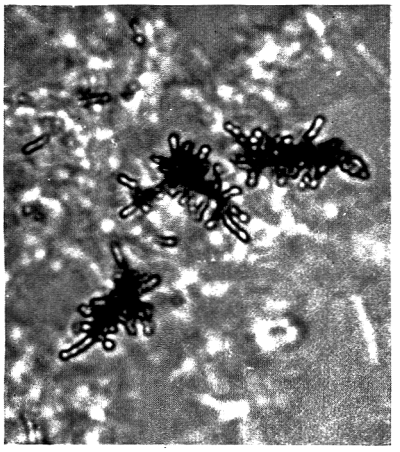

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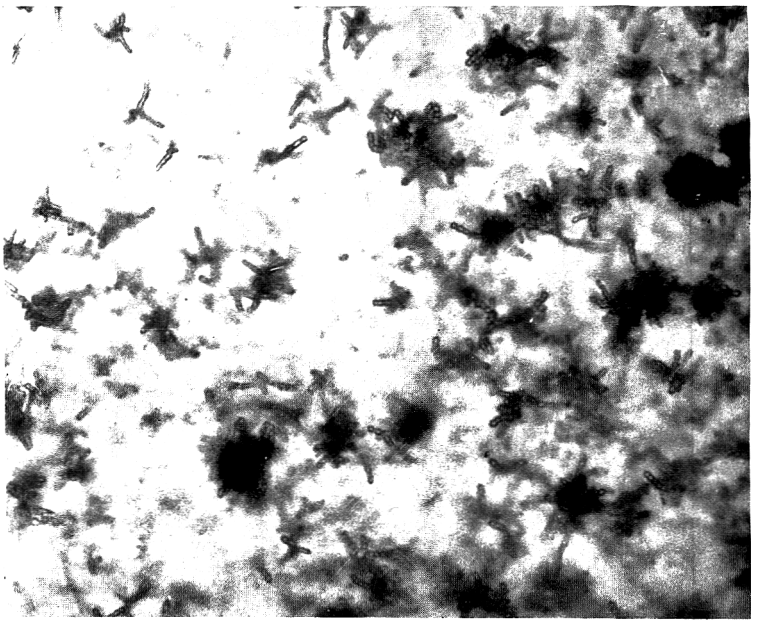

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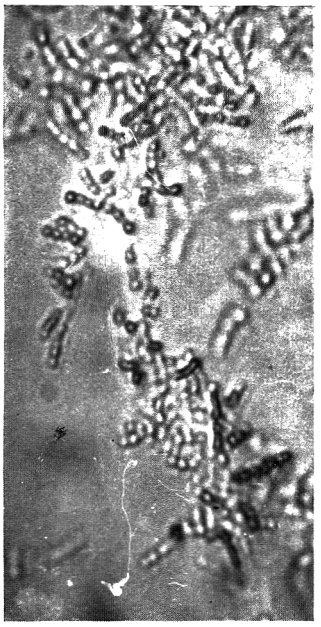

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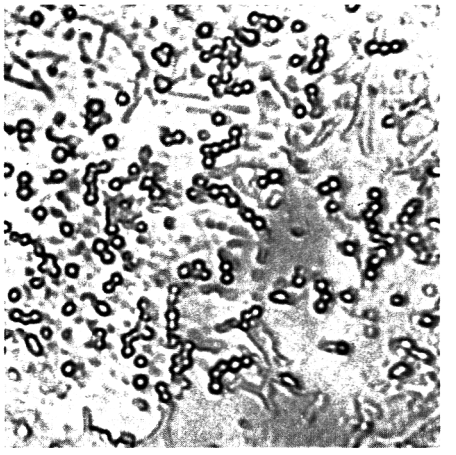

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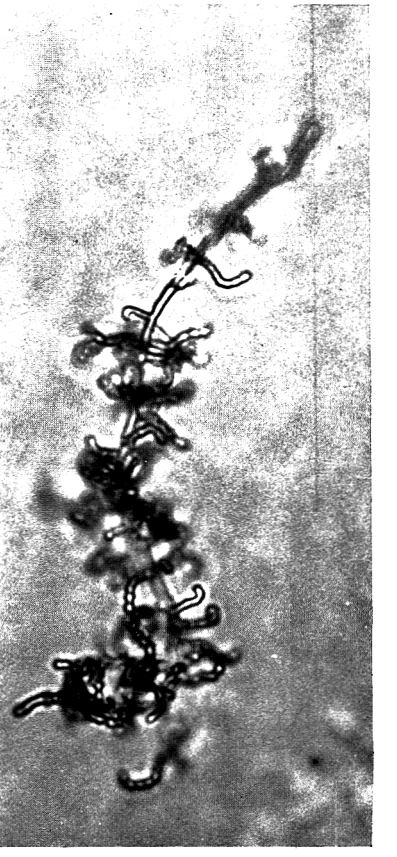

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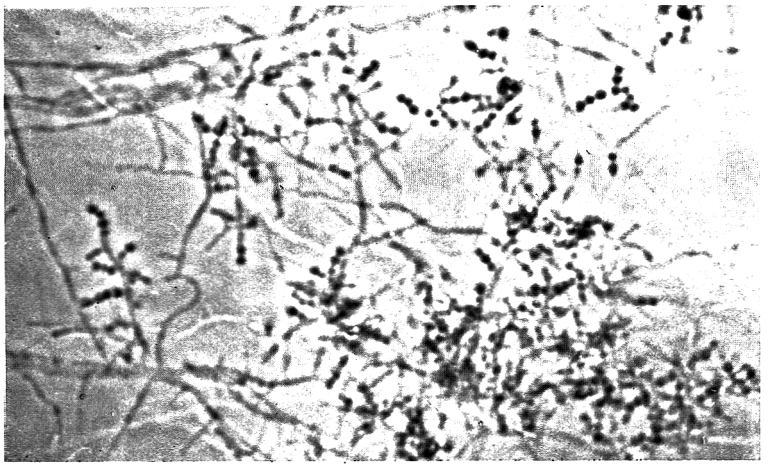

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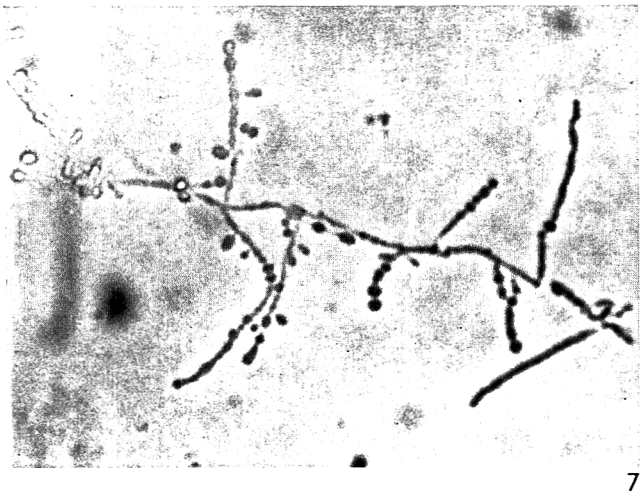

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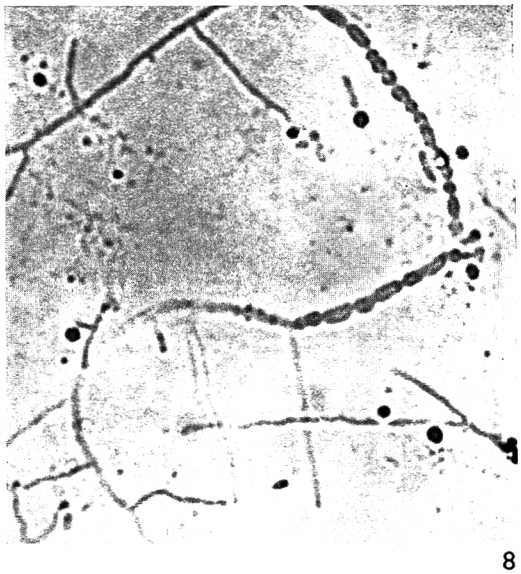

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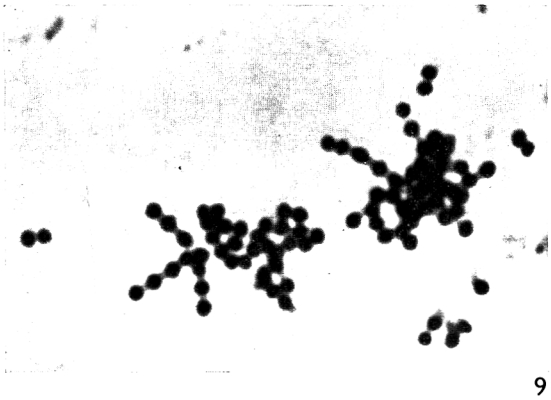

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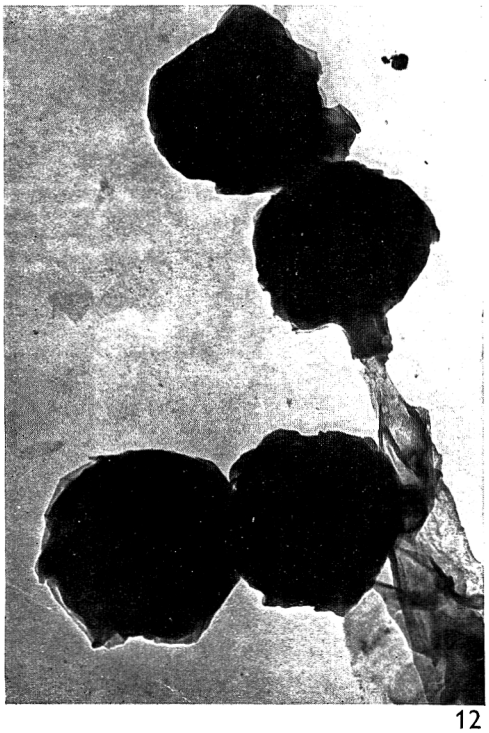

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Aerial mycelium on potato dextrose agar *Micropolyspora faeni* CUB 58 (× 500).

Fig. 2. Aerial mycelium on yeast extract agar *M. faeni* CUB 58 (× 800).

Fig. 3. Substrate mycelium beneath surface of agar. Nutrient agar. *M. faeni* CUB 58 (× 850).

Fig. 4. Substrate mycelium beneath surface of agar. Yeast extract agar. Phase contrast *M. faeni* CUB 58 (× 800).

Fig. 5. Spore chains on surface of colony growing in slide culture. V-8 agar. *M. faeni* A 94 (× 980).

Fig. 6. Aerial mycelium of *Nocardia madurae* A 443. Potato carrot agar (× 800).

PLATE 2

Fig. 7. Intercalary spore formation in slide culture. Nutrient agar. *M. faeni* A 94 (× 800).

Fig. 8. Intercalary spore formation in slide culture. Nutrient agar *M. faeni* A 94 (× 1600).

Fig. 9. Slide culture, dried and Gram stained. *M. faeni* A 94 (× 2000).

Fig. 10. Slide culture, dried and Gram stained *M. faeni* CUB 58 (× 2000).

Fig. 11. Electron micrograph of aerial mycelium spores *M. faeni* A 445 (× 7200).

Fig. 12. Electron micrograph of aerial mycelium spores. *M. faeni* A 94 (× 28,800).

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With 1 *plate*
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A Symptomless Carrier of Disease in *Helminthosporium victoriae*

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(*Accepted for publication* 14 *August* 1967)

SUMMARY

A mutant (DM-1 A) obtained from colonies of a diseased isolate (D-1 A) of *Helminthosporium victoriae* carried the causal agent of disease but showed almost no symptoms. Typical disease symptoms were produced in healthy *H. victoriae* after hyphal contact with the diseased mutant (DM-1 A). Hence, the character responsible for suppression of severe disease symptoms in the mutant did not appear to be transmitted with the agent of disease. Healthy mutant cultures that did not carry the causal agent of disease were obtained by isolation of hyphal tips from the diseased mutant. Such cultures inoculated with a second diseased isolate (D-1 B) remained nearly symptomless but were capable of transmitting the disease to healthy cultures. The diseased mutant (DM-1 A) inoculated with diseased isolate D-1 B usually transmitted only D-1 A to healthy cultures and the diseased mutant (DM-1 B) inoculated with D-1 A usually transmitted only D-1 B to healthy cultures. This indicates that the two agents tend to be mutually exclusive.

INTRODUCTION

In the course of cultivation of diseased isolate D-1 A of *Helminthosporium victoriae* M. & M. a mutant was observed and isolated. The mutant had a rate of growth approximately equal to that of healthy *H. victoriae* but had cultural characteristics markedly different from those of the healthy. Preliminary investigations have indicated that the mutant contained the causal agent of disease even though it showed no severe symptoms of disease (Lindberg, 1960). This investigation was undertaken to determine whether the absence of severe symptoms in the diseased mutant was due to a change in the causal agent of disease or whether the mutant behaved as a symptomless carrier of the causal agent. It was considered possible also that investigations utilizing the mutant might elucidate other characteristics of the disease in *H. victoriae*.

