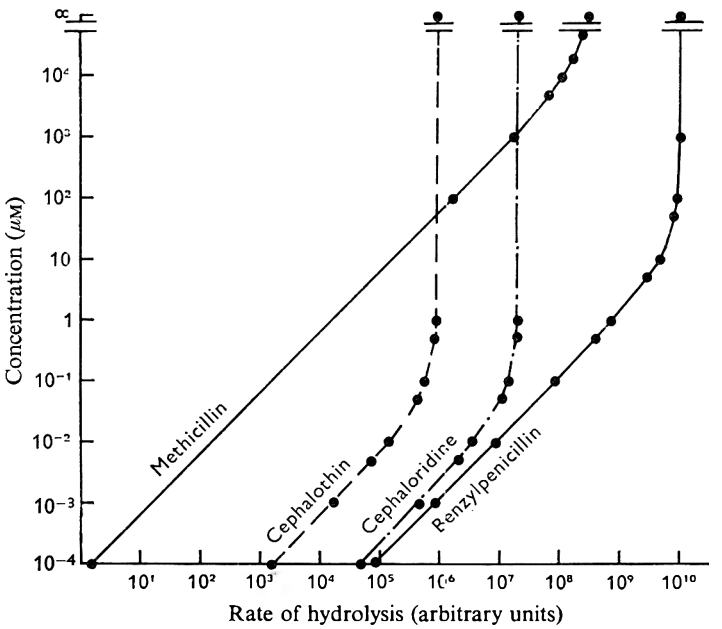

Fig. 1. Theoretical rates of hydrolysis of benzylpenicillin, cephaloridine, cephalothin and methicillin by penicillinase from *Staphylococcus aureus* 13. Rates calculated from Michaelis-Menten equation, $v = (V_{max}S)/(K_m + S)$, taking the following values of V_{max} and K_m: benzylpenicillin, 100, and 12·3 μM; cephaloridine, 0·18, and 0·045 μM; cephalothin, 0·007, and 0·056 μM; methicillin, 3, and 18 mM, respectively (Smith *et al.* 1962; Hamilton-Miller, unpublished results, and data presented in Table 3).

of methicillin (initial concentration 4·7 mM) was about 0·7 % that of the V_{max} for benzylpenicillin. The K_m for methicillin was about 18 mM. From these figures it is possible to calculate by assuming Michaelis-Menton kinetics that, at 10 μM (a therapeutically attainable concentration), methicillin will be hydrolysed at a rate which is only about 0·0019 % that of the V_{max} for benzylpenicillin. A similar argument applies to the isoxazolyl penicillins, but specific figures cannot be given, since K_m values for these compounds cannot be accurately measured (Richmond, 1965). As pointed out above, cephaloridine and cephalothin have extremely low K_m values, and hence will be hydrolysed at maximal rates even when at therapeutic concentrations. Thus, it is possible to draw up two completely separate lists to illustrate the order of lability of

these compounds. List 1, from data obtained at substrate concentrations of the order of 2 mg./ml., will show in descending order of lability: benzylpenicillin, cloxacillin, methicillin, cephaloridine, cephalothin. List 2, from data obtained at a therapeutically attainable concentration (e.g. 10 μM) will show in descending order of lability: benzylpenicillin, cephaloridine, cephalothin, cloxacillin, methicillin. It is clear that list 2 will be the more useful in working out correlations between penicillinase-lability and activity against penicillinase-producing staphylococci in a series of 'penicillinase-stable' compounds. The situation is represented graphically in Fig. 1.

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The Ultrastructure of the Cell Wall of *Bacillus megaterium*

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SUMMARY

The architecture of the cell wall of *Bacillus megaterium* strain M was studied by using electron microscopy of shadowed or negatively stained preparations, ultrathin sections and simple chemical methods. No conspicuous visible structure was found on the surface of whole organisms, isolated cell walls or the 'mucopeptide membranes'. Hot formamide treatment decreased the thickness of cell walls by about 50 %, leaving behind a rigid 'mucopeptide membrane'. The removed material is assumed to correspond with the 'teichoic acid' bound presumably by chemical linkage to the rigid layer, thus forming a plastic layer of the cell wall. The total thickness of the cell wall ranged from 200 to 240 Å, that of the rigid membranes from 90 to 110 Å. Poststaining of glutaraldehyde-fixed organisms or cell walls with osmium tetroxide, potassium permanganate or lead citrate produced a 'triple layered' cell-wall profile. This was not the case with uranyl acetate or phosphomolybdate staining, where the cell wall was represented by one homogeneous and dense track.

INTRODUCTION

The cell wall of *Bacillus megaterium* consists of two heteropolymers: (*a*) mucopeptide (or mucopolymer, glycosaminopeptide, murein) and (*b*) phosphomucopolysaccharide or X-teichoic acid (according to Ghuysen, Leyh-Bouille & Dierickx, 1962). The voluminous literature about the chemistry of *B. megaterium* cell wall as well as other Gram-positive bacteria has been summarized by Salton (1960, 1964), Work (1961), Colobert & Creach (1961), Rogers (1962) and Martin (1966). Mucopeptide represents the rigid part of the cell wall and is believed to form one bag-shaped molecule, the so-called 'murein sacculus' (Weidel & Pelzer, 1964). The plastic part of the cell wall is made up of teichoic acid. This is a polyol phosphate containing either ribitol or glycerol, alanine and an amino sugar, e.g. N-acetylglycosamine (in *B. megaterium*) or N-acetylgalactosamine in staphylococci (Ellwood, Kelemen & Baddiley, 1963). Archibald, Armstrong, Baddiley & Hay (1961) claimed that the teichoic acid was bound by salt linkage to the mucopeptide layer. This assumption was based on the fact that it could be extracted by cold trichloroacetic acid. But Ghuysen (1961) with *B. megaterium* and Krause & McCarty (1961) with streptococci showed that only a part of the teichoic acid was extracted by trichloroacetic acid. Both groups of authors used hot formamide extraction (Fuller, 1938) to isolate pure mucopeptide. This suggested that the linkage between teichoic acid and mucopeptide was of covalent nature (Czerkawski, Perkins & Rogers, 1963), namely a phosphodiester (Strominger & Ghuysen, 1963) or phosphomonoester (Knox & Hall, 1965). Recently Hay, Archibald & Baddiley (1965) suggested a phosphoramidate linkage, i.e. between the terminal phos-

phate group of the teichoic acid and an amino group of a mucopeptide amino sugar. There is less information about the ultrastructural arrangement of these two components. Till now most authors have been satisfied with stating that the cell wall of Gram-positive bacteria stains diffusely and homogeneously and has a thickness of 150–300 Å or more (see Salton, 1964).

Electron microscopic observations of shadowed as well as positively or negatively stained preparations of cell walls did not reveal any specific or regular arrangement of components (Gerhardt & Judge, 1964; Salton, 1964). An attempt has been made in the following experiments to clarify the localization and the mutual relationship of the rigid mucopeptide and the plastic teichoic acid layers, and to correlate the morphological findings with the results of chemical analysis (Ghuysen *et al.* 1962). Another task was to study the reliability of 'electron microscope stains' on a material which is chemically defined.

METHODS

Organism. Bacillus megaterium strain M (kindly provided by Dr C. Weibull, Stockholm) was used. An 18 hr meat infusion peptone agar culture was harvested into 0·06 M-phosphate buffer (pH 7·2), sedimented by centrifugation and used for further treatment.

Experiments with whole organisms. The pellet of bacilli was fixed with 3 % (w/v) glutaraldehyde (Eastman Kodak, biological grade) in 0·06 M-phosphate buffer (pH 7·2) for 3–5 hr at room temperature (Fahimi & Drochmans, 1965), washed many times (or overnight) with phosphate buffer, embedded in 2 % Noble agar, dehydrated and embedded in Vestopal according to Ryter & Kellenberger (1958*a*). Polymerization was done at 60° for 3 days, sections cut on an MT-1 Porter–Blum ultramicrotome and post-stained in the following ways: (*a*) 1 % (w/v) uranyl acetate (pH 4·5) for 50 min.; (*b*) 1 % (w/v) phosphomolybdate (pH 7·2) for 20 min.; (*c*) 1 % (w/v) osmium tetroxide for 60 min. or 4 % osmium tetroxide for 30 min.; (*d*) 0·5 % (w/v) potassium permanganate for 30 min.; (*e*) lead citrate according to Reynolds (1963); (*f*) 1 % (w/v) ruthenium red for 45 min. followed by differentiation with acetic acid (Luft, 1964); (*g*) 2 % (w/v) phosphotungstate (pH 7·2) for 20 min.; (*h*) 1 % (w/v) uranyl acetate for 50 min., followed by Reynolds lead citrate; (*i*) 4 % (w/v) osmium tetroxide for 30 min. followed by 1 % (w/v) uranyl ecetate for 30 min.; (*j*) the same as (*i*) followed by Reynolds lead citrate.

Experiments with isolated cell walls. The preparation, fixation and embedding of cell walls was done as described previously (Nermut, 1965). In some cases additional poststaining was done as described above. 'Mucopeptide membranes' were obtained by boiling bacilli in formamide at 200° for 4 min. (Nermut, 1965). In one experiment a shorter time of extraction was used to determine the localization of the extracted (sensitive) material.

Measurement of the thickness of cell walls or of mucopeptide membranes was done by means of a projector with a micrometric screw which enabled very exact measurements to be made (Profile projector, Nippon Kogaku, K.K.).

Electron microscopy. Preparations were shadowed with platinum or tungsten oxide. Positive staining of cell walls was done by 1 % (w/v) uranyl acetate treatment for 30 min. followed by washing. Negative staining was done with 1 % (w/v) phospho-

tungstate as in the author's modification (Nermut, 1964). Specimens were examined with an electron microscope JEM 6-c (Japan Electron Optics Laboratory) and with the Philips Em 100 or EM 200.

RESULTS

Experiments with whole organisms

Shadowed preparations of *Bacillus megaterium* organisms did not reveal any conspicuous structure similarly as negatively stained bacilli where only mesosomes were clearly seen. This showed that the cell wall was easily penetrated by phosphotungstate, which was not the case with Gram-negative bacteria (Nermut, 1964).

Sections of bacilli fixed with glutaraldehyde showed little or no contrast, but when the negatives were printed on an extra-hard photographic paper the structure of the cell wall and individual ribosomes could be distinguished. (Pl. 1, fig. 1). The cell wall consisted of two dark lines with a light interspace; the total thickness was about 160 Å.

However, sections poststained with uranyl acetate revealed homogeneous cell walls without any 'layering', 200–240 Å in thickness. The contours of the cell walls were often uneven and their thickness sometimes reached 300 Å or more (Pl. 1, fig. 2). This phenomenon may have been due to the presence of a microcapsule, which could be stained by uranyl acetate but not by osmium tetroxide or permanganate. In some cases the microcapsular layer was clearly seen (Pl. 2, fig. 6).