METHODS

A description of the two diseased isolates of *Helminthosporium victoriae* used in this investigation has been published (Lindberg, 1966). The diseased isolates were distinguished on the basis of symptoms and were designated D-1 A and D-1 B. Strain SN-1 of *H. victoriae* was the healthy fungus used throughout this investigation. The mutant isolated from diseased colonies of D-1 A contained the causal agent of that disease and was designated DM-1 A. Mutant cultures freed of the agent of disease by hyphal tip isolation were called healthy mutants. The mutant cultures that contained the agent of disease D-1 B were designated DM-1 B.

Two methods were used in attempts to transfer the disease agent to healthy fungus. The first, called 'hyphal contact inoculation', consisted of placing mycelial seedpieces of the diseased fungus and the fungus to be inoculated approximately 1 cm. apart in culture plates. In the second, called 'dip inoculation', cubes of mycelial mat plus agar of healthy *H. victoriae* were dipped into suspensions that contained viable fragments of the diseased fungus. Type symptoms of diseased isolates D-1 A and D-1 B were best expressed by the dip inoculation method. This method was often used to confirm the disease type after transmission by the hyphal contact method of inoculation.

RESULTS

The mutant (DM-1 A) isolated from colonies of diseased isolate D-1 A of *Helminthosporium victoriae* had a rate of growth approximately equal to that of the healthy fungus. The mutant, unlike the healthy fungus, however, sporulated very sparsely and had other characteristics of growth markedly different from those of the healthy fungus. Colonies of the diseased mutant (DM-1 A) were dark grey in colour and the only apparent symptom of disease was the premature collapse of aerial mycelium (lower colony Pl. 1, fig. 1 B). The mutant was observed in diseased colonies of D-1 A each year during the spring, but was never observed until the room temperature reached 28° or above. The mutant has never been observed during the winter at room temperatures of 23–26° or during the summer when cultures of diseased isolate D-1 A were incubated at artificial temperatures of 23–26°. The mutant has never been observed in cultures of diseased isolate D-1 B or in any cultures of healthy *H. victoriae*. The mutant has been maintained on culture plates or tubes of potato dextrose agar (PDA) for several years without having shown any signs of reverting to the diseased type growth. Mutant cultures were maintained continuously at room temperature (23–31°).

Test for the agent of disease in the mutant

Since the mutant was obtained from diseased fungus it was of interest to determine whether or not it carried the causal agent of disease. Pieces of mycelial mat of the mutant (DM-1 A) and of healthy *Helminthosporium victoriae* (SN-1) were placed approximately 1 cm. part in culture plates of PDA. Hyphae of the mutant and SN-1 touched after approximately 24–36 hr of growth. In 10 culture plates in each of the four experiments, symptoms identical to those of diseased isolate D-1 A developed in all colonies of SN-1 (upper colony, Pl. 1, fig. 1 B).

Even though the causal agent of disease was present in the mutant (DM-1 A), severe diseased symptoms typical of diseased isolate D-1 A were not expressed. It became of interest to determine whether the factor responsible for suppression of severe disease symptoms in DM-1 A could be transmitted to SN-1 along with the causal agent of disease. Following hyphal contact between DM-1 A and SN-1, portions of the colony margins of the latter which developed symptoms of disease were removed, ground in a mortar and diluted with sterile water. Small volumes of the suspension were placed on PDA plates with a wire loop. About 20–25 loops of the suspension, spaced approximately 1–2 cm. apart, were placed in each culture plate (Pl. 1, fig. 2). There were over 1500 colonies in 5 experiments and all developed symptoms identical to those of diseased isolate D-1 A. There was no suppression of severe diseased symptoms in any of the colonies nor was the mutant type growth ever detected.

Hyphal tip isolation of the normal mutant

The most severe symptoms in colonies of D-1 A occurred in the young cells at the colony margin (Lindberg, 1959). Such cells lysed and the colonies showed little further radial growth (Psarros & Lindberg, 1962). Hyphal tips isolated from D-1 A usually failed to grow but occasionally produced small diseased colonies.

The mycelial collapse characteristic of DM-1 A occurred first in the oldest portion or centre of the colony and gradually spread outwárd. The young hyphal cells at the colony margin appeared unaffected and continued to grow. Therefore, an attempt was made to obtain the mutant free of the causal agent of disease by isolation of hyphal tips. Twenty hyphal tips were isolated from 36- to 48-hr-old colonies of DM-1 A in each of four experiments. Fifty-five of the colonies showed no collapsed aerial mycelium and were tentatively classified as healthy mutants. Twenty-five of the colonies showed collapsed aerial mycelium and were classed as diseased (DM-1 A). Twenty-six of the colonies classified as healthy mutants were tested for the agent of disease by hyphal contact inoculation of SN-1. Symptoms of disease failed to develop in any of the inoculated SN-1 colonies and indicated that the mutant colonies classified as healthy did not contain the agent of disease. Eleven of the colonies classified as DM-1 A were tested against SN-1 and symptoms identical to those of D-1 A developed in all of the inoculated SN-1 colonies.

Inoculation of the healthy mutant with D-1B

A mutant similar to the one isolated from D-1 A has never been observed in colonies of D-1 B. Since healthy mutant cultures were obtained by isolation of hyphal tips of DM-1 A, it was of interest to determine what reaction the healthy mutant might have to inoculation with D-1 B. It was considered possible that the agent of diseased isolate D-1 B might produce more severe symptoms in the mutant than those produced by the agent of diseased isolate D-1 A. Ten culture plates of PDA were seeded with the healthy mutant and D-1 B in each of 4 experiments. Four or 5 days after hyphal contact with D-1 B the once-healthy mutant was subcultured. The colonies that developed had a light grey colour and the aerial hyphae collapsed only slightly (lower colony, Pl. 1, fig. 1 A). Such colonies were then tested for diseased type by hyphal contact inoculation of healthy *Helminthosporium victoriae* (SN-1). In 10 PDA plates in each of 4 experiments, diseased symptoms identical to those of diseased isolate D-1 B developed in the SN-1 test colonies of all 40 plates (upper colony, Pl. 1, fig. 1 A). Diseased mutant colonies that contained the agent of disease D-1 B were designated DM-1 B. It should also be stated that healthy mutant cultures inoculated with D-1 A were readily infected and transmitted only D-1 A to healthy *H. victoriae*.

Protection studies

It then became of interest to determine whether or not a mixture of the agents of the diseases, D-1 A and D-1 B, could be established in the mutant. If the agents of both diseases were present the mutant might show a synergistic reaction. The presence of one agent of disease in the mutant might, on the other hand, protect against infection by the other agent of disease.

To test these possibilities DM-1 A was confronted with inoculum of D-1 B and DM-1 B was confronted with inoculum of D-1 A. Ten of each of the above pairings were made in culture plates of PDA in each of 5 experiments. DM-1 A and DM-1 B challenged with

D-1 B or D-1 A, respectively, showed no change in symptoms. Nevertheless, pieces of the mycelial mats of the challenged DM-1 A and DM-1 B were subcultured on PDA plates. All subcultures of the DM-1 A appeared identical to the type colonies of DM-1 A and all subcultures of the DM-1 B appeared identical to the type colonies of DM-1 B.

The subcultures were then tested for disease type by hyphal contact inoculation of SN-1. Of 50 test colonies of SN-1 confronted with mycelium of the subcultures of challenged DM-1 A, 44 developed symptoms of D-1 A and 6 developed symptoms of D-1 B. Of 50 colonies of SN-1 confronted with mycelium of the subcultures of challenged DM-1 B, 45 developed symptoms of D-1 B and 5 colonies developed symptoms of D-1 A (Table 1).

Table 1. *DM-1A and DM-1B were inoculated by hyphal contact with D-1A and D-1B, respectively, and subcultured: the subcultures were tested for disease type against healthy Helminthosporium victoriae (SN-1)*

		Diseased type transmitted to SN-1			
		DM-1 A/D-1 B		DM-1 B/D-1 A	
Experiment	Date (1966)	D-1 A	D-1 B	D-1 A	D-1 B
1	6 Jan.	10/10	0/10	0/10	10/10
2	22 Jan.	10/10	0/10	0/10	10/10
3	15 Mar.	10/10	0/10	5/10	5/10
4	25 Mar.	10/10	0/10	0/10	10/10
5	9 May	4/10	6/10	0/10	10/10

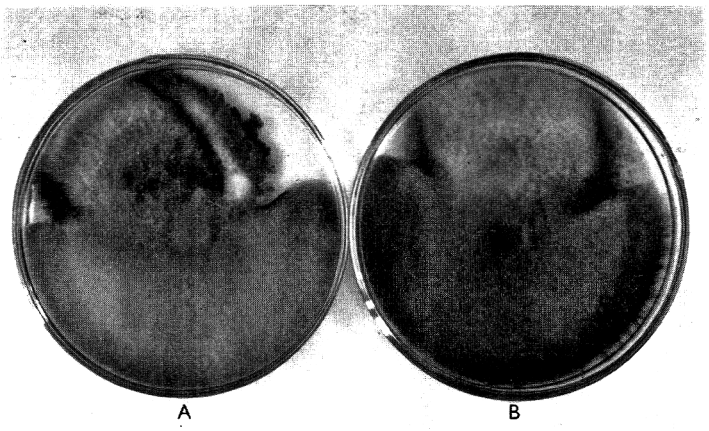
DISCUSSION

Except for the premature collapse of aerial mycelium DM-1 A and DM-1 B could be considered symptomless carriers.

The symptoms of disease that developed in healthy *Helminthosporium victoriae* (SN-1) inoculated by hyphal contact with DM-1 A were identical to those of diseased isolate D-1 A. This indicated that the absence of severe symptoms in DM-1 A was not due to a change in the agent of disease and that the character responsible for suppression of severe disease symptoms resided with the mutant.

Jinks (1964) has shown that when a normal homokaryon and a vegetative death variant of *Aspergillus glaucus* are grown side by side, heterokaryon sectors formed between them, but beyond these sectors ever widening sectors with the vegetative death phenotype replaced the normal homokaryon. Since the vegetative death phenotype penetrated well beyond the maximum point of penetration of the known chromosomally borne determinants, Jinks concluded that an unambiguous criterion of extrachromosomal heredity had been satisfied. The phenomenon was referred to as 'infective heredity'. Assays of diseased mycelium that developed in *Helminthosporium victoriae* inoculated by hyphal contact with the diseased mutant (DM-1 A) indicated that the factor responsible for suppression of severe diseased symptoms was not transmitted with the causal agent of disease. If the factor responsible for the mutant type of growth is controlled in the nucleus it seems likely that the agent of disease might be extranuclear or cytoplasmic.

DM-1 A inoculated by hyphal contact with D-1 B was subcultured and tested for disease type against SN-1. The SN-1 test colonies usually developed symptoms identical



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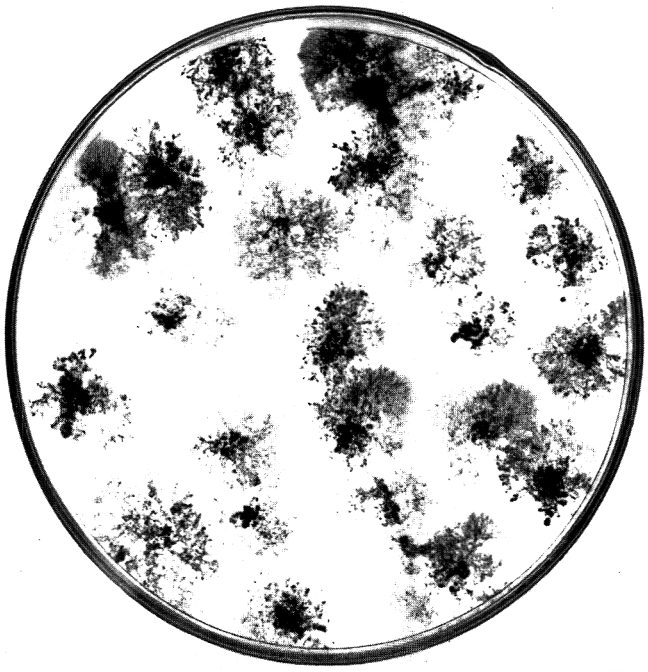

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to those of diseased isolate D-1 A. DM-1 B inoculated by hyphal contact with D-1 A was subcultured and tested for disease type against SN-1. The SN-1 test colonies usually developed symptoms identical to those of diseased isolate D-1 B. These results indicated that the presence of the agent of disease D-1 A may have protected the mutant against infection by the agent of disease D-1 B and vice versa. The protection, if indeed that was the case, was not complete.