Lead citrate did not stain the cell wall well; usually a double track was observed, but the ribosomes were very dark under those conditions (Pl. 1, fig. 3). Ruthenium red, reported by Luft (1964) to have affinity for polysaccharides, stained the cell walls and the ribosomes, even though not providing much contrast (Pl. 2, fig. 4). Interesting results were obtained with phosphotungstic acid and phosphomolybdic acid; in most cases the cell walls were stained homogeneously, as after uranyl acetate, and measured about 200–250 Å (Pl. 2, fig. 5). The cytoplasmic membrane was represented by a narrow light zone, demonstrating that the lipids were extracted during dehydration. In some cases a loose superficial material with holes could be seen which suggested a microcapsule (Pl. 2, fig. 6). The whole profile measured in this case about 400 Å, and the cell wall proper was only 200 Å thick.

With osmium tetroxide and with potassium permanganate the cell wall was limited by two distinct tracks about 160–200 Å apart (Pl. 2, fig. 7). With permanganate the interspace showed no electron-dense material. Further poststaining with uranyl acetate revealed a picture very like that after uranyl acetate alone; the cell wall measured then about 240 Å (Pl. 2, fig. 8).

Thus it was shown that the 'triple layered' cell wall in *Bacillus megaterium* was observed only when the walls were stained by osmium or permanganate. Better contrast was obtained by poststaining with lead citrate (Fig. 1). These results suggest a certain unreliability of the mentioned contrasting agents as far as the distribution of substance and the number of cell wall layers are concerned. Observations at high magnification made possible in some cases a detailed study of the cell walls; Pl. 2, fig. 9 and Pl. 3, fig. 10 are examples of this. On both photographs fine 'channels' could be observed, represented by light 'spaces' of about 15–25 Å on section, and of different lengths. They do not penetrate the cell wall in a direct way. They are very clearly seen, particularly in Pl. 2, fig. 9 (arrow). The dense granules or areas surrounding the 'channel' were of various shape and size. They usually measured 25–35 Å, but larger

structures were up to 50 Å. It is a question whether the dark structures represented sections through polymer fibres of the cell wall or only precipitated uranium or lead near the real fibres. In any case there were two clearly visible and distinguishable elements in the sections of cell walls: electron-transparent 'areas' and electron-dense granules, roclets and irregular areas. For comparison an oblique section through a 'mucopeptide membrane' is presented in Pl. 4, fig. 16. The dark granules measure about 20–30 Å. Thus, in sections there was no striking difference between the plastic layer and the rigid one. Therefore it is difficult to draw final conclusions about the fine arrangement of the 'building blocks' in the cell wall and one must be reserved when interpreting the electron micrographs.

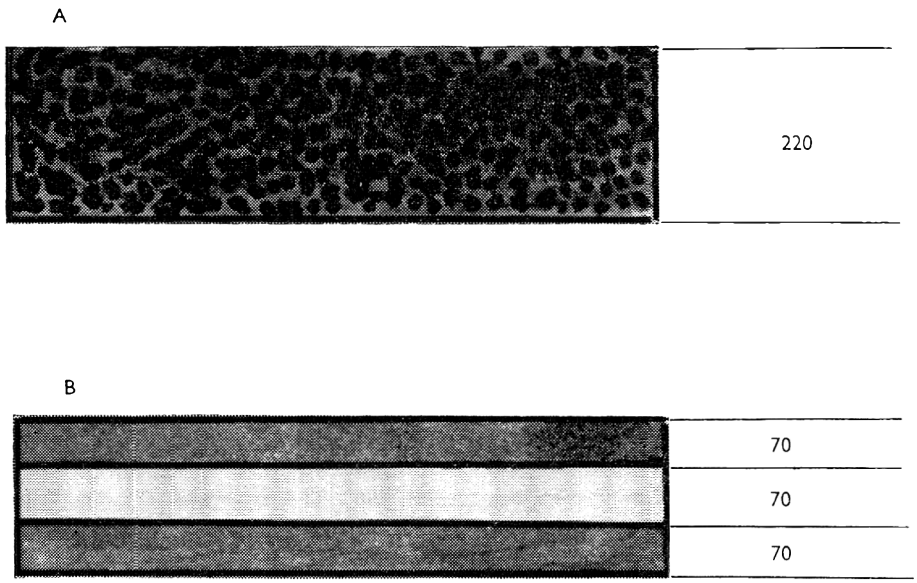

Fig. 1. Schematic representation of *Bacillus megaterium* cell wall fixed with glutaraldehyde and post-stained: A, with uranyl acetate, phosphotungstate, phosphomolybdate or ruthenium red; B, with osmium tetroxide, potassium permanganate or lead citrate.

No boundary could be seen between the two layers, supporting the concept that there is a firm chemical linkage between them. When interpreting the ultrathin sections one must be aware of the difficulties arising from the thickness and orientation of the section. The real arrangement of the cell-wall components could be seen only under ideal conditions, i.e. in very thin sections (~ 100 Å) which were exactly perpendicular to the cell body.

Experiments with isolated cell walls and mucopeptide membranes

The terms 'layer' and 'membrane' will be used in this paper in the following senses. A layer is a continuous part of the cell wall which can be separated from the outer components of the cell wall. For isolated layers the term 'membrane' has been used when they really form a membrane. Thus the mucopeptide forms a layer within the cell wall, but is called a membrane when isolated.

Shadowed preparations of isolated and purified cell walls did not reveal any con-

spicuous or regular structure. The same was the case with negatively stained cell walls. More interesting results were obtained when staining the cell walls positively with uranyl acetate. There was a striking difference between the outer and the inner side of the cell wall. The former was dark and 'rough', the latter light and smooth (Pl. 3, fig. 11). It is necessary to point out that this staining was done after first drying the cell walls on grids. This might account for the difference in electron density as between the outer and inner parts of the cell wall.

The observations made on sections can be summarized as follows: Uranyl acetate stained the cell wall homogeneously, whereas osmium tetroxide, potassium permanganate or lead citrate produced a 'unit membrane' image. Some sections were post-stained for a longer time with lead citrate so that a superficial loose layer was seen which might represent either the plastic layer or the above mentioned 'microcapsule' (Pl. 3, fig. 12). A detail of the same cell wall showing a 'unit membrane' about 100 Å thick is shown in Pl. 3, fig. 13. Thus the results with isolated cell walls were practically the same as with whole bacilli.

Boiling bacilli or cell walls in formamide at 200° for 4 min. was effective in producing delicate rod-shaped membranes (Pl. 4, fig. 14). They were lysed within 8 min. by 200 μg. lysozyme/ml. (observed by nephelometry) and therefore were presumed to be mucopeptide. To determine the location of the plastic layer (removed by formamide), shorter extraction times were used (1·5 and 2·5 min.). Shadowed preparations made from specimens so treated showed irregular remnants or a coarse surface of the cell walls, but it was not easy to make any definite decision about the location of the remnants. Better results were obtained with sections, where it was evident (in some cases) that the material was being extracted from the outermost zone (Pl. 4, fig. 15). After extraction for 4–5 min. the mucopeptide membranes were free from remnants. There was no basic difference among the contrasting agents in staining the mucopeptide membranes (in sections). Mostly only one dark track was observed, about 100 Å thick.

With a high-resolution electron microscope (EM 200) we hoped to find structural details in the rigid layer, particularly in oblique sections (Pl. 4, fig. 16), but it was difficult to find a regular pattern. The most important question is whether the dark points or spots represented the cross-sections of polymer fibres or only granules of osmium or lead. This is not yet clear and our conclusions about the ultrastructure of the rigid layer remain uncertain.

The measurement of thickness of the cell wall in sections is charged with error, in spite of accurate measurement by means of a projector. The difficulties arise from the fact that not all the measured cell walls were cut perpendicularly. Every deviation from the perpendicular plane distorts the results (Lickfeld, 1965). Although more than 50 cell walls and 50 mucopeptide membrane were measured, the results were not statistically evaluated. The thickness of the cell wall ranged from 210 to 240 Å, that of the 'mucopeptide membranes' from 80–110 Å. The average thickness was near 220 Å for the cell wall and near 100 Å for the 'mucopeptide membranes'. This means that the mucopeptide membrane occupied about 45 % of the cell-wall width

DISCUSSION

Extraction of *Bacillus megaterium* cell walls with hot formamide decreased their thickness by about 50 %. This supports the concept that the cell wall is made up of two layers which can be separated. The mucopeptide rigid layer is resistant to hot formamide extraction and forms a rod-shaped membrane about 100 Å thick. The plastic mucopolysaccharide layer, which is dissolved by hot formamide, is situated outside the rigid layer. This type of result has not always been obtained. For example Archibald *et al.* (1961) did not find any difference in 'submicroscopic appearance' of cell-wall sections of *Bacillus subtilis* and a *Lactobacillus* sp. before and after extraction with cold trichloroacetic acid. In both cases the walls were 'triple-layered' after permanganate fixation, however, no exact data about their thickness was given. In addition, permanganate is not very reliable in staining the cell-wall substance, and it

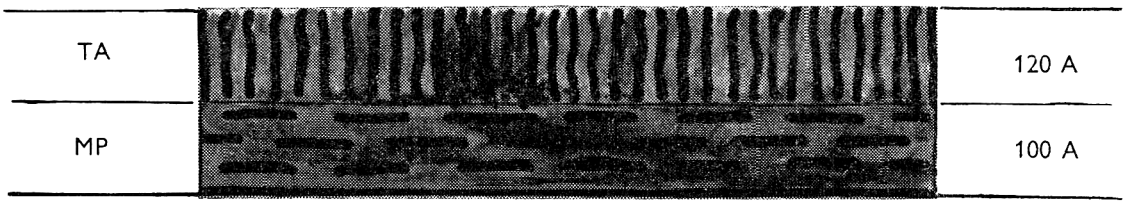

Fig. 2. A tentative scheme of the cell wall of *Bacillus megaterium* showing a rigid mucopeptide layer (MP) and a 'plastic' layer formed by teichoic acid bristles' (TA).

seems to give a precipitate on the surface of membranes, irrespective of their chemical composition (Bradbury & Meek, 1960). Ghuysen *et al.* (1962) found that the teichoic acid of *B. megaterium* was not completely extracted by cold trichloroacetic acid and that complete removal required specific enzyme or hot formamide. Krause & McCarty (1961) made a similar observation with streptococci. For the same reason the results obtained by using the method of Park & Hancock (1960), were not satisfactory as the hot formamide extraction of membranes obtained by this procedure solubilized some more hexosamines (about 40 % according to Nermut & Svobodová, 1965). In the present experiments, sections through cell walls extracted for shorter times showed that the teichoic acid lay on the outer side of the rigid layer. Thus, there is some analogy with Gram-negative cell walls, where the innermost position of mucopeptide layer has been demonstrated by Bladen & Mergenhagen (1964) and Murray, Steed & Elson (1965).

Uncertainties surround the presence of microcapsular material, which could be seen in some cases and which very probably caused the increase in thickness up to 300–400 Å. No special structured protein layer could be found as was observed in *Bacillus polymyxa* (Nermut & Murray, 1967). For this reason it is assumed that the cell wall of *B. megaterium* represents a 'basic Gram-positive cell wall' built up of two closely placed layers, one of them being rigid and the other plastic (Fig. 2). This is supported by the chemical analysis by Ghuysen *et al.* (1962), which showed that the mucopeptide and the mucopolysaccharide were in equimolar proportion. The plastic layer of *B. megaterium* is represented by short chains perpendicular to the cell surface. There are several reasons for this type of arrangement. The plastic layer cannot be isolated as a membrane, as can the mucopeptide one. This means that there are probably no,

or very weak, side links between the fibres of the teichoic acid. The surface of the cell walls is coarse rather than smooth. No regularity was observed in sections, either longitudinal or transverse. This again speaks in favour of the plasticity, or at least the non-rigidity, of this layer.

The idea of the perpendicular orientation of teichoic acid fibrils is supported by the results of Ghuysen *et al.* (1962), who established that the teichoic acid from *Bacillus megaterium* cell wall was built up of 10 subunits. Similar results with other organisms were reported by Armstrong, Baddiley & Buchanan (1960) and Hay *et al.* (1965). Thus in lactobacilli the teichoic acid is made up of 7·6 units, in staphylococci of 8·1 and in *B. subtilis* of 8·9 units. It seems very probable that in *B. megaterium* the chain of a 10-unit teichoic acid is attached to the mucopeptide by one phosphate group (phosphoramidate linkage according to Hay *et al.* 1965) and the other group lies free on the surface, so contributing to the negative charge of the cell wall (Rogers, 1962). There is no direct evidence for this idea from our sections. It is very difficult to find an exactly perpendicular section (either to the long or short axis) which is also very thin where the parallel 'bristles' of teichoic acid could be seen. The other difficulty arises from the flexibility or plasticity of the teichoic acid fibres, which can collapse upon drying so that any regularity is disturbed. The length of teichoic acid composed of 10 units is estimated to be about 100–120 Å (Dr H. J. Rogers, personal communication), which corresponds very well with our findings. Application of freeze-etching method could probably help to solve this problem. Fibrils in the cell walls of *B. cereus* were recently described by Remsen (1966).

The presence of irregular 'channels' accounts for the good permeability (or penetrability) of *Bacillus megaterium* cell walls for phosphotungstic acid or larger molecules (Gerhardt & Judge, 1964). As the chemistry is not the main topic of this paper, the question of the type of linkage of teichoic acid to the mucopeptide has not been discussed in more detail.

Post-staining of glutaraldehyde-fixed bacilli or cell walls with various metal compounds showed that one cannot speak of a 'triple layered' or 'trilaminate' cell wall if this is based on staining by osmium, permanganate or lead only. We were able to show that 'unit membranes' can be produced by all these agents, particularly by post-staining with lead (Pl. 3, fig. 13); this happens even with material which does not contain any lipids or lipoproteins. Obviously other chemical groups are responsible for the reduction of osmium tetroxide or potassium permanganate in this case (Bahr, 1954). The most reliable view of the disposition of cell-wall material is given by uranyl acetate because of its low specificity (binding occurs with both phosphate groups and carboxyl groups; Elbers, 1964). A similar conclusion was drawn from parallel observation on *Bacillus polymyxa* (Nermut & Murray, 1967).

This paper is dedicated to the memory of Professor F. Herčík, my first teacher in science.

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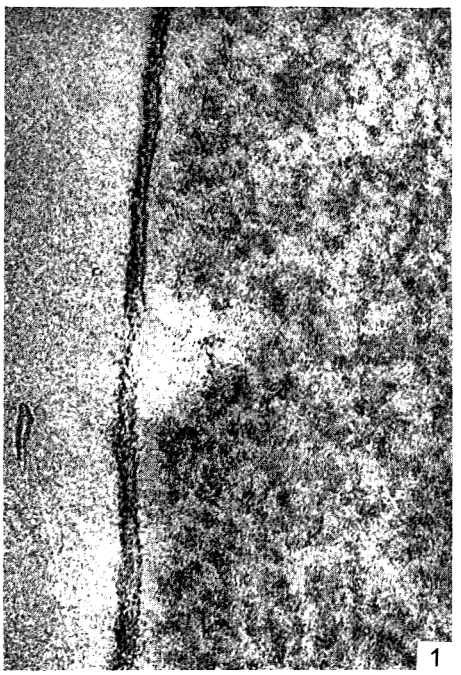

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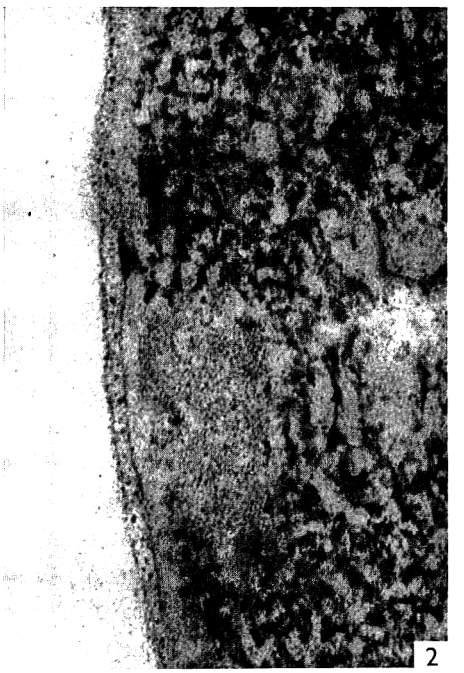

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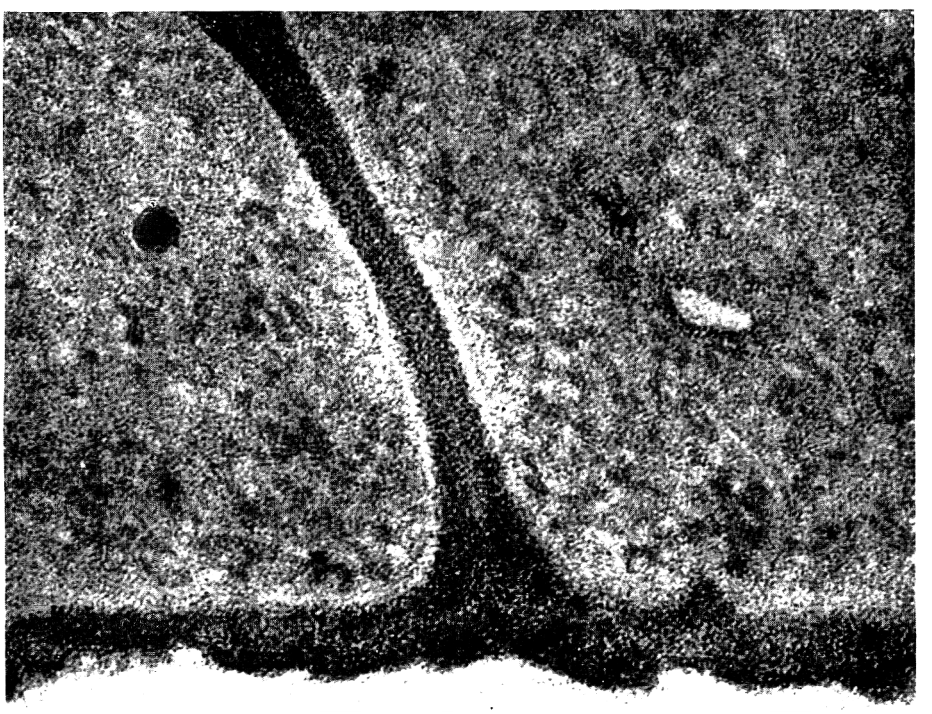

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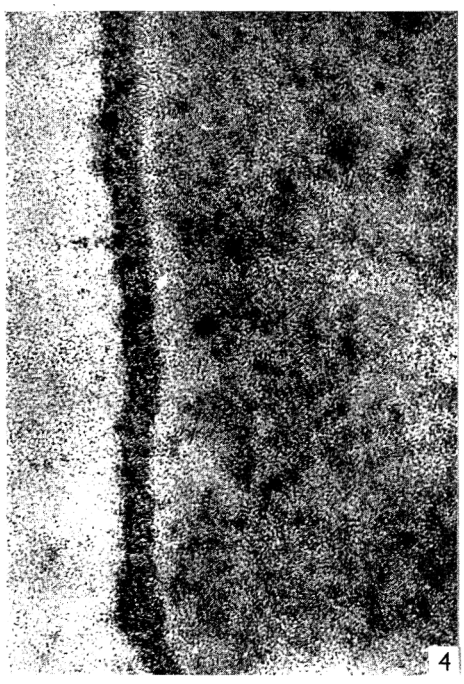