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EXPLANATION OF PLATE

Fig. 1. A. Diseased mutant (DM-1B, lower colony) originally plated with healthy *Helminthosporium victoriae* (SN-1). Diseased symptoms identical to those of diseased isolate D-1B developed in the latter (upper colony). B. Diseased mutant (DM-1 A, lower colony) originally plated with healthy *H. victoriae*. Diseased symptoms of D-1 A developed in the latter (upper colony).

Fig. 2. Colonies are from *Helminthosporium victoriae* that developed symptoms of disease following hyphal contact with the diseased mutant (DM-1 A). The colonies show symptoms of disease D-1 A but the factor responsible for suppression of disease symptoms in the mutant was not detected.

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Survival of *Streptococcus lactis* in Starvation Conditions

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SUMMARY

The survival characteristics of washed *Streptococcus lactis* ML3 organisms, suspended in phosphate buffer, have been examined at the growth temperature. Mg^{2+} markedly prolonged survival. High bacterial concentrations extended survival times, probably because Mg^{2+} was excreted by the bacteria. Surviving organisms in some conditions showed prolonged division lags, especially in the absence of Mg^{2+}. Salt concentration had little effect on survival within wide limits; the optimum pH value for survival was near 7·0. Survival times increased considerably at lower temperatures. Agitation and aeration tended to decrease survival. Death was not influenced by the phase of growth at which cells were harvested from a lactose-limiting medium. Casamino acids increased survival markedly in the presence of Mg^{2+}; arginine was almost as effective as the complete mixture of amino acids while other individual amino acids tested gave only slight increases in survival times. Fermentable carbohydrates accelerated death of starving organisms irrespective of the growth phase from which they were harvested and of the limiting nutrient; the accelerated death was reduced by addition of Mg^{2+}.

INTRODUCTION

Apart from the extensive findings of Postgate, Strange, and collaborators on the survival characteristics of *Aerobacter aerogenes* (Postgate & Hunter, 1962, 1963*a*, 1964; Strange, Dark & Ness, 1961; Strange & Dark, 1965; Strange & Hunter, 1966), the literature contains few precise measurements of the survival of bacteria held at growth temperatures in buffered suspensions. Postgate & Hunter (1962, 1963*a*) stressed the pitfalls in interpreting bacterial survival measurements and the ambiguities which may arise from toxic elements in suspending solutions, cryptic growth and growth on impurities.

Postgate & Hunter (1962, 1963*a*, *b*, 1964) observed increased survival times in the presence of Mg^{2+} and Ca^{2+} and the phenomenon of 'substrate-accelerated death' which occurred when starved *Aerobacter aerogenes* were exposed to the growth-limiting substrate. Exceptions were the response of sulphate- and magnesium-limited cells to the addition of sulphate and magnesium ions respectively. However, Strange & Dark (1965) repeated these experiments and concluded that this phenomenon was less general than was claimed by Postgate & Hunter (1964) and was in fact restricted to the lethal effect of carbon energy sources metabolized by *A. aerogenes* in the absence of added Mg^{2+}. Strange & Hunter (1966) later withdrew this conclusion, having succeeded in observing nitrogen-accelerated death. Death of *A. aerogenes* in starved sus-

pensions did not involve alteration or degradation of the osmotic barrier (Postgate & Hunter, 1962). Harrison (1960) first observed the extension of survival times at high cell concentrations and claimed there was an optimum density for survival above which the death rate increased. Postgate & Hunter (1962, 1963*a*) confirmed these findings in general but did not observe the reversed death rate at high bacterial concentrations. Extended survival and maintenance of respiratory activity of *Escherichia coli* resulting from the metabolism of a carbon energy source in the presence of Mg^{2+} was observed by Clifton (1966). McGrew & Mallette (1962, 1965) maintained *E. coli* at constant viability with a very low threshold concentration of glucose, but Clifton (1966) pointed out that their experimental conditions did not preclude regrowth.

The industrial importance of lactic streptococci has led to several studies on their survival, mainly in milk cultures at low temperatures (Gibson, Landerkin & Morse, 1965, 1966; Lamprech & Foster, 1963; Cowell, Koburger & Weese, 1966). No detailed measurements have been reported on the survival of lactic streptococci, or closely related bacteria, at growth temperatures under starvation conditions. The present paper describes a study of factors which affect the survival of *Streptococcus lactis* under starvation conditions.

METHODS

Organism. Streptococcus lactis ML 3 was obtained from the cheese starter collection of the New Zealand Dairy Research Institute. The master culture was maintained in skim-milk media as described by Whitehead, Briggs, Garvie & Newland (1956) for *Streptococcus cremoris* HP (D.R.I.).

Medium. The culture medium, developed for this work from known nutritional requirements of *Streptococcus lactis*, contained (g./l.): lactose monohydrate, 5·0; sodium acetate, 1·0; glycerol, 1·0; Casamino acids (Difco), 5·0; peptone (Difco), 0·5; L-asparagine, 0·1; DL-tryptophan, 0·05; L-arginine, 0·1; adenine, 0·005; guanine, 0·001; uracil, 0·005; xanthine, 0·005; pyridoxine hydrochloride, 0·001; pyridoxal hydrochloride, 0·0002; nicotinic acid, 0·001; calcium pantothenate, 0·001; biotin, 0·0001; riboflavin, 0·0001; thiamine hydrochloride, 0·0001; folic acid, 0·0001; *p*-aminobenzoic acid, 0·0002; $NaHCO_3$, 4·2; Na_2HPO_4, 4·25; KH_2PO_4, 2·7; $MgSO_4.7H_2O$, 0·1; $FeSO_4.7H_2O$, 0·001; $MnSO_4.4H_2O$, 0·001; disodium ethylenediaminetetra-acetate (EDTA), 0·004. The lactose, $NaHCO_3$, and vitamin components were sterilized separately using a Seitz filter, which had been prewashed with 200 ml. de-ionized water, and added aseptically to the other components which had been autoclaved at 115° for 20 min. The medium had a final pH of 7·3. This medium is not completely defined because peptone is a component; in subsequent descriptions it will be referred to as the routine medium.

Growth conditions and resuspension procedures. All growth cultures and washed suspensions were incubated at 30° in static conditions unless otherwise stated. The routine medium was inoculated from the skim-milk culture and, after 20 daily transfers of 1 % inocula in this medium, a number of small tubes of routine medium were inoculated. These cultures were frozen rapidly (solid CO_2 + ether) towards the end of the growth phase and stored at −75° to −80°. When required, cultures were removed, thawed in a 30° water bath (thawed cultures had 50–80 % viable organisms after 5 months storage), and used to inoculate 10 ml. fresh routine medium. Organisms were prepared for survival studies as follows. After 10–20 daily transfers (70–140

generations), growth phase organisms were inoculated into 25 ml. medium and growth was followed turbidimetrically. At the end of the growth phase a sample (usually 6 ml.) was removed and the organisms were centrifuged down (30,000*g* for 1 min.). The packed organisms were rinsed thoroughly and then dispersed in sterile phosphate buffer (see below), centrifuged (30,000*g* for 1 min.), rinsed and finally suspended in 5 ml. sterile phosphate buffer (pH 7·0). All washings and resuspension buffers were at 30°. Manipulations were done aseptically and were completed in about 15 min., thus minimizing changes in temperature and chemical environment. These procedures left insignificant traces of growth medium (equivalent to a 10^{-7} dilution) and repeated centrifugation had no measurable effect on the survival curves of the organisms.

The phosphate buffer used was 0·075M-$Na_2HPO_4 + KH_2PO_4$, (pH 7·0) containing 10^{-5}M-EDTA, unless otherwise stated. No measurable change in pH value occurred with suspensions of equiv. 10 mg. dry wt organisms/ml. Small volumes (0·2 ml.) of the washed suspension were used to inoculate 10 ml. phosphate buffer (+substrates, etc., where specified) in metal-capped test-tubes which were incubated in a water bath at 30°. Suspensions prepared in this manner contained equiv. 20 ± 2 μg. dry wt organisms/ml. (equivalent to about 6×10^7 chains/ml. or about $1{\cdot}6 \times 10^8$ cocci/ml.) and had an initial viability of about 99%. Usually samples were removed at intervals and placed directly on agar slides for viability measurements. In experiments with dense populations some dilution was necessary before doing viability determinations. Where the effect of a toxic metal or growth inhibitor was being examined the organisms were centrifuged down and washed in phosphate buffer before the viability determination.

Growth measurement and cell-counting procedures. Growth was followed by taking samples at intervals from the culture and measuring the turbidity at 600 mμ with a Beckman DB Spectrophotometer. Dilutions were made in distilled water where necessary, equivalent dilutions in phosphate buffer gave the same turbidities. Bacterial dry weights were calculated from the turbidities of bacterial suspensions after calibration with suspensions of known dry wt/ml. Bacterial dry weights were obtained by taking known volumes of suspensions in distilled water and drying to constant weight at 100°. All cell masses are recorded as mass units dry wt bacteria/ml. Total counts were made in a Petroff-Hausser chamber with a phase-contrast microscope (magnification ×310). The 4 large squares at each corner of the grid were counted for each sample (400–500 chains each). Counts are of individual chains separated by at least 2 coccal diameters unless otherwise specified. The number of cocci/chain was determined by counting the individual cocci in a total of 250 chains for each sample (magnification ×700).

Viability measurement. The percentage of viable organisms in resuspended systems was determined by the slide-culture method of Postgate, Crumpton & Hunter (1961). The only modification to the method found necessary was the use of stainless-steel annuli instead of brass annuli, to avoid copper toxicity. The agar medium used had the following composition (g./100 ml.); lactose monohydrate, 2·0; Casamino acids (Difco), 1·0; peptone (Difco), 0·8; yeast extract (Difco), 0·1; agar (Davis), 1·0; Na_2HPO_4, 0·25; $MgSO_4 \cdot 7H_2O$, 0·05; final pH 7·0. The agar was filtered (Whatman no. 15) before autoclaving and centrifuged hot before slide preparation to remove debris. Slides were incubated at 30° for times sufficient to allow 4–5 generations which provided adequate differentiation between live and dead organisms. *Streptococcus*

lactis ML3 occurred mainly as diplococci, but chains with up to 6 cocci were observed. Slides were counted in green light using a phase-contrast microscope with an eyepiece grid (magnification $\times 310$). As a routine, 8 typical fields with 40–60 objects/field were counted on duplicate slides; on slide cultures of organisms with long division lags, 10 fields were counted with 20–35 objects/field to minimize errors from overgrowth of dead organisms. Conventional colony counts were made by the standard pour-plate method (Cruickshank, 1965) with phosphate buffer diluent and the agar medium described above.

Analytical methods. Magnesium and copper were measured in cell-free supernatant fluids and buffer solutions respectively, by using a Techtron AA3 atomic absorption spectrophotometer (Techtron Pty. Ltd., Australia) under standard operating conditions. Standard magnesium solutions and samples contained in phosphate buffer were diluted 1/10 with de-ionized water to appropriate concentrations. Phosphorus interference was suppressed by using lanthanum. Cellular magnesium content was determined by the method of Webb (1966). Perchloric acid was added to copper standards and samples to a final concentration of 10% (v/v). Ammonium pyrrolidine dithiocarbamate (1%) and methyl isobutylketone were added (Allan, 1961) and the copper determined in the organic phase after shaking. Lactose was measured by the anthrone method of Richards (1959). Lactic acid was determined by the method of Barker & Summerson (1941).

Materials. Reagents of analytical grade were used when available. All water was distilled and then de-ionized by passage through a mixed bed ion-exchange resin (Permutit 'Biodeminrolit').

RESULTS

Growth of organism. The mean generation time (doubling of turbidity) of *Streptococcus lactis* ML3 during the logarithmic growth phase in batch culture was 46 ± 4 min. Analyses showed that lactose was the growth-limiting substrate. The pH changed from 7·3 to $6{\cdot}3 \pm 0{\cdot}1$ at the end of growth; the yield was $0{\cdot}98 \pm 0{\cdot}03$ mg. dry wt organism/ml., corresponding to about 8×10^9 cocci/ml.

Numbers of organisms in resuspended systems. It was important in the present investigation to determine whether the total number of cocci in a given suspension remained constant on prolonged incubation. For this reason total counts/ml. were made as well as slide counts by the method of Postgate *et al.* (1961), which determines only the ratio of viable/total organisms. Four washed suspensions (equiv. 50 μg. dry wt organisms/ml.) in phosphate buffer were sampled over a 28 hr period. For total chain counts, quadruplicate samples from one suspension and single samples from the other suspensions were counted. The viable chain counts given in Table 1 are the means of plate counts from samples of all flasks diluted in duplicate and plated in duplicate (i.e. 16 plates). The number of cocci/chain and the % viability figures from slide culture are means of duplicate counts from each suspension (see Methods). The results in Table 1 show that *Streptococcus lactis* ML3 organisms had a tendency to clump in phosphate buffer on prolonged storage. This was the reason for the decrease in total chain counts but there was no significant decrease in total numbers of cocci for at least 28 hr. The 20% decrease in turbidity was probably a result of endogenous metabolism and leakage of cellular material into the suspending buffer. There appeared to be a constant systematic error in the % viability results obtained by

normal methods, similar to that noted by Norris & Powell (1961), Postgate *et al.* (1961), and Strange *et al.* (1961). These low figures were probably the result of errors in the total count determination, since the survival curves were very similar when plate count viabilities were expressed as percentages of the initial plate count (taken as 99 %).

Table 1. *Constancy of total cell numbers and comparison of survival measurements on Streptococcus lactis* ML3 *in washed suspensions at 30°*

Organisms were harvested at end of growth phase, washed twice, and resuspended in 0·075 M-phosphate buffer (pH 7·0, containing 10^{-5} M-EDTA) at equiv. 50 μg. dry wt organism/ml. with gentle agitation provided by a magnetic stirrer.

Suspension incubation time (hr)...	1	3	5	7	10	24	28	S.D.*
(1) Total chain count ($\times 10^{-8}$/ml.)	1·52	1·47	1·36	1·45	1·42	1·12	1·10	±0·07 (7)
(2) Viable chain count ($\times 10^{-8}$/ml.)	1·19	1·16	1·10	0·52	0·22	0·03	—	±0·08 (16)
(3) % viability ((2)/(1) × 100)	78	79	81	36	15	3	—	—
(4) % viability (slide count)	99·5	98	96·5	65	28	6	—	—†
(5) Cocci/chain or clump	—	2·64	—	2·71	—	3·56	3·45	±0·05 (8)
(6) Total coccal count ((1).(5) × 10^{-8}/ml.)	—	3·88	—	3·93	—	3·99	3·79	±0·22
(7) Turbidity	0·173	0·165	0·157	0·149	0·143	0·140	0·139	—

* Standard deviation (no. of measurements/sample). † See Fig. 1.

The slide culture technique of Postgate *et al.* (1961) was evaluated for *Streptococcus lactis* ML3 in the following way. Six washed suspensions were prepared in phosphate buffer (see Methods). Duplicate samples were removed from each suspension at times designed to give measurements at high, medium and low viabilities. Slide preparation and counting was performed as described in Methods. An analysis of variance was carried out on the % viability figures for each sampling time. The standard deviation from the mean was computed together with the 95 % confidence interval for two slides prepared from one suspension (Fig. 1). At high viabilities (94–99 %), variance between tubes was negligible and the accuracy of the method was high, but variance increased with moribund populations which had variable cell division times. Variance was largely a function of division lag time. The survival curve of *S. lactis* ML3 starved in buffered suspension was essentially sigmoid in form.

Survival in resuspended systems

Survival measurements on all resuspended systems were repeated at least three times. Survival curves showed small fluctuations with different batches of organisms but trends between systems were always the same.

Effect of ethylenediaminetetra-acetate (EDTA). Early experiments on survival in phosphate buffer produced irregular results and rapid death rates, these were decreased on addition of 5×10^{-6} M-EDTA (Fig. 2). Lower concentrations had only a slight effect while high concentrations of EDTA (10^{-2} M) accelerated death, possibly as a result of its capacity to destabilize cell walls (Gray & Wilkinson, 1965; Asbell & Eagon, 1966) and ribosomes (Wade, 1961). A toxic metal impurity was thus probably present in the buffer (Postgate & Hunter, 1962). Extraction of phosphate buffers with organic metal complexing reagents (see Methods) revealed an average of 0·06 p.p.m. copper (about 10^{-6} M-copper). When trace amounts of Cu^{2+}(10^{-5} M) were added to suspensions

containing 5×10^{-6} M-EDTA the survival curve was similar to that for buffer without EDTA (Fig. 3*a*).

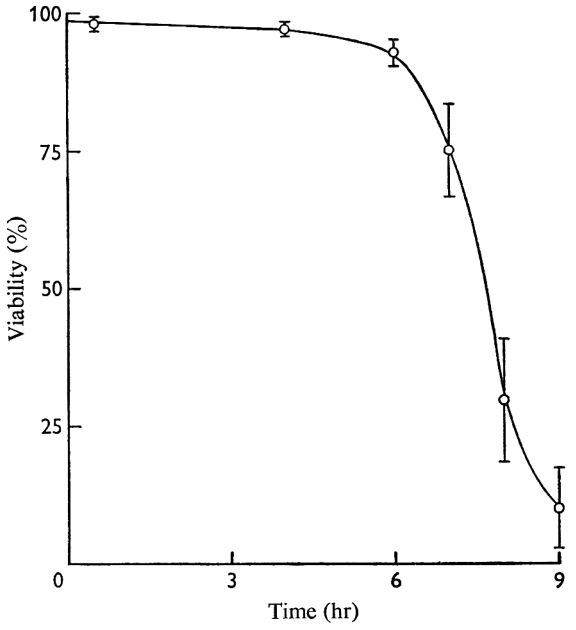

Fig. 1

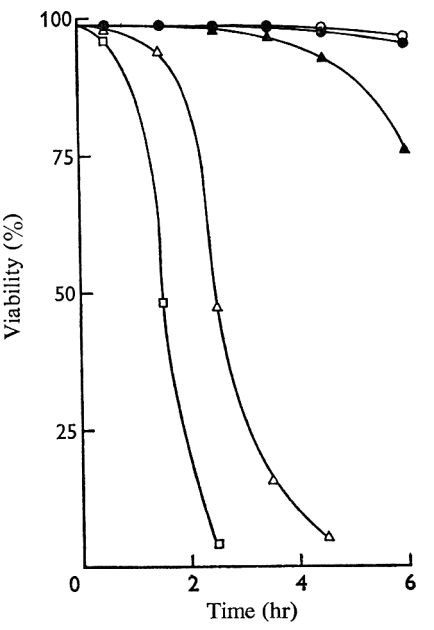

Fig. 2

Fig. 1. Variance in viability determinations on washed suspensions of *Streptococcus lactis* for 95% confidence limits. Duplicate counts on six similar populations were determined using the slide culture technique. For details see text.

Fig. 2. Effect of EDTA on survival of *Streptococcus lactis*. *Streptococcus lactis* harvested at the end of the growth phase was washed and resuspended in 0·075 M-phosphate buffer at 20 μg./ml. and incubated at 30°, pH 7·00 ± 0·05 in static culture. Tubes contained the following concentrations of EDTA: ▲, 10^{-2} M; ○, 10^{-5} M; ●, 5×10^{-6} M; △, 10^{-6} M; □, no EDTA. Viabilities were determined by slide culture.

Effect of divalent metal ions. The effect of divalent metal ions was studied with suspensions containing sufficient EDTA to remove the toxic effect of contaminant copper. Hg^{2+} (10^{-6} M) caused complete death within 5 min. while the toxicity of other ions tested decreased in the order $Cu^{2+} > Ni^{2+} > Fe^{2+} > CO^{2+} > Zn^{2+} > Pb^{2+}$ (Fig. 3*a*). From literature reports (Perrin, 1964) this would also appear to be the approximate order of decreasing stability of the metal-EDTA complexes so that it is unlikely that any of these ions displaced the contaminant copper from its complex. Ca^{2+}, Mn^{2+}, and Sn^{2+} (10^{-5} M and 10^{-4} M) were without effect.

Addition of Mg^{2+} produced greatly increased survival times (Fig. 3*b*), maximum survival being afforded with about 10^{-4} M-Mg^{2+}. Mg^{2+} also prolonged survival of organisms in phosphate buffer without EDTA. When incubated with $Mg^{2+} + Cu^{2+}$ the toxic effect of Cu^{2+} was decreased (Fig. 3*b*). These observations are similar to those made by MacLeod & Snell (1950) for *Lactobacillus arabinosus* and by Abelson & Aldous (1950) for *Escherichia coli*. The experiments described show two effects of Mg^{2+}: (i) a protective effect decreasing Cu^{2+} toxicity, (ii) a stabilizing effect (see Discussion).

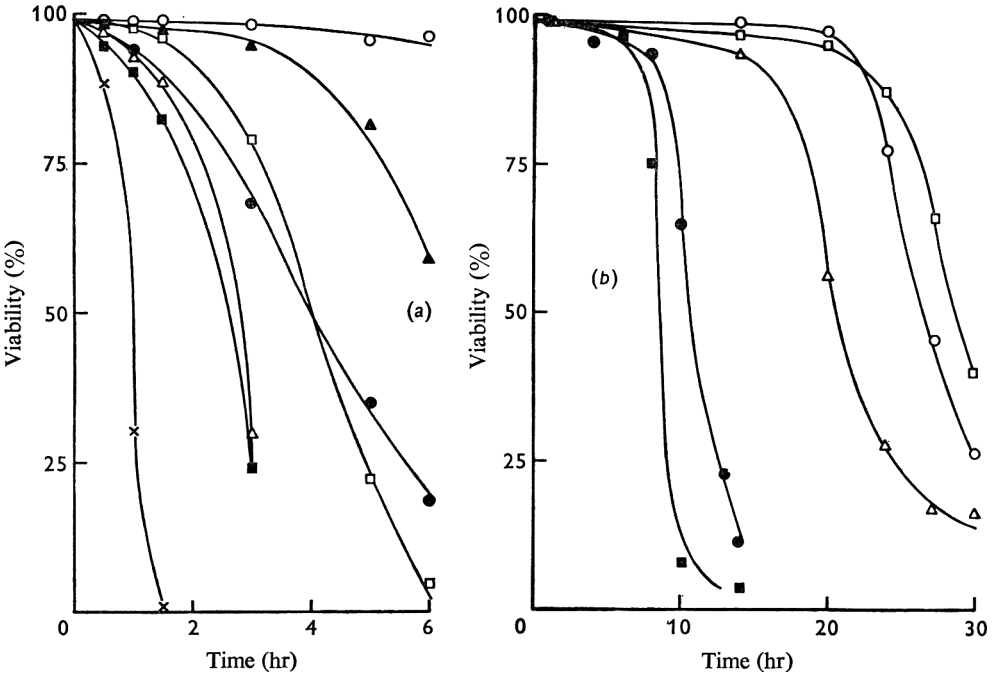

Fig. 3. Effect of divalent metal ions on survival of *Streptococcus lactis*. Suspensions were prepared as for Fig. 2. Tubes contained 5×10^{-6} M-EDTA in 0·075 M-phosphate buffer. Part (*a*): ○, no addition; ▲, $+Pb(NO_3)_2$; □, $+ZnSO_4$; ●, $+CoCl_2$; △, $+FeSO_4$; ■, $+NiCl_2$; ×, $+CuSO_4$; all salts 10^{-5} M. Part (*b*): ■, no addition; remaining tubes contained $MgSO_4$ at the following concentrations: □, 10^{-3} M; ○, 10^{-4} M; △, 10^{-5} M; ●, 10^{-4} M + $CuSO_4$ (10^{-5} M).

Effect of bacterial concentration. Very dense suspensions (equiv. 7·8 mg. dry wt organism/ml. or about 6×10^{10} cocci/ml.) survived best, with decreasing survival times at lower bacterial concentrations (Fig. 4*a*). Measurements of Mg^{2+} excretion by the organisms (see Methods) showed that at high bacterial concentrations Mg^{2+} leakage from the organisms was sufficient to produce stabilizing concentrations (Fig. 4*a*). Analyses of whole bacteria immediately after washing in buffer indicated 0·41 % (w/w) magnesium. The concentration (7×10^{-4} M) of Mg^{2+} in the supernatant fluid with equiv. 7·8 mg. dry wt organisms/ml. represented a loss of 0·22 % (w/w) magnesium from the organisms (i.e. 54 % of the cellular magnesium). From these results it seemed likely that Mg^{2+} excretion by the organisms was the cause of extended survival at high bacterial concentrations. Additional support for this conclusion was obtained in a similar experiment where the addition of 10^{-4} M-Mg^{2-} gave almost identical survival surves at each bacterial concentration (Fig. 4*b*). High bacterial concentrations also prolonged survival in phosphate buffer without EDTA, presumably because the copper toxicity was decreased by the excreted Mg^{2+} in a similar manner to that described in the preceding section.