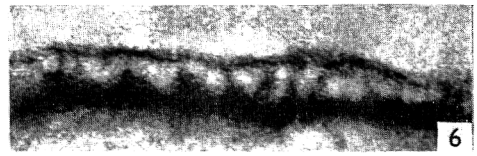

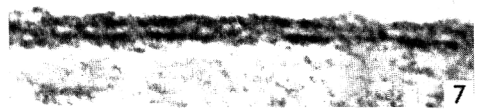

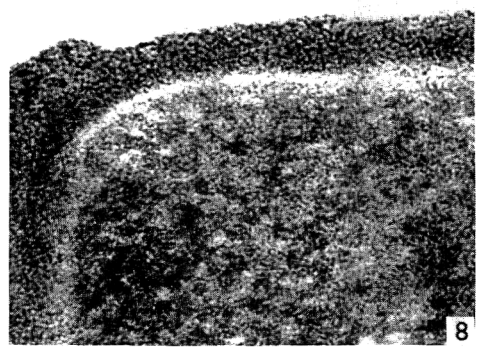

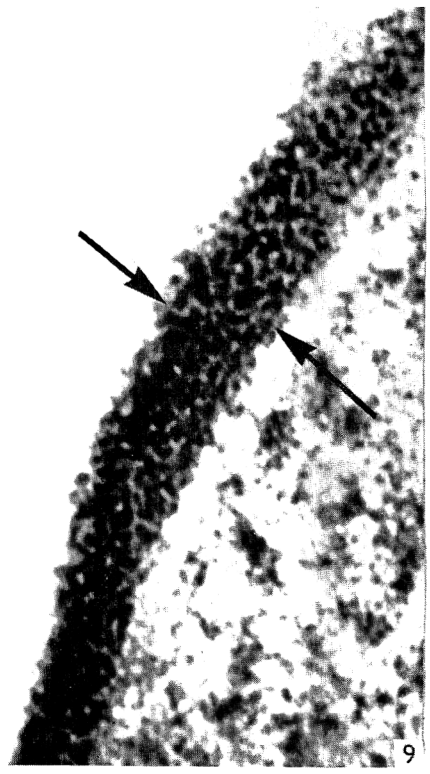

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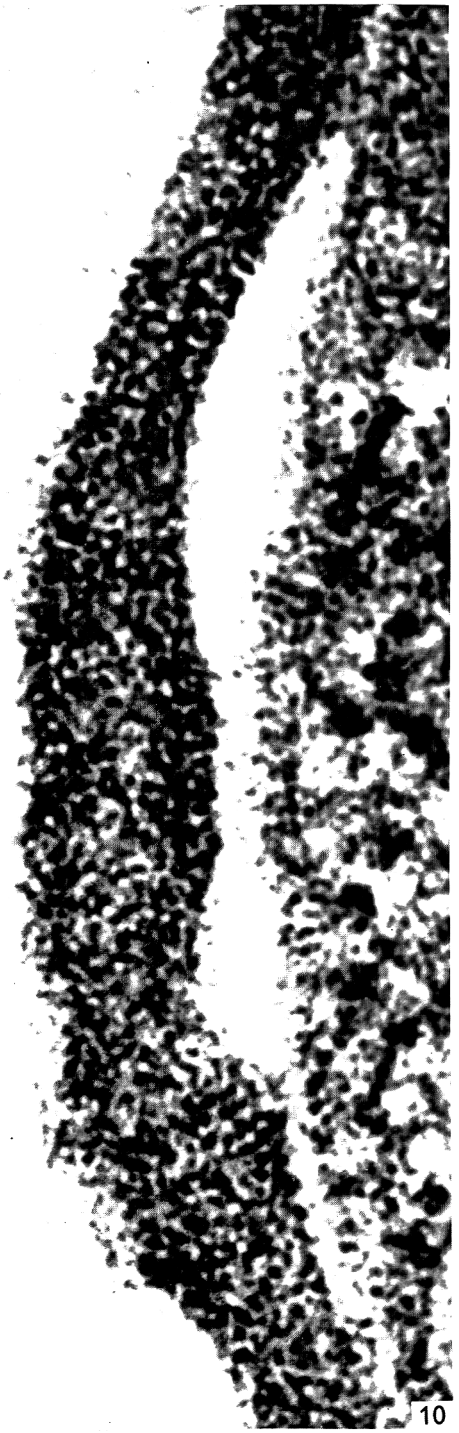

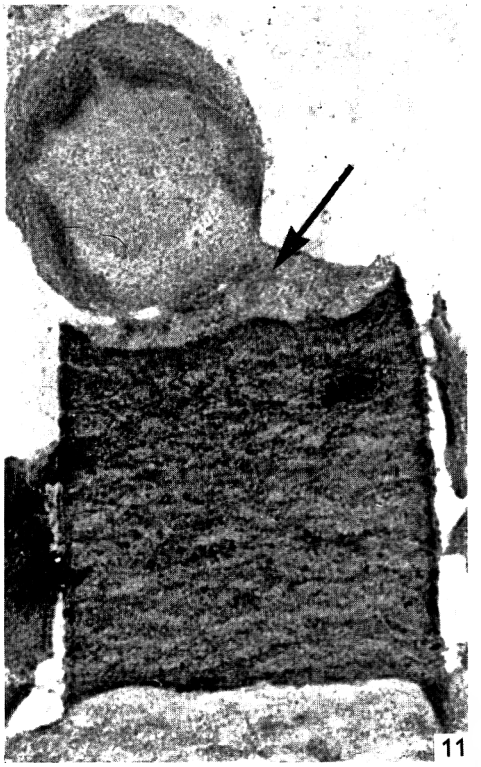

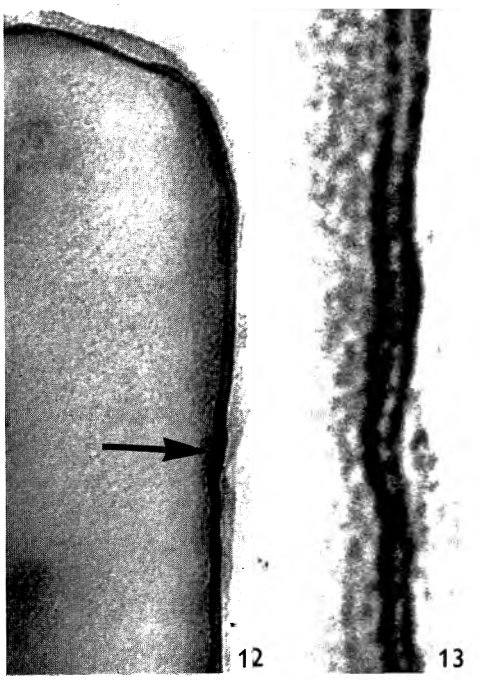

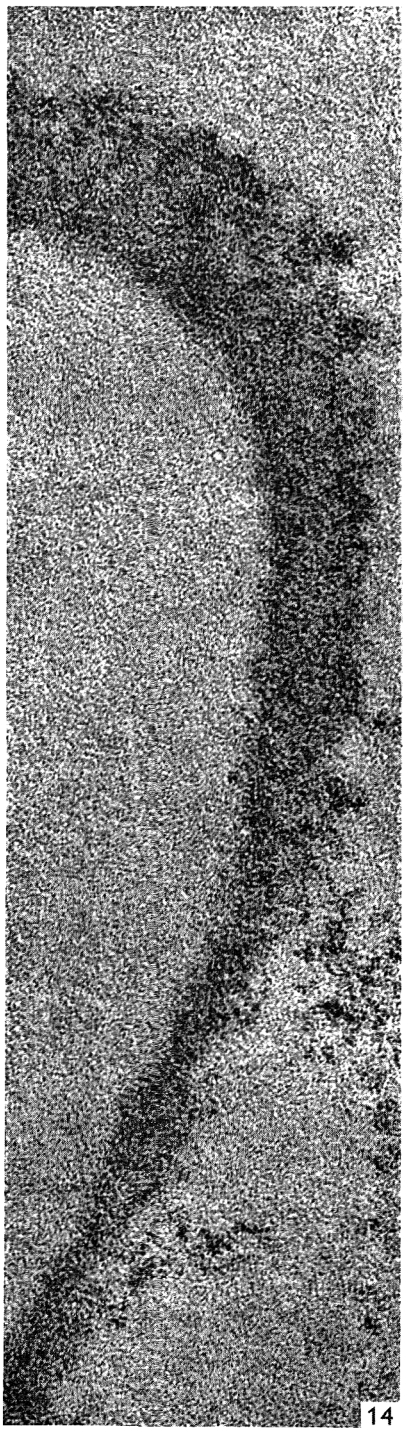
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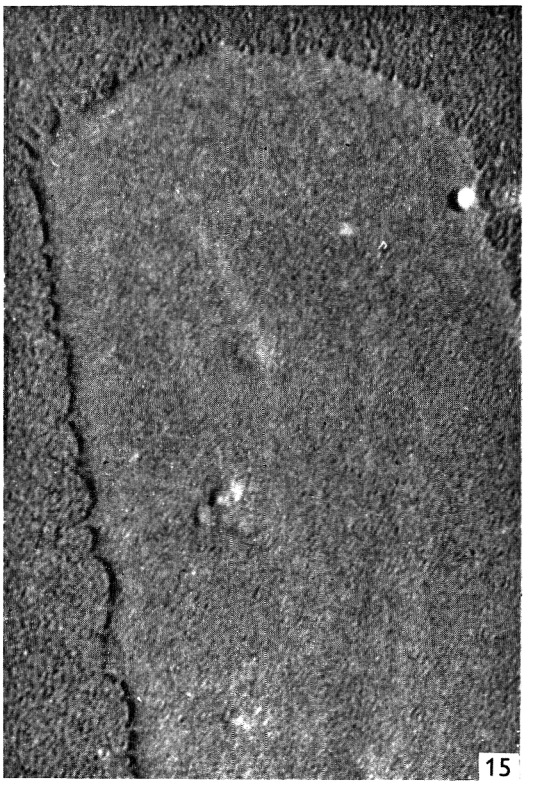
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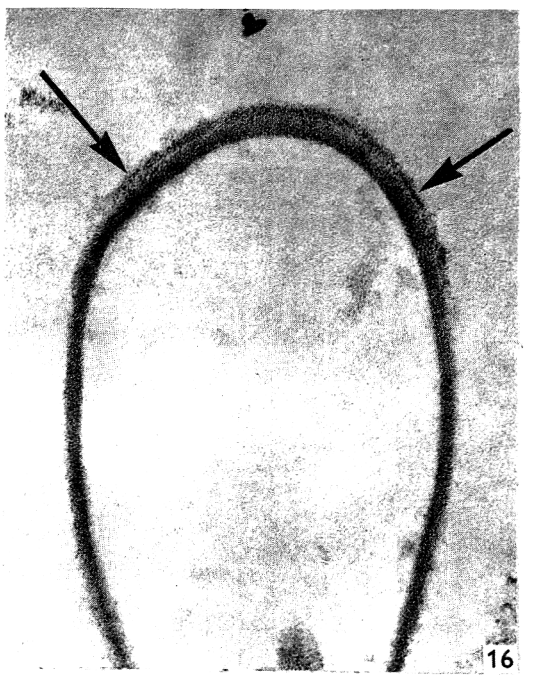
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EXPLANATION OF PLATES

All micrographs are of *Bacillus megaterium* or its fractions.

PLATE 1

Figs. 1–9. Sections of *Bacillus megaterium* fixed with glutaraldehyde and post-stained on sections as indicated.

Fig. 1. No post-staining, printed on an extra-hard photographic paper. × 120,000.

Fig. 2. Post-stained by lead citrate. × 120,000.

Fig. 3. Post-stained by uranyl acetate. The cell wall and the septum are dense (dark); no layering observable. The outer edge is uneven. × 200,000.

PLATE 2

Fig. 4. Post-stained by ruthenium red. × 170,000.

Fig. 5. Post-stained by phosphomolybdate. General appearance is very similar to that of uranyl acetate stained cells. × 200,000.

Fig. 6. Post-stained by phosphotungstate. The proper cell wall is dark and homogeneous. The holey part is probably a microcapsule. × 200,000.

Fig. 7. A part of the cell post-stained by osmium tetroxide showing a 'triple layered' cell wall. × 200,000.

Fig. 8. Post-staining by osmium tetroxide and uranyl acetate. × 200,000.