Effect of growth phase and media composition. The survival of organisms taken from different growth phases in the lactose-limiting medium were compared. Organisms from the mid-logarithmic phase, the end of the growth phase and from 1 and 2 hr after growth had ceased showed no measurable differences in the survival curves. This is in

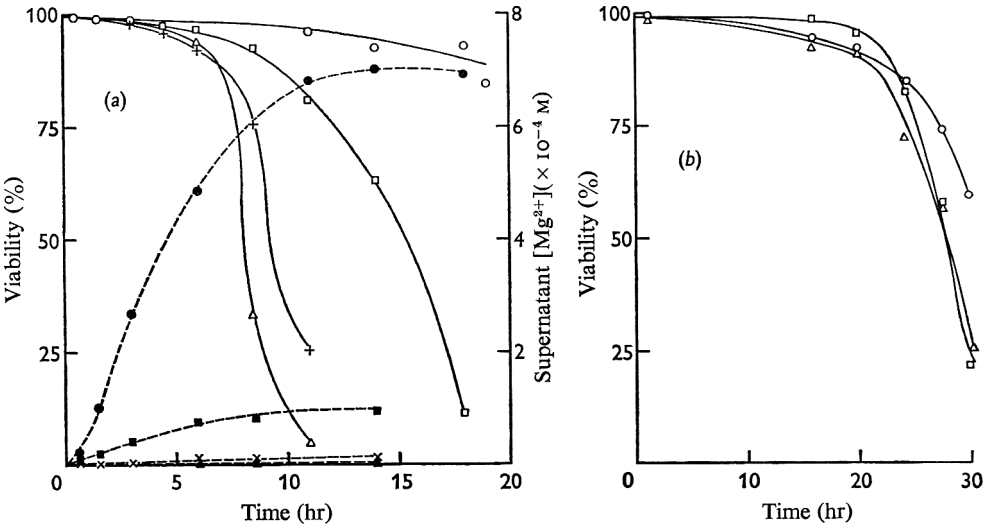

Fig. 4. Effect of cell concentration on survival of *Streptococcus lactis*. Cells were harvested from 500 ml. of culture at the end of the growth phase, washed and resuspended in 60 ml. 0·075 M-phosphate buffer with 10^{-5} M-EDTA. Part (*a*) At intervals viability determinations were made, after sample dilution where necessary, and 5 ml. samples withdrawn from each suspension. The samples were centrifuged and the cell-free supernatants carefully removed and filtered (sintered glass, porosity 5) into tubes. These samples were frozen and later analysed for magnesium (see Methods). Viabilities of washed suspensions (50 ml.) containing 7·8 mg. dry wt bacteria/ml. (○—○), 0·77 mg./ml. (□—□), 0·078 mg./ml. (×—×) and 0·019 mg./ml. (△—△) are shown. Supernatant Mg^{2+} concentrations at the same cell densities were ●---●, ■---■, × ---×, and ▲---▲ respectively. Part (*b*). Cell suspensions were prepared as in part (*a*), containing: ○, 8·1 mg./ml.; □, 0·80 mg./ml.; △, 0·079 mg./ml. Each suspension contained 10^{-4} M-Mg^{2+}.

agreement with the observations of Postgate & Hunter (1962), who stressed the importance of the nutritional status of the population and suggested that for a genetically uniform population the growth phase has only a small effect on the survival time during starvation. Attempts to find a satisfactory non-carbohydrate-limiting medium were unsuccessful. When the lactose concentration was increased to 1% growth ceased, presumably because of the inhibitory pH value produced (about pH 4·7); analyses indicated the presence of 0·08% lactose at the end of growth. These organisms, when washed and resuspended, showed a slightly increased death rate as compared with organisms from the routine medium. When the amino acid concentration was decreased from 0·5% to 0·05% the growth rate was substantially decreased (doubling time 2–2·5 hr as compared with about 46 min. for routine medium), and resuspended organisms from this medium also showed higher death rates than normal (50% decrease in viability in 4·5–5 hr).

Effect of salt concentration. In de-ionized water containing 10^{-5} M-EDTA the viability of resuspended organisms decreased to 50% 3·6 hr. In $Na_2HPO_4 + KH_2PO_4$ buffer (pH 7·0) containing 10^{-5} M-EDTA, survival times decreased slightly with increasing buffer concentration. Organisms in phosphate buffer at 0·075 M, 0·150 M and 0·300 M decreased to 50% viability in 7·2 hr, 6·1 hr, and 6·0 hr, respectively. Ringer solution, with and without 10^{-5} M-EDTA, gave 50% viabilities after 6·7 hr and 3·8 hr, respectively. All times were ±0·3 hr for replicate samples.

Effect of pH value. In phosphate + citrate buffer at 30° *Streptococcus lactis* ML 3 had a sharp optimum for survival near pH 7·0 (Fig. 5). Survival times decreased sharply on decreasing the pH value and 50% decrease in viability occurred in 0·5 hr at pH 4·0. It was therefore of interest to re-examine the observations of Harvey (1965), who concluded that *S. lactis* ML 3 organisms held in broth culture at pH 4·2 were damaged but maintained complete viability for 5 hr. Organisms were grown and placed in a growth medium at pH 4·2 as described by Harvey (1965). Measurements showed that while the turbidity increased by 20% in 3 hr there was no increase in viable or total

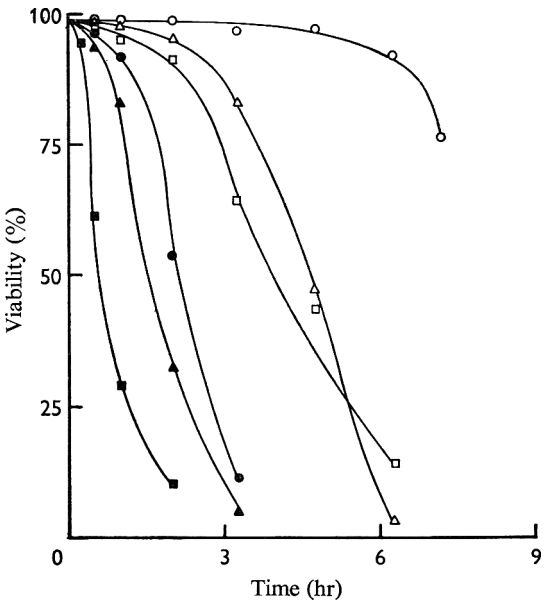

Fig. 5. Effect of pH on survival of *Streptococcus lactis*. Buffers were prepared from 0·1 M-Na_2HPO_4 and 0·05 M-citric acid containing 10^{-5} M-EDTA. Cocci were harvested from 20 ml. culture at the end of the growth phase, washed and resuspended in 5 ml. phosphate-citrate buffer (pH 7·0), 0·5 ml. of this suspension was added to 10 ml. buffer at 30° to give 190 μg./ml. and final pH's (±0·05) of: △, 7·93; ○, 6·98; □, 6·55; ●, 6·00; ▲, 4·90; ■, 4·03. Before viability determination a 1/10 dilution was made in phosphate-citrate buffer, pH 7·0.

bacterial numbers, contrary to the interpretation of Harvey (1965), and 97–99% of the organisms survived for 6 hr. This indicated a very marked protective effect of nutrients at low pH values. Organisms harvested from the same broth medium (pH 6·5) and starved in phosphate buffer gave similar survival times to washed organisms from the routine medium starved in phosphate buffer.

Effect of temperature. The death rate decreased markedly on lowering the incubation temperature of washed suspensions from 45° to 3° (Fig. 6*a*, *b*). Note the time scale for Fig. 6*b* is ten times that for Fig. 6*a*. In contrast, Postgate & Hunter (1962) observed decreased death rates on lowering the incubation temperature from 50° to 20°, but found accelerated death again below 20°.

Effect of atmosphere and agitation. Gentle agitation produced by a magnetic stirrer had no measurable effect on survival curves as compared with those of static suspensions, but vigorous agitation (magnetic stirrer) generally gave more rapid death

(50% decrease in viability in 2–3 hr). Both air and O_2-free N_2 increased the death rate when gently bubbled through washed suspensions (50% decrease in viability in 4·5–5 hr for air, and in 3–3·5 hr for N_2). These observations are difficult to explain but it is possible that agitation washed out soluble intracellular components. Vigorous agitation did not produce significant chain breakage. The results imply that special E_h values were required for survival, as would befit a micro-aerophilic organism.

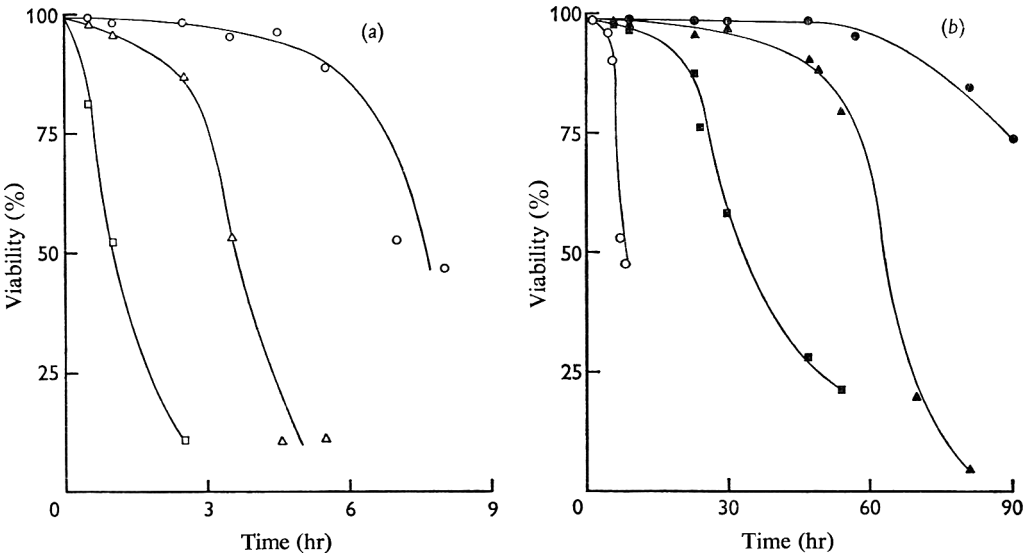

Fig. 6. Effect of temperature on survival of *Streptococcus lactis*. Suspensions were prepared as for Fig. 2, the washed cocci being inoculated into phosphate buffer equilibrated to the storage incubation temperature. Part (*a*): □, 45°; △, 37°; ○, 30°; part (*b*): ○, 30°; ■, 22°, ▲, 16°; ●, 3°. Temperatures are ± 1°, slide cultures were incubated 30°.

Effect of metabolic inhibitors. The addition of 0·01 mM-iodoacetate decreased survival times sharply (Table 2). Methylene blue at concentrations above 0·02 mM showed an immediate bactericidal effect, while at 0·001 mM the death rate increased slightly. The addition of 2·7 mM sodium arsenate and 2·4 mM-sodium fluoride also produced a slight increase in death rate. Sodium azide, 2,4-dinitrophenol, potassium cyanide and sodium malonate did not significantly influence the survival curves, although all experiments were made under conditions of low O_2-tension. Before slides were prepared for viability determination the inhibitors were removed by washing (see Methods), this procedure had no noticeable effect on the viability of control populations.

Effect of substrates

Carbohydrates. Lactose, glucose, galactose and fructose (10^{-2} M; Fig. 7*a*) produced markedly increased death rates in washed suspensions (50% decrease in viability in 1–1·5 hr) irrespective of the growth phase of the organisms and limiting nutrient (50% decrease in viability in 0·5–0·7 hr for organisms from amino acid limited medium). The suspension remained at pH 7·0 and analyses for lactic acid indicated that fermentation of all carbohydrates took place. Sodium lactate (10^{-2} M) had no effect on

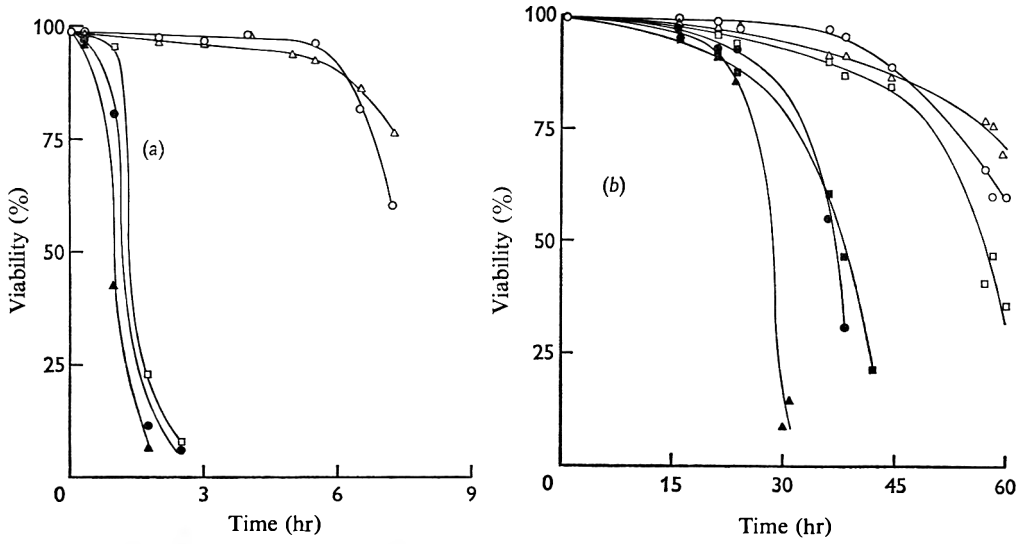

Fig. 7. Effect of nutrients on survival of *Streptococcus lactis*. Suspensions were prepared as for Fig. 2 in phosphate buffer containing 10^{-5} M-EDTA. Part (*a*): ○, no addition; ▲, +lactose; ●, +glucose; □, +galactose; △, +lactose+Mg^{2+} (10^{-3} M). All carbohydrate supplements were 10^{-2} M. Part (*b*): ▲, +Mg^{2+}; ○, +Casamino acids (Difco) (0·5%) +Mg^{2+}; □, +arginine (10^{-2} M)+Mg^{2+}; ■, +alanine, aspartic acid, glutamic acid (each 10^{-2} M) +Mg^{2+}; ●, +glycerol (10^{-2} M)+Mg^{2+}; all Mg^{2+} supplements 10^{-3} M; △, growth medium minus lactose, vitamins and $NaHCO_3$.