Fig. 9. A detail of a cell wall; post-stained by uranyl acetate followed by lead citrate. Irregular 'channels' connecting the outer and inner surface can be found in the wall (arrow). × 500,000.

PLATE 3

Fig. 10. A detail of a cell wall fixed and post-stained as in Fig. 9. × 500,000.

Fig. 11. Isolated cell wall dried on grid and stained 30 min. with 1 % uranyl acetate. Note the difference between the surface and the inner side (arrow). × 30,000.

Fig. 12. Isolated cell wall fixed with glutaraldehyde, post-stained by lead citrate. After prolonged lead staining two parts can be distinguished. The inner one has a unit membrane structure (arrow). × 100,000.

Fig. 13. Enlarged portion of the cell wall of fig. 12 indicated by arrow. × 500,000.

PLATE 4

Fig. 14. Detail of an oblique section of an incompletely extracted cell wall treated with hot formamide for 2·5 min. Same fixation as in fig. 15. × 300,000.

Fig. 15. 'Mucopeptide membrane' obtained by formamide extraction. Shadowed. × 100,000.

Fig. 16. Section of a cell wall extracted 1·5 min. only with hot formamide. The formamide sensible 'layer' remains on the outer part of the cell wall (arrows). Fixed with Ryter & Kellenberger fixative (1958*b*). × 100,000.

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The Formation of Protoplasts and Quasi-spheroplasts in Normal and Chloramphenicol-pretreated *Bacillus subtilis*

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SUMMARY

The lytic action of lysozyme upon *Bacillus subtilis* walls was studied by following the disappearance of bacillary-colony-forming units and the appearance of L-colony-forming-units. The rapidity of cell wall removal by lysozyme fluctuated markedly during growth in a chemically defined medium, presumably because subtle changes in the cell wall were constantly occurring. When lysozyme-sensitive bacilli were grown with chloramphenicol 10 μg./ml. for 3 hr they showed a notable increase in lysozyme resistance; at the same time, their walls almost doubled in thickness. As lysozyme attack proceeded in a given culture, the bacilli passed first through a rod-shaped osmotically sensitive stage, and then a spherical stage characterized by incomplete removal of cell wall before finally reaching the naked protoplast stage. The spherical forms with adherent wall residues formed L colonies on a medium containing the reversion inhibitor D-methionine and bacillary colonies on the same medium without D-methionine. Under the latter conditions, the cell wall residue served as a starting point for rebuilding of complete wall, much as residual wall permits reversion of Gram-negative spheroplasts to the bacillary state. In the presence of D-methionine, the feedback sequence required for wall formation was severed, resulting in heritable propagation of the protoplast state.

INTRODUCTION

The enzyme lysozyme hydrolyses the β-1,4 linkages between the muramic acid residues and the *N*-acetylglucosamine residues of bacterial cell-wall peptido-glycan. Peptido-glycan, a rigid material which holds bacilli to their characteristic shape and helps to protect them from osmotic shock, is found in almost all bacteria and in the blue-green algae. In the Gram-positive bacteria, peptido-glycan is generally a principal cell-wall component, while in Gram-negative bacteria the peptido-glycan or rigid layer is only one of several components which make up the complex wall (Salton, 1964). When Gram-positive bacteria such as *Micrococcus lysodeikticus* or *Bacillus subtilis* are subjected to lysozyme action, the wall is extensively depolymerized so that teichoic acid, the other major wall constituent, is freed and released into the supernatant fluid along with fragments of the peptido-glycan (Miller & Landman, unpublished; Young, 1967). A spherical protoplast results which is bounded only by membrane and is apparently devoid of cell wall (Ryter & Landman, 1964). When Gram-negative *Escherichia coli* organisms are treated with lysozyme, presumably only the rigid (innermost?) layer is attacked. The lipoprotein-containing outermost layer and the lipopolysaccharide-containing middle layer remain (Salton, 1964; Weidel, Frank &

Martin, 1960). Nevertheless, having lost much of their rigid layer, these forms, called spheroplasts, are spherical in shape and sensitive to osmotic shock. Superimposed on this relatively simple and perhaps overgeneralized picture, there are numerous complexities due to differences among bacterial species. Further, as we shall show in this paper, the response to lysozyme of a single strain is clearly different at different times during growth.

Even greater changes in lysozyme sensitivity are produced by incubation of bacteria with antibiotics. It is well known that an important group of antibiotics attacks bacteria principally through damage of the cell envelope. This group includes penicillin, bacitracin, novobiocin, ristocetin, vancomycin, cycloserine, tyrocidin, gramicidin-S, and polymixin (Newton, 1965). Incubation of bacteria in the presence of any one of these substances could plausibly be expected to produce damage to the cell wall. In the present paper, we are concerned with changes in the cell wall produced by incubation with chloramphenicol (CAP), an antibiotic which is primarily an inhibitor of protein synthesis (Newton, 1965). In agreement with previous reports (Hash & Davies, 1962; Shockman, 1965), we have observed that prolonged inhibition of protein synthesis leads to a marked increase in the thickness of the cell wall. A sharp increase in lysozyme resistance was also noted in conjunction with CAP incubation or tryptophan starvation.

In studying the lysozyme-catalysed removal of cell wall from bacteria incubated with CAP it was found that a notable proportion of the bacteria from incompletely treated populations exhibited a plating behaviour intermediate between naked protoplats and intact bacilli. Like protoplasts, these forms gave rise to heritably stable L colonies on one type of plating medium (Landman & Halle, 1963) and, like bacilli or spheroplasts of Gram-negative bacteria (Landman, Altenbern & Ginoza, 1958), they produced bacillary colonies on a more favourable medium. The intermediate forms are thus on the borderline to commitment to the stable L state. A similar form has been detected in streptococcal populations incompletely treated with a wall-removing enzyme (King & Gooder, 1965). A preliminary report of the present findings has already been published (Miller & Landman, 1965).

METHODS

Organism. Bacillus subtilis strain 168 (indole$^-$) was used in all experiments.

Media. L forms and bacillary forms were grown on two plating media, DP and SDM. DP medium had the following composition: 0·021 M-K_2HPO_4; 0·011 M-KH_2PO_4; 0·5% acid-hydrolysed casein (Nutritional Biochemicals); 0·2% glucose; 0·002% L-tryptophan; 0·5 M-sodium succinate, pH 7·3; 0·005 M-$MgCl_2$; 0·5% horse serum or human plasma; 0·9% agar. SDM medium was as DP medium except that 0·1% NH_4NO_3 replaced the casein hydrolysate as nitrogen source and 0·06% D-methionine was added to retard reversion of L forms. For dilutions of bacilli and protoplasts a hypertonic dilution fluid (DFR) was used; it had the following composition: 0·25 M-sucrose; 0·25 M-sodium succinate, pH 7·0; 0·021 M-K_2HPO_4; 0·011 M-KH_2PO_4; 0·01 M-$MgCl_2$; 0·0005 M-EDTA. The bacilli were grown successively on media SL1 and SL2 (Landman & Halle, 1963) before treatment to form protoplasts. These media were the same as the competence media of Anagnostopoulos & Spizizen (1961) except that medium SL2 contained 0·5 M-sucrose to stabilize protoplasts.

Growth of cultures. The procedure followed to prepare bacilli for treatment to give protoplasts paralleled the procedure used by Anagnostopoulos & Spizizen (1961) to render bacteria competent for transformation: spores of *Bacillus subtilis* 168 were inoculated onto plates of blood agar base medium (DIFCO), germinated, and incubated overnight at 37°. The confluent growth was harvested into a small volume of medium SL1 and adjusted with fresh medium to 45–50% transmission (at 670 mμ) in the Bausch & Lomb Spectronic 20 Spectrophotometer; this turbidity corresponded to a colony count of about $1{\cdot}5 \times 10^8$ bacilli/ml. This culture was incubated with shaking at 37° for 4 hr, then diluted 1/10 in medium SL2 and incubated with shaking at 37° for a further 90 min. These incubations were usually followed by further incubation in SL2 medium without or with chloramphenicol 10–15 μg./ml. Samples of these bacilli were treated to give protoplasts at various times during these incubations, by adding lysozyme as indicated to 5 ml. volumes of bacillary suspension in SL2 medium in 125 ml. Erlenmeyer flasks which were kept stationary in a water bath at 32–34°.

Colony counts quoted are averages based on 3–4 plates. Plates inoculated with protoplast-containing suspensions were incubated at 30°; plates from bacillary suspensions were incubated at 30° or 37°.

Arrest of lysozyme action for differential phase microscope counts of spheres and rods. To assess the progress of sphere formation by phase microscopy at intervals during lysozyme treatment, it was important to arrest enzyme action abruptly. Formation of protoplasts in SL2 medium was stopped instantly upon adding an equal volume of 1% (w/v) OsO_4 in 0·5M-sucrose. Differential counts in the Petroff-Hausser chamber had to be performed promptly since the spheres and osmotically sensitive bacilli began to lyse about 5 min. after the OsO_4 addition.

Procedure for electron microscopy. Five ml. of suspension of bacilli, protoplasts or 'protoplasting' bacilli were placed into a plastic centrifuge tube at 4° and centrifuged for 20 min. at 8000 *g*. The pellet was rinsed 3 times with fresh SL2 medium and suspended in 0·1 ml. SL2 medium. Then 0·05 ml. of 4% (w/v) glutaraldehyde (redistilled) in 0·1M-cacodylate buffer (pH 6·0) was added and the tube warmed to 45°. Molten 3% agar (0·2 ml.) containing a 0·5 × concentration of SL1 salts and 0·5M-sucrose was then mixed gently into the slurry. After the agar had cooled to room temperature, the plastic tube was cut open and the agar with the suspended organisms cut into 2–3 mm.[3] blocks which were fixed overnight in cacodylate-buffered 4% glutaraldehyde. The following day the agar blocks were washed in 3 changes of veronal buffer (pH 6) for a total of 3 hr, according to the procedure of Kellenberger, Ryter & Séchaud (1958). Overnight fixation with OsO_4, washing, treatment with uranyl acetate and further washing all were now followed according to the prescription of these authors. Finally, the blocks were dehydrated in graded concentrations of ethanol, washed twice for 15 min. in propylene oxide and embedded in Epon (Luft, 1961). Sections, approximately 500 Å in thickness, were cut with the MT 1 Porter–Blum microtome and stained for 1 min. in 0·2% lead citrate in 0·1N-NaOH. The sections were examined in the RCA EMU 2D electron microscope.

RESULTS

Changes in lysozyme sensitivity during growth

When lysozyme is added to a suspension of *Bacillus subtilis* in hypertonic medium, the bacilli are gradually converted to protoplasts. These protoplasts give rise quantita-

tively to L colonies when plated on certain soft agar media containing 0·5M-Na succinate (Landman & Halle, 1963). The rate of cell wall removal—and hence the rate of disappearance of bacillary-colony-forming elements and the rate of appearance of L-colony-forming units, 'commitment', depends on several factors. ('Commitment' was earlier defined as 'The point in time at which a cell loses its ability to give rise to a bacillary colony and gains its capacity to give rise to an L colony' (Landman & Halle, 1963). With the demonstration in the present paper that a given cell may persist for some time in a borderline state from which it may give rise to either a bacillary colony or an L colony, this definition of commitment requires further refinement.) For example, lysozyme acts much more rapidly in sucrose-stabilized media than in succinate-containing media. Further, both lysozyme concentration and temperature markedly affect the rate of protoplast formation. Table 1 exhibits the effect of a more subtle variable on lysozyme sensitivity, namely, the previous growth history of the

Table 1. *Bacillus subtilis 168. Changes in sensitivity to lysozyme during competence incubation*

Organisms were grown for 4 hr in medium SL1, diluted 1/10 into medium SL2 and incubated further. At the times shown, samples were taken, subjected to 25 min. treatments with lysozyme 100 μg./ml. and plated on DP medium. The % survival was obtained by comparing the total count of colonies (bacillary+L colonies) appearing on these plates with counts of untreated culture samples. A high % of L colonies on the experimental plates is a reflexion of a high degree of sensitivity to lysozyme.

Time of growth in medium SL1 (hr)	Experiment 1		Experiment 2	
	Survival (%)	L colonies (%)	Survival (%)	L colonies (%)
0	120	21	93	13
1	68	65	75	98
2	117	58	92	93
3	106	18	84	54
4	99	6	105	43
In medium SL2				
0·5	.	.	102	16
1	.	.	91	47
1·5	.	.	106	99·8
3	81	96	.	.

bacteria. In the experiments shown, samples of culture were protoplasted at various times during the usual 'competence incubations' in SL1 and SL2 media (see Methods). Table 1 indicates that in SL1 medium the susceptibility to lysozyme increased to a maximum after 1–2 hr of growth and then decreased again after 1–2 hr of further incubation. During growth in SL2 medium, lysozyme sensitivity increased again. After 90 min. of incubation (when the bacilli are highly competent for transformation) lysozyme sensitivity also reached a very high value. The two experiments presented in Table 1 show the same pattern of fluctuation of lysozyme sensitivity, but markedly different absolute values for the % of L colonies formed at different times. Further instances of consistent reproducibility of sensitivity patterns in the face of poor reproducibility of absolute sensitivity values are shown in subsequent Tables. We can offer no certain explanation for this phenomenon.

Effect of incubation in chloramphenicol media upon lysozyme sensitivity

Changes in the lysozyme susceptibility of *Bacillus subtilis* 168 were particularly marked after prolonged incubation in media containing chloramphenicol (CAP). For example, in the experiment shown in Table 2, CAP 10 μg./ml. was added to highly sensitive competent bacilli after the 90 min. incubation in SL2 medium. At the time of CAP addition (zero time) both experimental and control suspensions were attacked equally by lysozyme since, as expected, CAP did not affect the activity of this enzyme. However, as incubation in the presence of CAP proceeded, the bacilli became increasingly resistant to lysozyme. For example, after 3 hr in SL2 medium + CAP, 40 % of the organisms still gave bacillary colonies on DP plates after a 25 min. lysozyme

Table 2. *Bacillus subtilis 168: development of lysozyme resistance during incubation in chloramphenicol*

Organisms were grown for 4 hr in medium SL1 and then for 90 min. in medium SL2. At this time the colony count was $1{\cdot}1 \times 10^8$/ml. The culture was divided and chloramphenicol (10 μg./ml. final concentration) was added to one of the subcultures. Samples from both cultures were taken immediately and at hourly intervals and treated with lysozyme 100 μg./ml. for 25 min. The treated samples and lysozyme-free controls were diluted in DFR and plated on DP and SDM plates. The development of lysozyme resistance during incubation in chloramphenicol medium is reflected in the increasing % bacillary colonies remaining after lysozyme treatment.

	With chloramphenicol				Without chloramphenicol			
Time of incubation (hr)	DP medium		SDM medium		DP medium		SDM medium	
	Survival (%)*	B colonies (%)†	Survival (%)	B colonies (%)	Survival (%)	B colonies (%)	Survival (%)	B colonies (%)
0	80	2	81	0·7	87	1·1	71	0·8
1	100	7	83	1·3	110	1·7	82	0·8
2	99	26	119	3	108	0·8	96	0·2
3	99	40	104	7	92	0·7	76	< 0·1

* % = Ratio of total counts of bacillary + L colonies after lysozyme action to the counts before lysozyme treatment × 100.

† % B = Ratio of number of bacillary colonies total number of colonies × 100.

Table 3. *Bacillus subtilis 168: percentage of bacillary-colony formers among survivors of lysozyme treatment*

Organisms were pretreated and incubated in the presence or absence of chloramphenicol as shown for the 3 hr samples in Table 2. A chloramphenicol-treated and a control culture were then incubated with lysozyme 200 μg./ml. and samples taken at intervals and plated on DP and SDM media. Survival averaged 93 %. Note the striking difference in lysozyme sensitivity of the two cultures. However, after 1 hr of lysozyme treatment the vast majority of the organisms in the resistant cultures had also become committed.

	No treatment with chloramphenicol				Treated with chloramphenicol for 3 hr.			
	Time of lyzosyme treatment (min.)				Time of lyzosyme treatment (min.)			
	25	30	35	60	25	30	35	60
Plating medium	bacillary-colony formers (%)							
DP	< 0·1	< 0·1	< 0·3	< 0·1	80	63	52	4
SDM	< 0·2	< 0·2	< 0·1	< 0·2	44	34	19	2

treatment. By contrast, sensitivity to lysozyme continued to increase in the control populations incubated in parallel in SL2 medium without CAP. It should be stressed that the lysozyme resistance which was manifest in the 3 hr CAP-incubated culture was not absolute: when the lysozyme treatment was continued long enough, virtually all of the bacilli even in this population become committed to the L-state. This is shown in Table 3.

The marked increase in lysozyme resistance due to incubation with CAP suggested that it might be possible to observe by electron microscopy correlated changes in the thickness of the cell walls. Accordingly, thin sections of CAP-incubated and of control bacilli were examined in the electron microscope. The results (Pl. 1, fig. 1, 2, Table 4) clearly indicate that a thickening in the cell wall occurred during the 3 hr incubation in SL2 medium+CAP. On the average, the wall of the CAP-incubated bacilli was 1·9 times as thick as that of the controls (Table 4). The electron micrographs of the CAP-incubated organisms show that the thickness of the cell wall had increased fairly uniformly all around the bacilli.

Table 4. *Bacillus subtilis 168*: *average wall thickness of chloramphenicol-treated and untreated bacilli*

Electronmicrographs × 103,000 of 30 randomly chosen bacilli were used to compile each of the 2 sets of figures. Each number is an average of ten measurements. The deviations shown are standard deviations. The difference between the two sets of measurements is significant ($P < 0{\cdot}001$).

	Chloramphenicol treated	Untreated
	Wall thickness (Å)	
	268	150
	295	160
	275	142
	305	143
	284	145
	295	140
	292	160
	268	155
	289	150
	289	155
Average thickness	268±47	150±29

Effect of tryptophan deprivation on lysozyme resistance

Blockage of protein synthesis by CAP presumably causes accumulation of unused amino acids (Hancock & Park, 1958) and, possibly, diversion of some of these amino acids into cell-wall synthesis. If this view be correct, starvation for tryptophan, which is not a constituent of *Bacillus subtilis* cell walls (Young, Spizizen & Crawford, 1963) should mimic the effect of incubation with CAP. As shown in Table 5, omission of tryptophan from SL2 medium did cause the development of increased resistance to lysozyme. However, tryptophan starvation was notably less effective than incubation with CAP and, in a combination of the two treatments, the CAP effect predominated. Examination of thin sections of tryptophan-starved and control bacilli in the electron microscope has shown that thickening of the cell wall also occurs as a result of tryptophan starvation (L. J. Archer & G. B. Chapman, unpublished).

Table 5. *Bacillus subtilis 168*: *effect of tryptophan deprivation and chloramphenicol on lysozyme sensitivity*

Organisms were grown in SL1 medium for 4 hr and then in SL2 medium for 90 min. After removal of the 0 time sample, the culture was divided into 4 portions (± tryptophan, ± chloramphenicol) as shown and incubated for another 4 hr. All cultures were treated with lysozyme 200 μg./ml. for 25 min. immediately after sampling and then plated on DP and SDM media.

Time of sampling (hr)	Treatment: Trypto-phan	Treatment: Chloram-phenicol	Plating medium	Experiment 1: Survival (%)*	Experiment 1: B colonies (%)†	Experiment 2: Survival (%)	Experiment 2: B colonies (%)
0	−	−	DP	125	1·60	50	0·30
			SDM	73	0·57	33	0·22
4	+	−	DP	23	< 0·56	30	0·44
			SDM	29	< 0·45	21	0·11
4	−	−	DP	92	14	77	5·2
			SDM	86	12	60	< 0·2
4	+	+	DP	91	80	71	32
			SDM	94	43	62	7·1
4	−	+	DP	95	77	74	19
			SDM	96	43	60	5·8

* % = ratio of counts of bacillary + L colonies after lysozyme action to counts before lysozyme treatment × 100.

† % B = ratio of number of bacillary colonies to total number of colonies × 100.

Transient appearance of a spheroplast-like form during protoplasting of Bacillus subtilis 168

A conspicuous feature of the data in Tables 2, 3 and 5 is the discrepancy between the percentage of bacillary colonies observed on DP medium and those counted on SDM medium. For example, Table 2 shows that after 3 hr incubation in SL2 medium + CAP, a 25 min. lysozyme treatment produced a suspension which gave 40% of bacillary colonies on DP medium and 7% of bacillary colonies on SDM medium. Since survival was complete, it must be concluded that 33% of the total population scored as 'committed to the L state' on SDM medium and as 'bacillary' on DP medium. Control experiments with media lacking either casein hydrolysate or D-methionine have shown that D-methionine is responsible for the difference in the plating behaviour of the intermediate form on the two media. The D-amino acid, a reversion inhibitor (Landman & Halle, 1963), apparently prevents repairs in the lysozyme-damaged walls which are required for the continued maintenance of the bacillary state (Landman, 1967). The intermediate form is characteristic of suspensions incompletely changed to protoplasts and is not restricted to populations which have been pretreated by incubation with CAP. In more lysozyme-sensitive cultures, a briefer lysozyme treatment can be used to show the phenomenon (Table 6).