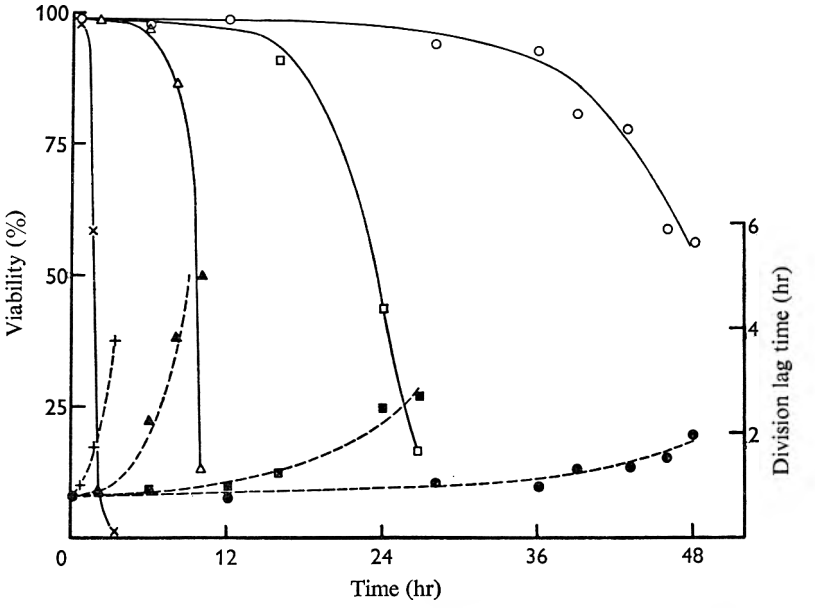

Fig. 8. Division lag times for *Streptococcus lactis* from various resuspended systems. Suspensions were prepared as for Fig. 2 in phosphate buffer containing 10^{-5} M-EDTA. Viabilities for the suspensions: +0·5% Casamino acids (Difco)+10^{-3} M-Mg^{2+} (○—○), +10^{-3} M-Mg^{2+} (□—□), no addition (△—△), +10^{-2} M-lactose (×—×). Division lags of cells in the same systems are indicated. ●---●, ■---■, ▲---▲, × ---×.

survival times. Accelerated death was markedly reduced in all cases on addition of 10^{-3} M-Mg^{2+}, giving survival times similar to control systems without carbohydrate or Mg^{2+} (Fig. 7*a*). Addition of 10^{-2} M-ribose had no effect. Analysis for lactic acid showed *Streptococcus lactis* ML 3 did not ferment exogenous ribose to lactate in washed suspensions.

Table 2. *Effect of metabolic inhibitors on survival of Streptococcus lactis in buffered suspensions at 30°*

Organisms were harvested at end of growth phase, washed twice, and resuspended in 0·075 M-phosphate buffer at equiv. 20 μg. dry wt. organism/ml. Buffer (pH 7·0, except where indicated) contained 10^{-5} M-EDTA and inhibitor as below.

Inhibitor	Concentration (mM)	Death rate*
None (pH 7·0)	—	7·2
None (pH 6·5)	—	6·0
2,4-Dinitrophenol	1·10	6·8
	0·11	6·6
Iodoacetic acid†	0·11	1·1
	0·01	1·9
Methylene blue	0·010	2·9
	0·005	5·0
	0·001	6·7
Potassium cyanide	1·54	7·0
	0·77	6·8
Sodium arsenate	2·70	6·1
	0·54	6·8
Sodium azide	1·54	6·8
	0·15	7·5
Sodium fluoride	2·38	6·9
	0·24	7·3
Sodium malonate†	6·75	6·3
	0·67	6·0

* Time (hr) for 50% decrease in viability (±0·3 hr).
† Buffer adjusted to pH 6·5.

Amino acids and other growth media components. Organisms resuspended in routine medium without lactose, vitamins and $NaHCO_3$, survived for a longer period than organisms resuspended with Mg^{2+} only (Fig. 7*b*). In the former system no detectable change in total numbers occurred up to 48 hr in 2 suspensions containing equiv. 20 μg. dry wt organism/ml., one suspension contained penicillin G (100 units/ml.) to prevent cell growth and provide a control. Casamino acids + Mg^{2+} gave equivalent survival as in routine medium without lactose, vitamins and $NaHCO_3$, while arginine + Mg^{2+} was almost as effective (Fig. 7*b*). Alanine, aspartic acid and glutamic acid, which were found to comprise most of the free amino acid pool in these organisms, produced a marginal increase in survival time when incubated together with washed organisms + Mg^{2+}. Glycerol + Mg^{2+} also produced a marginal increase in survival time while 10^{-2} M-sodium acetate + 10^{-3} M-Mg^{2+} was ineffective (Fig. 7*b*). Washed organisms incubated with the vitamins, purines and pyrimidines supplied at the routine medium concentrations were without effect in the presence of 10^{-3} M-Mg^{2+}.

Growth characteristics of survivors

In some resuspended systems surviving organisms had considerably longer division lags than did exponentially growing organisms. These lags were measured (Postgate & Hunter, 1964) in four resuspended systems giving the maximum scatter in survival times. Division lags tended to increase just before the onset of bacterial death, the increase being particularly sharp in systems without Mg^{2+} (Fig. 8). In almost all experiments where organisms were incubated in the absence of Mg^{2+}, surviving organisms in slide cultures showed a large proportion of morphologically aberrant colony forms once the organisms started to die; instead of the typical circular colonies many organisms produced filamentous colonies and these organisms always showed longer division lags.

DISCUSSION

The slide culture method of Postgate *et al.* (1961), is well suited to survival measurement of short-chain lactic streptococci such as *Streptococcus lactis* ML3 and the present results show many similar trends to those found for *Aerobacter aerogenes* by Postgate, Strange and collaborators. Subsidiary stresses were minimized by washing and resuspending cells in a constant chemical environment at a constant temperature, and by incubating suspensions near the optimum survival pH.

Addition of Mg^{2+} considerably extended survival times of starving cells of *Streptococcus lactis*, in agreement with the observations of Postgate & Hunter (1962) for *Aerobacter aerogenes*. Apart from its potential action in reducing the toxic effect of certain metal cations (MacLeod & Snell, 1950; Abelson & Aldous, 1950), Mg^{2+}, together with Na^{+} and K^{+}, has been established as an important stabilizer of ribonucleoprotein in bacterial cells (Bowen, Dagley & Sykes, 1959; Tempest, Dicks & Hunter, 1966). In experiments not reported here, addition of Mg^{2+} to washed *Streptococcus lactis* ML3 organisms in phosphate buffer markedly suppressed the excretion of pentose and soluble u.v.-absorbing substances. It is probable that maintenance of the integrity of cell ribonucleoprotein is important for cell survival of *S. lactis*.

Streptococcus lactis organisms in dense suspensions (equiv. 7·8 mg. dry wt organisms/ml.) lost 54% of their cellular magnesium, producing a stabilizing Mg^{2+} concentration (7×10^{-4} M) in the suspending buffer. With equiv. 0·77 mg. dry wt organisms/ml. a stabilizing concentration of Mg^{2+} was reached (10^{-4} M) but death was more rapid than at the higher cell density. It is possible that a considerable amount of irreparable damage was done to the organisms before this concentration was reached. Results suggest that Mg^{2+} excretion by starving organisms was a major factor in prolonging survival of dense populations and further support for this conclusion was obtained when the addition of 10^{-4} M-Mg^{2+} produced almost identical survival times at all population densities. This result contrasts with the observation of Postgate & Hunter (1963*a*) who showed a population effect in the presence of optimal Mg^{2+} concentration. Cryptic growth (Harrison, 1960; Postgate & Hunter, 1962) in dense populations is unlikely for organisms with such exacting nutritional requirements as *S. lactis*. In contrast to the present results Postgate & Hunter (1964) could not detect magnesium by chemical methods in concentrates of cell-free supernatants in which 20 μg. dry wt organisms/ml. populations of *Aerobacter aerogenes* had died. Harrison (1960) first observed the effect of bacterial concentration on survival and reported a

'reversal' of this phenomenon at high concentrations although possible errors are not discussed. If death of *A. aerogenes* at high concentrations is due to anoxia as Harrison suggested, then it is not unexpected to find that *S. lactis*, a facultative anaerobe, did not show a reversed death rate at high bacterial concentrations. Postgate & Hunter (1962, 1963*a*) confirmed Harrison's findings in general but did not observe the 'reversed' population density effect. This discrepancy is explained if anoxia was the cause of the increased death rate at high concentrations of organisms since Harrison (1960) did not aerate the washed suspensions, in contrast to the experimental procedure of Postgate & Hunter (1962).

All the fermentable carbohydrates tested produced accelerated death of washed cells of *Streptococcus lactis* irrespective of the growth phase or growth-limiting nutrient. Accelerated death was markedly reduced on addition of Mg^{2+}, and was not due to a toxic effect of the lactate produced. These results contrast with the observations of McGrew & Mallette (1962, 1965) and Clifton (1966), who reported extended survival of *Escherichia coli* without growth in the presence of glucose and Mg^{2+}. Postgate & Hunter (1963*b*, 1964) observed that addition of certain growth-limiting nutrients to starving suspensions of certain Gram-negative bacteria increased the death rate. This applied to nitrogen-, phosphorus-, and carbon-limited populations and it was suggested that this 'substrate-accelerated death' was a fairly general phenomenon for Gram-negative bacteria. Glycerol-accelerated death was slower, the more dense the population and addition of Mg^{2+} prevented glycerol-accelerated death. This suggests that Mg^{2+} excretion by *Aerobacter aerogenes* might extend the survival of these bacteria in dense populations, but Postgate & Hunter stated that because of interference it was not possible to perform satisfactory magnesium analyses on cell-free buffers in which dense populations had died.

Arginine, which *Streptococcus lactis* converts to ornithine with the production of ATP (Korzenovsky & Werkman, 1953, 1954), produced a remarkable increase in survival time in the presence of Mg^{2+}, indicating that the nature of the energy source was critical for survival. Walker & Forrest (1964) showed maintenance of glycolytic activity of starved *S. faecalis* with exogenous arginine and showed that restoration of glycolytic activity in aged organisms was possible on incubation with Casamino acids and an energy source. The extended survival of *S. lactis* ML3, without growth, in routine growth medium without lactose, vitamins and bicarbonate, was attributable to its Mg^{2+} and amino acid content. Casamino acids presumably prolonged survival of *S. lactis* by providing a limited energy source for cell maintenance and supplying amino acids for cell turnover minimizing breakdown of essential components, and giving the observed short division lags of survivors. It would appear that the division lags for *S. lactis* in various resuspended systems are a function of the extent of cell damage which eventually leads to death.

The insignificant death rate observed in the initial incubation period of *Streptococcus lactis* ML3 in most resuspended systems indicates that intrinsic differences of resistance between individual bacteria are slight. The duration of this period of almost complete viability compared with the phase of rapid death varied widely, depending on the resuspension system and the biological history of the population. The death rate then begins to increase and generally continues at a rapid rate. The actual cause or causes of death of *S. lactis* in those non-toxic buffers which provide maximum stability are probably complex and diverse.