Since the intermediate form is obtained when the removal of cell wall is incomplete, it became of interest to know whether such forms appeared as spheres or as rods in the phase microscope. The experiments recorded in Table 7 were made to answer this question. In the first of the experiments, microscopic counts of spherical, spheroidal and rod forms were made for a population which, according to its subsequent plating

behaviour, contained about 16% intermediate forms and 3% completely committed forms. The microscopic counts gave 18% 'true' spheres and 82% rods+spheroids; clearly, therefore, the intermediate forms appeared to be completely spherical in the phase microscope. In the second experiment the microscopic observations and platings were made after lysozyme action had proceeded much further. However, the results

Table 6. *Bacillus subtilis 168*: *appearance of intermediate form during brief exposure of sensitive organisms to lysozyme*

Organisms were grown in SL1 medium for 4 hr and then in SL2 medium for 90 min. Lysozyme was then added to 250 μg./ml., samples were taken at times shown, then immediately diluted, and plated on DP and SDM media. Intermediate forms are those organisms which form L colonies on SDM medium and bacillary colonies on DP medium (e.g. 29% of the organisms were in the intermediate form after 10 min. of incubation.)

	Duration of lysozyme treatment (min.)				
Plating medium	10	15	20	25	30
	bacillary colony formers (%)				
DP	63	38	24	9	4
SDM	34	17	7	4	2

Table 7. *Bacillus subtilis 168*: *relationship between plating behaviour and morphology in populations forming protoplasts*

The bacilli were subjected to the usual competence procedure, then chloramphenicol was added and incubation continued in SL2 medium for 3 hr more (2·5 hr in Expt. 2). After 0 time platings, lysozyme was added to a final concentration 200 μg./ml.; 20 (or 25) min. later samples were taken for platings. OsO_4 was added to block instantly further changes in morphology and Petroff-Hausser total counts were made immediately.

Columns							
1	2	3	4	5	6	7	8
		Plating data					
					Microscope counts		
Expt. no.	Protoplasting time (min.)	L colonies on DP medium (%)	L colonies on SDM medium (%)	intermediate forms (%)	rods (%)	spheroids (%)	spheres (%)
1*	0	0	0	0	100	0	0
	20	3	19	16	77	4	18
2†	0	0	0	0	100	0	0
	25	91	99	8	1	3	96

* The number of perfect spheres seen in the phase microscope (col. 8) far exceeded the number of completely committed protoplasts (col. 3). Further, the % spheres (col. 8) was about the same as the combined % of committed forms and intermediate forms (col. 3 and 5 or col. 4). Hence it is concluded that the intermediate forms were spherical in shape.

† The % intermediate forms (col. 5) considerably exceeded the combined count of rods and spheroids (cols. 6 and 7). Hence it is concluded that the intermediate forms were spherical in shape.

led to the same conclusion, namely, that the intermediate forms were spherical. In Expt. 2 the number of intermediate forms was considerably greater than the sum of remaining rod and spheroid forms counted in the microscope.

Plate 2, figs. 3–5 show electron micrographs of spherical forms from populations

which had been treated with lysozyme for, respectively, 20 and 30 min. and which contained 29% or 23% intermediate forms. Considerable portions of the surface of the pictured forms are covered with a thinned residual layer of wall. In an earlier experiment, in which a larger number of organisms was photographed, 33 out of a total of 60 spherical forms retained residual cell-wall material. In this latter experiment, 33% of the population behaved as intermediate forms, and 46% as committed forms. In the three electron-microscopic surveys of intermediate populations which were made, the residual wall usually adhered fairly closely to the underlying protoplasts in a manner similar to that shown in Pl. 2, figs. 3–5. However, we also observed separation of cell-wall 'casings' from protoplasts, a phenomenon which had been seen earlier in studies of protoplasting on solid media (Ryter & Landman, 1964). A typical form from an extensively (80 min.) lysozyme-treated population is shown in Pl. 2, fig. 6. Of this population, 90% behaved as committed forms; no cell-wall residues were observed.

DISCUSSION

The observations described in this paper concern several different topics, namely, changes in the cell wall of *Bacillus subtilis* 168 during normal growth, changes in the cell wall produced by inhibition of protein synthesis by chloramphenicol, and the relationship of cell-wall removal to commitment to the L state. These topics will be discussed in turn.

There are reports that, in a given bacterial species, changes in the cell wall may be produced not only by changing the medium of cultivation (Martin, 1966; Whitney & Grula, 1964; Young, 1965), but differences can occur also in the course of the regular growth cycle (Young, 1965). Further, subtle changes in the cell surface which develop under some conditions during growth are apparently responsible for competence in transformation. In competence development in Pneumococcus and Streptococcus, chemically and serologically identifiable materials appear quite abruptly in growing cultures, and again disappear, at least partly as a result of enzymic action (Tomasz & Hotchkiss, 1964; Tomasz & Beiser, 1965; Pakula & Walczac, 1963). With *Bacillus subtilis*, wall-surface modifications also have been reported to accompany changes in competence (Charpak & Dedonder, 1965; Jensen & Haas, 1963; Young, 1965; Young, 1966); again these variations occur well within the compass of a few divisions or, indeed, in the absence of division (Archer & Landman, 1967). The changes in lysozyme sensitivity which we have observed sometimes run parallel with changes in competence. In lysozyme sensitivity, as in competence, the changes are quite rapid and substantial variations may occur without division or in 1 or 2 divisions. It is likely that the wall-associated autolytic enzyme acetylmuramyl-L-alanine amidase (Young, 1965), which has been tentatively implicated in competence development, plays a role in effecting these variations. Further, enzymes of this type may also enhance or modify the protoplast-formation action of lysozyme in the various samples (Shockman, 1967). In any case, if the varying rates of commitment of organisms growing in SL1 and SL2 media are indeed a reflexion of changes in the structural and enzymic constitution of the cell wall, it must be concluded that the walls of growing *B. subtilis* are in a state of almost continual change.

When the metabolism of *Bacillus subtilis* is altered by inhibition of protein synthesis, cell wall changes become even more pronounced. In the terminology of Shockman

(1965), inhibition of protein synthesis produces a state of unbalanced growth which permits continued increase in cell-wall substance while blocking an increase in protein. The increase in cell-wall substance relative to cytoplasmic substance is clearly indicated by the increase in wall thickness observed in the electron microscope. Analogous increases in wall thickness in *Streptococcus faecalis* during both CAP treatment and starvation for threonine or valine were described by Shockman (1965). Tetracycline, another protein synthesis inhibitor, has been shown to produce thickening of cell walls in *Staphylococcus aureus* (Hash & Davies, 1962). (Incubation in tetracycline media also causes development of lysozyme-resistance in *B. subtilis*, I. L. Miller, unpublished.)

We know little about the comparative chemical make-up of normal cell walls and the abnormal walls produced under conditions of inhibition of protein synthesis. However, it seems to us unlikely that the great differences in lysozyme sensitivity which we have observed could be due exclusively to even a 2- to 3-fold increase in thickness of cell wall; it seems much more probable that changes in cell-wall composition are also involved. For one thing, the formation of wall-associated autolytic enzymes (Shockman, 1965; Young, 1966) is almost certainly blocked by chloramphenicol (CAP). If autolytic enzymes play a complementary role with lysozyme depolymerization of normal wall, the slowed lysis of presumably autolysin-depleted CAP-treated organisms may be more easily understood. The assumed lowering in the concentration of autolytic enzymes may also be invoked to explain the unexpected observation that wall thickening in CAP-incubated bacilli was almost uniform all-round the periphery of the organism. A localized thickening might have been anticipated on the basis of the observations of Chung, Hawirko & Isaac (1964) and of Cole (1965) that cell-wall growth in Gram-positive bacteria occurs in localized regions. The partial absence of localized autolysins required for the loosening of expanding cell walls (Shockman, 1965; Young, 1966) might explain an abnormal uniformity in the deposition of wall during CAP treatment.

In addition to modifying the enzyme content of cell walls, the inhibition of protein synthesis alters the pool concentration (Hancock, 1960) and metabolic flow of amino acids. As a result, the peptide content of the wall, the relationship of peptide content to teichoic acid content, and the cross-linking between wall constituents may well be changed. In *Streptococcus faecalis*, a quantitative difference between the amino acid content of the walls of threonine-deprived and of normal cocci has been described (Toennies, Bakay & Shockman, 1955). However, in *Staphylococcus aureus*, growth in presence of CAP did not appear to modify the composition of the cell wall, although wall synthesis continued throughout the CAP treatment period (Hancock & Park, 1958).

In their discussion of the process of protoplast formation, Landman & Halle (1963) distinguished an intermediate between bacillus form and protoplast, namely a rod-shaped osmotically sensitive form which did not survive in hypotonic media but gave bacillary colonies on hypertonic plating medium. In the present paper a succeeding intermediate form is recognized: a sphere-shaped form which gave a bacillary colony on DP medium and produced an L colony on SDM medium. In the upper portion of Table 8 the characteristics of the various forms are summarized. The lower portion of Table 8 shows the appearance and disappearance of the different forms as observed during a typical experiment on protoplast formation. The gradual progression from the bacillary state through the first and then the second intermediate form to the protoplast

state is clearly evident from the shifting percentage composition of the population undergoing lysozyme treatment (Table 8, lower right).

From the point of view of commitment, the second intermediate forms are very interesting since they are evidently able to re-initiate cell-wall formation on DP medium, but not on SDM medium. The primer mechanism which is responsible for this wall re-initiation (Landman, 1967; Landman & Halle, 1963) is apparently present vestigially in these forms. We suggest that the residues of cell wall which are visible in

Table 8. *Bacillus subtilis 168*

Stages of protoplast formation: schematic

Stage	Osmotic sensitivity	Morphology in phase microscope	Colony form on DP medium	Colony form on SDM medium
Bacillus	Insensitive	Rod	Bacillary	Bacillary
1st intermediate form	Sensitive	Rod	Bacillary	Bacillary
2nd intermediate form	Sensitive	Sphere	Bacillary	L-form
Protoplast: committed form	Sensitive	Sphere	L-form	L-form

Stages of protoplast formation: experimental

		Colony counts ($\times 10^{-6}$/ml.)					Among survivors: approximate			
			On DP med.		On SDM med.					
Predominant stage	Time of lysozyme treatment*	On low salt†	B‡	L§	B	L	B (%)	1st form (%)	2nd form (%)	P (%)
Bacillus	0	95	101	—	89	—	100	—	—	—
1st intermediate form	20	4·2	79	7·5	53	32	5	57	29	9
1st and 2nd intermediate forms	25	1·2	67	16	33	42	2	40	38	20
2nd form and protoplast	30	0·8	48	32	15	43	1	20	33	46
Protoplast	35	1·5	37	38	8	50	2	10	31	57
Protoplast	40	0·5	25	39	5	50	< 1	8	27	65
Protoplast	45	0·3	12	42	3	52	< 2	4	17	76

* Organisms incubated with chloramphenicol (15 μg./ml.) medium for 3 hr before protoplast formation with lysozyme concentration 200 μg./ml.

† Hypotonic medium was DP medium without succinate. Colony counts shown are actual counts $\times 10^{-6}$.

‡ Bacillary colonies.

§ L-form colonies.

Pl. 2, figs. 3–5 constitute the vestigial primer. How this vestigial primer accomplishes its priming functions is not known; it might do so directly by serving as a nucleus of attachment for further wall synthesis or wall fragments might indirectly serve as primers by facilitating other priming events in adjacent areas of the cell membrane. In any case, re-initiation of cell-wall formation in these incompletely formed protoplasts, unlike the reversion of protoplasts, takes place promptly and efficiently in ordinary media. The difficult initial event required for the reversion of protoplasts and stable L-forms is avoided. However, it is possible that in later phases of protoplast reversion the reverting forms pass through a stage resembling the second intermediate form (see Landman, 1967; Ryter & Landman, 1967).

The second intermediate form presents an interesting parallel with the spheroplasts

of Gram-negative bacteria. Both forms are osmotically sensitive spheres, both retain important quantities of cell-wall material and both revert promptly to the bacillary form as soon as the removal of cell wall is interrupted (i.e. both retain priming activity). In both forms, further removal of wall leads to loss of primer and thereby to an heritably persistent L-state (Landman & Ginoza, 1961; Landman & Halle, 1963). The second intermediate form of *Bacillus subtilis* 168 differs from spheroplasts of Gram-negative bacteria mainly by its lack of persistence under present experimental conditions.

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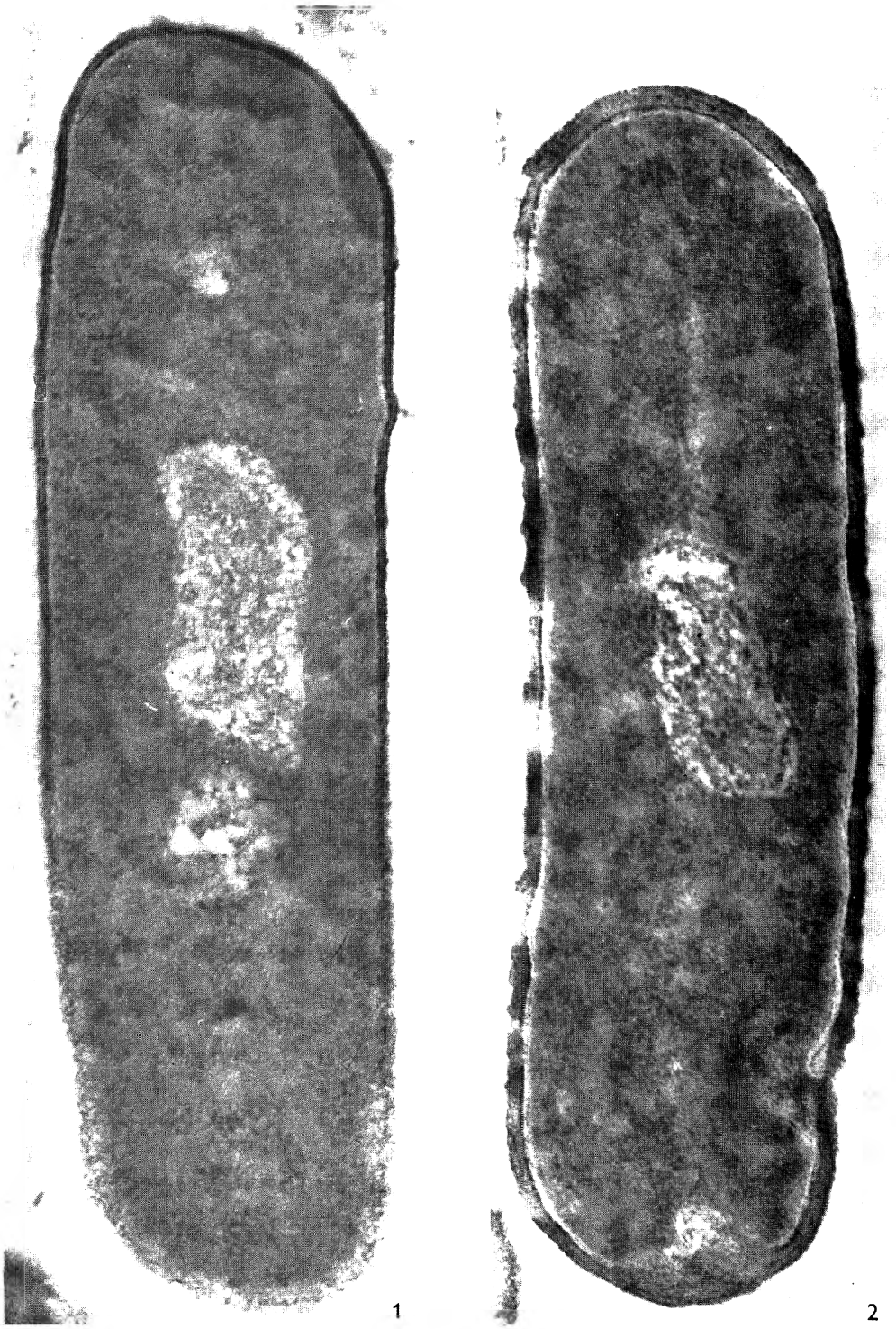

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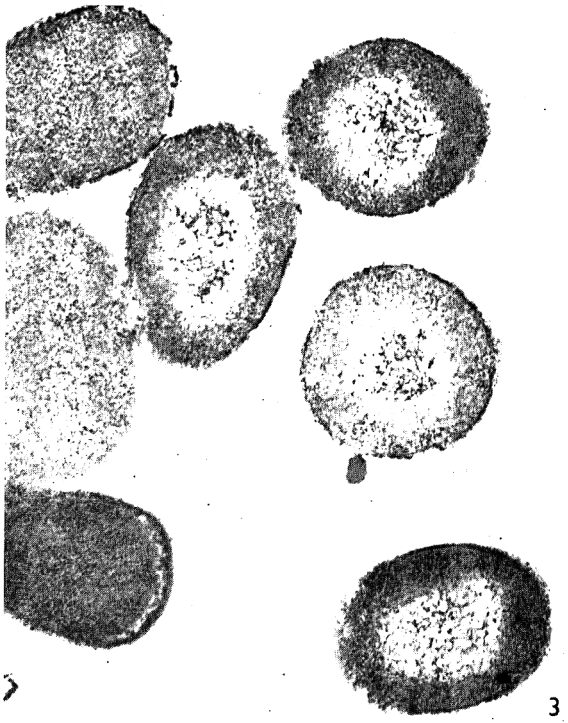

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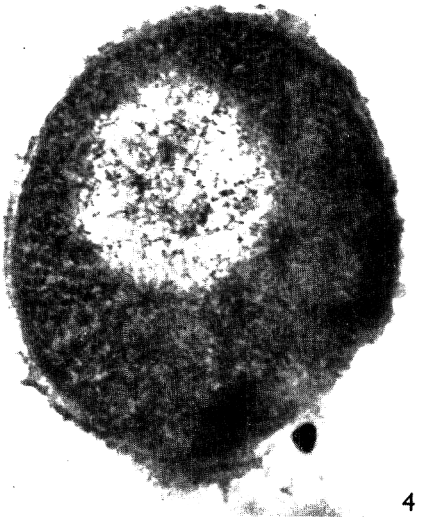

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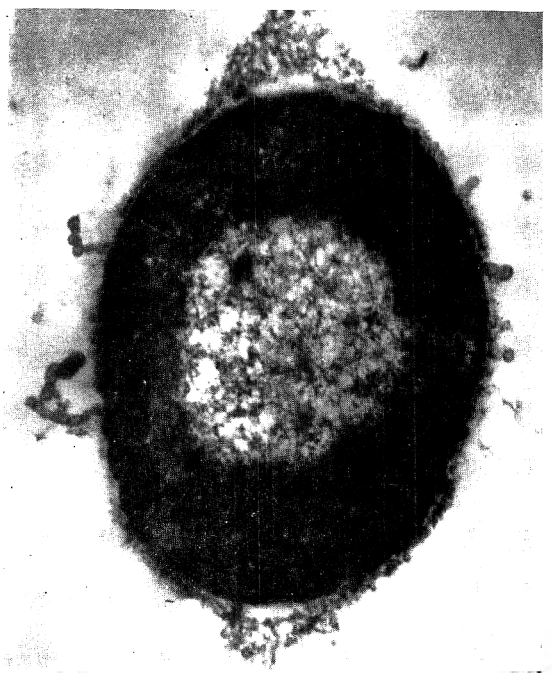

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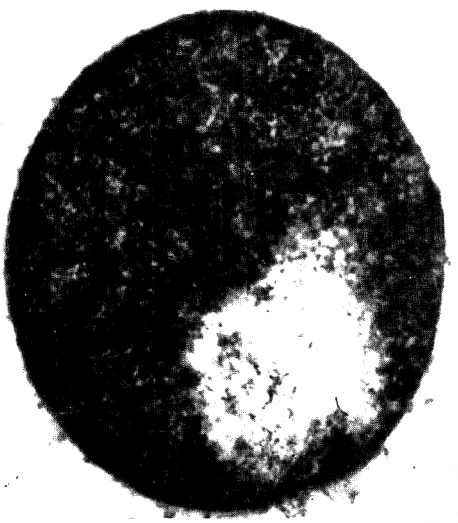

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Section of normal *Bacillus subtilis* 168 fixed after competence incubation followed by 3 hr additional incubation in SL2 medium. × 93,000.

Fig. 2. Section of chloramphenicol (CAP)-treated *Bacillus subtilis* 168 fixed after competence incubation followed by a 3 hr treatment in SL2 medium + CAP 15 μg./ml. × 93,000.

PLATE 2

Figs. 3, 4, 5. Sections of organisms from CAP-preincubated bacteria.

Fig. 3. Organisms from a population which had been treated with lysozyme 200 μg./ml. for 20 min. and which contained 29% intermediate forms (see Table 8). × 19,000.

Figs. 4 and 5. Spherical forms from a population which had been treated with lysozyme 200 μg./ml. for 30 min. and which contained 23% intermediate forms. Thin remnants of cell wall cover a large proportion of the surface of the spheres. The strings of small bodies clinging to the cell surface (fig. 5) are probably residues of expelled mesosomes. × 46,500. (See Ryter & Landman, 1967; Fitz-James, 1967.)

Fig. 6. Typical organism from the same suspension as figs. 4 and 5, taken after 80 min. of lysozyme treatment. No wall residues are visible in this protoplast. At the time of fixation, 5% of the population still behaved as intermediate forms. × 51,000.

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