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The Relation of the Length of Lag Phase of Growth to the Synthesis of Nisin and Other Basic Proteins by *Streptococcus lactis* Grown Under Different Cultural Conditions

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SUMMARY

A nisin-producing strain of *Streptococcus lactis* was grown in batch culture and at intervals during growth secondary cultures were derived from the parent culture and the length of lag of growth was estimated. Two media were used: a highly buffered 'medium 22' and an unbuffered (LTB) medium both without pH control and with pH controlled at about 6·7. In 'medium 22' the dry weight doubling time was 95 min. and at the end of growth the pH was 5·2. Neutralization of the culture in this medium did not markedly affect nisin production or dry weight. The total and cell-bound nisin formed/unit dry wt of organism tended to correlate with the length of lag phase of growth. In LTB medium the dry wt doubling time was 45 min. and the terminal pH was 4·5. Of the total nisin formed > 80% was in the culture fluid when the pH was allowed to decrease below 6·0; total nisin in cultures without pH control did not correlate with length of lag phase of growth. Neutralization of the culture increased the dry wt of organism about threefold. Neutralization also increased the cell-bound nisin/unit dry wt organism in stationary phase and the length of lag of growth of such organisms was longer than comparable stationary-phase organisms grown in unneutralized culture. Chloramphenicol (1 μg./ml. medium) decreased the nisin/unit dry wt organism at stationary phase and the length of lag of growth of such cultures was shorter than in comparable cultures without chloramphenicol. Frequently the start of growth was characterized by a decrease of bio-assayable nisin and a shortening of the lag phase; these changes could be mimicked by a culture which had reached the stationary phase through glucose depletion. The characteristics of early growth were reproduced after the addition of more glucose.

INTRODUCTION

The biosynthesis of nisin is sensitive to inhibitors of protein synthesis and messenger RNA appears to be involved (Hurst, 1966*a*). The molecular weight of nisin was first reported to be about 7000; this figure was challenged by Bodansky & Perlman (1964) who suggested that the molecular weight was more likely to be one third of the original estimate. However, recent work on the migration of peptides and small proteins in polyacrylamide gels (Ingram, Tombs & Hurst, 1967) has confirmed the original estimate of 7000. Thus both the method of biosynthesis of nisin and its molecular weight suggest that it is a small basic protein rather than a polypeptide. Growing

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cultures of the nisin-producing streptococcus synthesize nisin only before the stationary phase (Hurst, 1966*b*). These results led to the tentative conclusion that basic proteins and especially nisin have a regulatory function for *Streptococcus lactis*, possibly being concerned with initiation and halting of growth.

Our first growth experiments were done without control of pH value in batch fermentations in medium 22. This was a buffered medium in which the nisin-producing streptococcus grew slowly. We now report results in which we attempted to alter the ratio of nisin to dry weight of organism by growing the organism under pH-controlled conditions in medium 22 and in another medium (LTB) which permits faster growth. The neutralization technique did not have much effect with the buffered medium 22 but it had considerable effect with LTB medium. Stationary-phase organisms obtained from LTB medium with pH control contained more nisin/dry wt organism than comparable cultures grown without pH control, and the length of the lag phase was increased. When chloramphenicol was added in small amounts, nisin produced/dry wt organism was decreased and, as expected, the resulting cultures had a shorter lag phase. These results support the earlier suggestion that in the nisin-producing strain of *S. lactis* the antibiotic may have a regulatory function.

METHODS

Media, organism, culture conditions, preparation of samples, estimations, extinctions, nisin bio-assay, electrophoresis and estimation of the length of lag phase of growth were all as described previously (Hurst, 1966*b*). As defined previously, total nisin was the amount which could be estimated by bio-assay of cocci and medium. Cell-bound nisin was the amount of nisin retained by the cocci after discarding the culture fluid.

Media. Medium 22 and the LTB medium were as described before (Hurst, 1966*a*). Previously we used medium 22 for growth of the nisin-producing strain, and medium LTB for growing the bio-assay test organism. In the experiments in this paper both media were used for growing the nisin-producing strain; the much faster growth rate in LTB medium permitted complete growth of a batch culture in about 8 hr as compared to about 14 hr in medium 22. In experiments in which the culture was maintained at pH 6·7, media contained 2·5% (w/v) glucose unless otherwise stated; neutralization of the lactic acid formed by the growing cultures was by periodic addition of 10 N-NaOH with gentle stirring to avoid aeration.

Basic proteins were prepared as described by Hurst (1966*b*). Basic proteins were extracted from whole cocci with hot 0·05 N-HCl, followed by propanol extraction followed by ion-exchange chromatography on columns of Amberlite resin CG 50 (British Drug Houses Ltd., Poole, Dorset, England). Total basic protein was defined as the fraction eluted after exhaustive washing of the columns with 50% (v/v) acetic acid in water; this was measured either by weighing or by densitometry after electrophoresis in polyacrylamide gels. Weighing tended to give an underestimate of the basic proteins because of loss of material during the manipulations involved in freeze-drying. With densitometry, the concentration of basic proteins was expressed in terms of a nisin marker after staining with amido-black. Provided samples were run on the same gel and stained at the same time the results were reproducible to within 15%. It was recently shown with ribosomal proteins that densitometry after staining gives the same quantitative and qualitative results as radio-activity measurements (Traut, 1966).

RESULTS

Distribution of nisin between cocci and culture fluids in LTB medium at different stages of fermentation

During a lactic fermentation the pH value decreased as lactic acid accumulated and this increased the solubility of basic proteins. Figure 1 shows that at pH > 6·0 more than 80% of the nisin was associated with the cocci, at pH < 6·0 more than 80% was in the culture fluid.

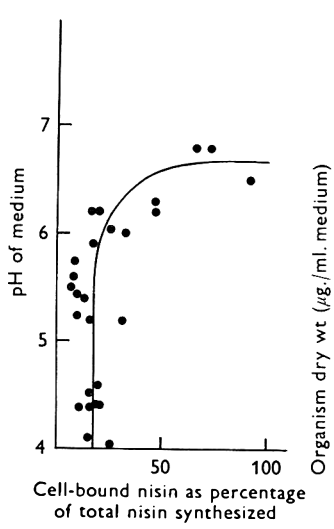


Fig. 1

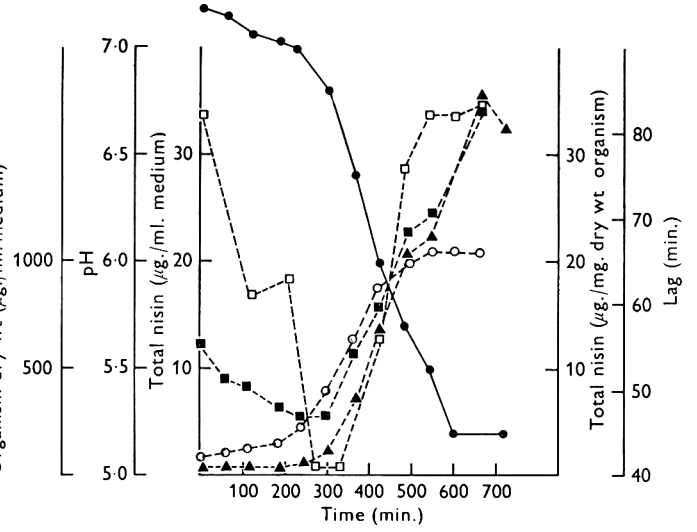


Fig. 2

Fig. 1. Synthesis and binding of nisin by *Streptococcus lactis* as a function of the pH value of the medium.

Fig. 2. Growth of *Streptococcus lactis* in a buffered complex medium. Dry wt organism O---; lag □---; total nisin/ml. medium ▲---; total nisin/mg. dry wt. ■---; pH ●—. The length of the lag phase of growth was estimated in LTB medium.

Growth in medium 22 without pH control

An example of this type of experiment is shown in Fig. 2. The initial pH 7·2 decreased to pH 5·2 in 10 hr; during the same time the dry wt of organism increased from 80 μg./ml. medium to 1050 μg./ml. During the exponential phase of growth the dry wt doubling time was 95 min. Nisin was not synthesized during the lag phase and the nisin/unit dry wt organism decreased during this time. Coincident with this change in the nisin concentration the lag phase of growth of secondary cultures became shorter. The inoculum had a lag of 80 min. which decreased to 40 min. at early exponential phase. During this phase of growth nisin synthesis started and the length of lag of secondary cultures progressively increased until it returned to its original value of 80 min. A maximum of 33 μg. nisin/mg. dry wt organism was obtained.

Growth in medium 22 with pH controlled to 6·7

The initial pH 7·3 of the medium was allowed to decrease to pH 6·7 and then maintained at this value. The final dry wt of cocci was 1100 μg./ml., only slightly higher than that of the pH-uncontrolled fermentation. Nisin synthesis started after a delay of 100 min.; the inoculum had a lag of growth of 176 min. which decreased to 126 min. at early exponential phase, to reach a maximum of 206 min. at stationary phase. A maximum of 44 μg. nisin/mg. dry wt cocci was obtained (Fig. 3). The data of Fig. 2 and 3 have been redrawn in Fig. 4. This shows that there might be a correlation between total nisin (μg./mg. dry wt cocci) and time of lag in the controlled and in the pH-uncontrolled fermentation.

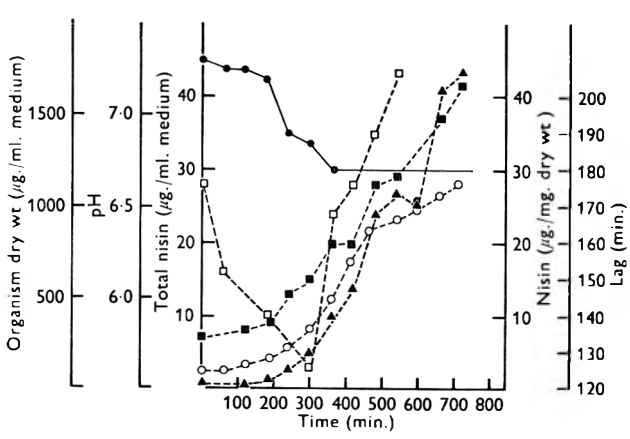


Fig. 3

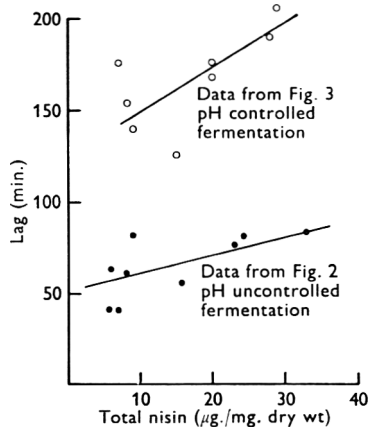


Fig. 4

Fig. 3. Growth of *Streptococcus lactis* in a buffered complex medium with pH control. Dry wt O---; lag □---; total nisin/ml. medium ▲---; total nisin/mg. dry wt ■---; pH ●—.

Fig. 4. Correlation between length of lag and total nisin/unit dry wt cells in pH-controlled and uncontrolled fermentations in buffered medium.

Growth in LTB medium with and without pH control

Figure 5 is a record of an experiment in LTB medium which enabled the organism to grow about twice as fast as in medium 22 (doubling time 45 min.). In these experiments cell-bound as well as total nisin was estimated; the uncontrolled medium decreased from pH 6·8 to pH 4·4 in 6 hr. Fig. 5 shows that neutralization had the following effects: dry wt of stationary-phase organisms increased about threefold from 800 μg./ml. medium to 2500 μg. Total nisin/unit dry wt organism was higher in the uncontrolled than in the pH-controlled fermentations; cellular nisin was about the same for the first 4 hr but at stationary phase the cellular nisin/unit dry wt organism in the pH-controlled fermentation was about double that of the uncontrolled fermentation. Accompanying the twofold increase in cellular nisin, the length of lag increased about 2·5-fold. The difference in the length of lag in the pH-uncontrolled fermentation was from 65 to 80 min. (15 min.) whereas with pH-controlled cultures the difference was from 65 to 102 min. (37 min.).

Examination of data obtained in four separate experiments showed that total nisin

did not correlate with length of lag of growth in pH-uncontrolled experiments. On the other hand, cell-bound nisin and length of lag were related even in pH-uncontrolled-experiments; to show this the data of Fig. 5 have been replotted in Fig. 6. The correlation coefficient between length of lag phase of growth and cellular nisin was highly significant ($\rho = 0{\cdot}886$).

Effect of inhibitors of protein synthesis on nisin synthesis by cultures of Streptococcus lactis growing in LTB medium

Previous results in a defined reaction mixture with washed suspensions of *Streptococcus lactis* showed that nisin synthesis was more sensitive to inhibition than was protein synthesis (Hurst, 1966*a*). Inhibitors of protein synthesis therefore appeared a

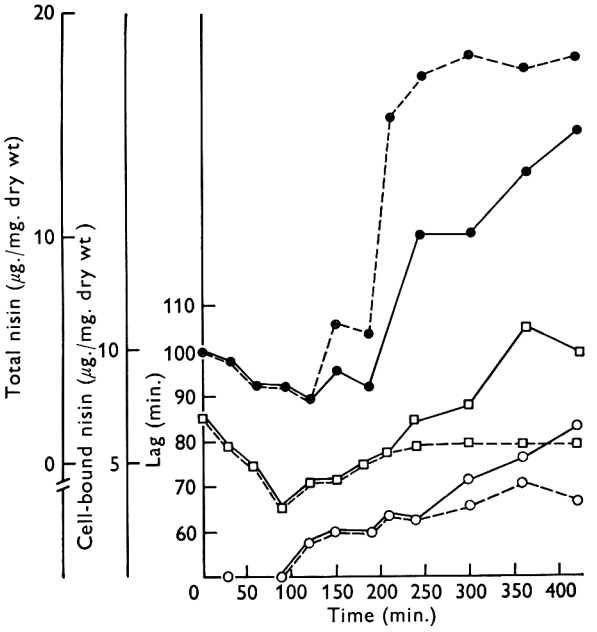


Fig. 5

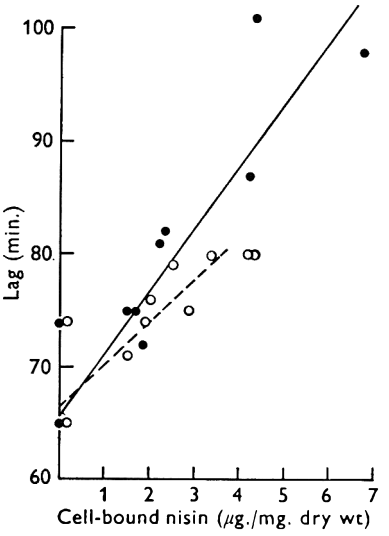


Fig. 6

Fig. 5. Total nisin and cell-bound nisin per unit dry wt of organism and length of lag of growth of *Streptococcus lactis* grown in LTB medium with and without pH control. The total nisin scale has been moved for the sake of clarity. pH controlled ———; pH uncontrolled -----; total nisin ●; cell-bound nisin ○; minutes of lag of growth □.

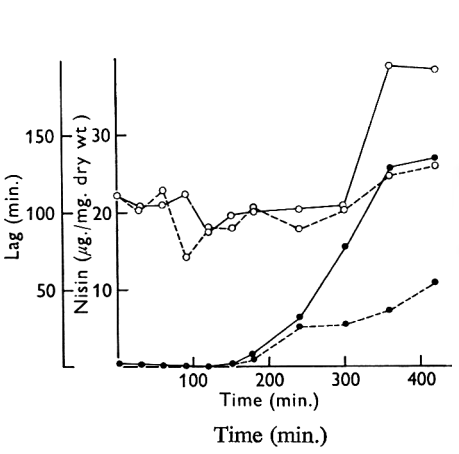
Fig. 6. Correlation between cell-bound nisin and length of lag of *Streptococcus lactis* grown in LTB medium with and without pH control. pH controlled ●——. pH uncontrolled ○----.

possible means of producing cocci with a decreased nisin content, and chloramphenicol was chosen for further work because it has the advantage of being loosely bound by ribosomes from which it can be washed off readily (Vazquez, 1966). The selective inhibition of nisin synthesis was reproduced with cultures growing in LTB medium when the chloramphenicol was added at zero time at 1 μg./ml. medium.

Effect of chloramphenicol on nisin synthesis and length of lag phase of growth

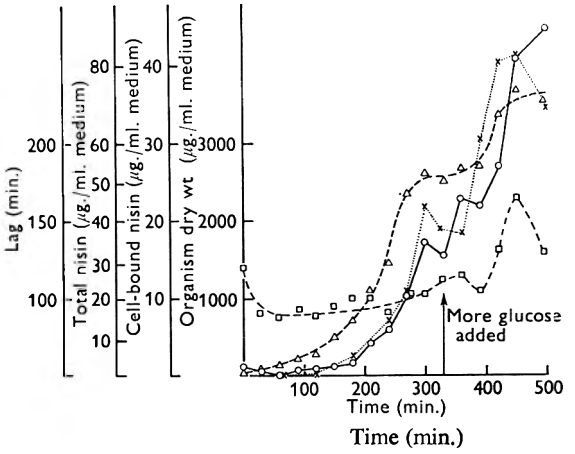
Chloramphenicol was tested in four experiments which gave similar results. At zero time 10 l. of inoculated culture in LTB medium was divided into two equal portions and to one portion chloramphenicol was added at 1 μg./ml. The results of one of these experiments are shown in Fig. 7. At stationary phase the control cocci contained 27 μg. nisin/mg. dry wt organism; the cocci grown in presence of chloramphenicol contained 11 μg. nisin/mg. dry wt organism (60% inhibition). The length of lag of the control cocci was 193 min; that of the cocci grown in presence of chloramphenicol was 132 min. When all the data were considered, the correlation coefficient between the length of lag phase and the nisin content of control- and chloramphenicol-grown cocci was highly significant (ρ = 0·884).

The basic protein content of cocci obtained in another experiment is summarized in Table 1. The ratio of nisin to other basic proteins was lowest in the pH-uncontrolled



Time (min.)

Fig. 7



Time (min.)

Fig. 8

Fig. 7. The effect of chloramphenicol on nisin synthesis and length of lag. Control —; chloramphenicol ----; cellular nisin μg./mg. dry wt ●; length of lag of growth ○.

Fig. 8. The effect of glucose depletion on nisin synthesis and length of lag of growth. Dry wt △---; cell-bound nisin ○—; minutes of lag of growth □---; total nisin × ··.

Table 1. *Basic protein content of stationary-phase Streptococcus lactis batch-grown under different experimental conditions in LTB medium*

Cultural condition*	Basic protein % of dry weight	Nisin % of dry weight	Nisin % of basic protein
No pH control	1·4	0·22	15
With pH control	3·6	0·75	21
With pH control + chloramphenicol	2·6	0·55	21

* Chloramphenicol was used at 1 μg./ml. of medium added before inoculation. Basic proteins were estimated by densitometry after polyacrylamide gel electrophoresis as described under 'Methods'.

fermentation and these cocci also had the lowest total content of basic proteins. In the pH-controlled fermentation nisin was the same % of the basic protein whether or not chloramphenicol was used. However, chloramphenicol inhibited the synthesis of all basic proteins by 26%.

Growth in LTB medium with pH control with limiting glucose

When the glucose concentration was decreased to 1·5% (w/v) and LTB medium was used for pH-controlled fermentation, glucose became exhausted and growth stopped after 5 hr (Fig. 8). At this time more glucose was added to bring the total concentration to that normally used. At inoculation the lag was about 120 min., which decreased to about 90 min. at the beginning of the exponential phase. It increased to the original value when glucose became exhausted; after addition of more glucose growth was resumed and the lag decreased to 102 min.; and at the end of the experiment at 495 min., the lag again increased to 130 min. The time course of the nisin biosynthesis followed that of the length of lag of growth. Cellular nisin present at time zero was not detected again until the lapse of 60 min. It increased to about 17 μg./ml. medium when glucose became exhausted and then increased irregularly until growth was resumed. At the end of growth the concentration reached was 45 μg./ml. medium. The synthesis of total nisin followed a time course which was also close to the length of lag (Fig. 8).

DISCUSSION

The evidence presented here supports the suggestion (Hurst, 1966*b*) that nisin and other basic proteins are synthesized for cellular-control purposes in the *Streptococcus lactis* strain which produces it. The length of time to growth initiation (lag phase) was correlated with the nisin content of the organisms.

Two media were used; in the buffered medium 22 total nisin or cell-bound nisin tended to correlate with length of lag. Keeping the cultures at constant pH value did not change greatly the yield of organism or the nisin content of the organisms. In the unbuffered LTB medium, however, the results were more striking. The growth-limiting effect of the acidity which developed in this medium was removed by periodic neutralization. This enabled the culture to continue growth when the pH-uncontrolled culture had already reached stationary phase. The nisin/unit dry wt organism approximately doubled, and such cultures showed a longer lag phase (Fig. 5). With chloramphenicol we succeeded in reversing this situation and obtained cocci which contained less nisin/unit dry wt organism than did control cocci; such cultures had a shorter lag phase (Fig. 7). When cultures were grown with limiting glucose and more glucose was added at about stationary phase, growth was resumed and changes in nisin and length of lag phase occurred which were similar to those observed during the lag and early exponential phase (Fig. 8).

In uncontrolled LTB medium cell-bound nisin rather than total nisin correlated with length of the lag phase (see Fig. 8). Lack of correlation could be due to most of the nisin being in the culture fluid and not being cell-bound (Fig. 1). When the pH was controlled total nisin could also correlate with length of lag (see Fig. 8). Even so, the correlation between cellular nisin and length of lag phase was not perfect. In the course of normal growth most of the nisin is excreted; when the culture is grown with pH control most of the nisin is probably non-specifically bound. Thus cellular nisin may

have two components, a non-specifically bound part and an additional functional form; the bio-assay does not distinguish between these two forms. It is possible that the other basic proteins play a part in cellular-control mechanisms. This also appears rather likely since nisin forms only about 20% of the total basic proteins (Table 1) and attention has so far been focused on it simply because of the ease of estimating it by bio-assay.

It is interesting to note that the composition of the cell could be altered by the cultural conditions used (Table 1). Leaver & Cruft (1966) found that the basic proteins of *Bacillus megaterium* and *Escherichia coli* constituted up to 1% of the dry weight of the organisms. We have found similar figures for our nisin-producing organism when grown without pH control. The changes in the cell composition were in concordance with the length of the lag phase of growth, confirming our earlier suggestion that nisin had a regulatory function. Not all strains of *Streptococcus lactis* produce nisin, so that the regulatory function is either unimportant or nisin-like molecules are more widely distributed than has been appreciated so far. A small survey of different strains of *S. lactis* showed that nisin production was the norm, and strains which did not produce nisin produced, nevertheless, small molecular weight basic proteins which resembled nisin by Sephadex chromatography and electrophoretic behaviour (Hurst 1967).

It is hoped that this work will draw attention to the possible importance of small-molecular-weight basic proteins. They may or may not be antibiotics and are widely distributed within the *Streptococcus lactis* species. In one strain we have shown a probable correlation between length of lag phase of growth and basic protein content of the organism. We have not yet shown whether these phenomena are causal or coincidental.

We wish to thank Mr M. Pugh for estimations of nisin and Miss M. Hirst for calculation of correlation coefficients.

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The Effect of Chloramphenicol on Growth and Mitochondrial Structure of *Pythium ultimum*

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SUMMARY

Chloramphenicol was found to inhibit the growth of *Pythium ultimum*, showing a maximum dose-response at a concentration of 100 μg./ml. Mycelium grown in the presence of chloramphenicol 100 μg./ml., was devoid of the mitochondrial cytochromes aa_3 and *b* but contained increased cytoplasmic cytochrome *c*. Mitochondria in thin sections of hyphae grown in the presence of chloramphenicol resembled mitochondria in normal hyphae. In negatively stained preparations of isolated normal mitochondria stalked particles were observed on the cristae. These particles could not be demonstrated in mitochondria from hyphae grown in the presence of chloramphenicol 100 μg./ml.

INTRODUCTION

Until recently chloramphenicol was considered to be a fairly specific inhibitor of protein synthesis in prokaryotic organisms (bacteria, blue-green algae), with little or no effect on eukaryotic cells at concentrations which completely inhibit bacterial growth (Vazquez, 1966). Several authors have reported that chloramphenicol can uncouple phosphorylation (Hanson, Stoner & Hodges, 1964) or inhibit protein synthesis and utilization of ATP (Morris, 1966*a*, *b*) in eukaryotic cells. These effects were, however, only observable at concentrations of chloramphenicol 1 mg./ml. or more, whereas most bacterial systems are inhibited by 2–10 μg./ml. The role of chloramphenicol as a specific inhibitor of protein synthesis in intact eukaryotic cells is therefore in doubt. Several workers (Mager, 1960; Kroon, 1963) have reported that chloramphenicol inhibits amino acid incorporation by intact mitochondria from several sources. However von Ehrenstein & Lipmann (1961), So & Davie (1963) and Bretthauer *et al.* (1963) showed that chloramphenicol did not inhibit amino acid incorporation by isolated cytoplasmic ribosomes from animal and yeast cells. It would seem therefore that chloramphenicol ought to cause a specific inhibition of mitochondrial protein synthesis *in vivo* at low concentrations if it were able to penetrate the cells sufficiently well. Huang *et al.* (1966) reported a seemingly specific inhibition by chloramphenicol of mitochondrial cytochrome synthesis in yeast and have coupled this with observations on the disorganization of the mitochondrial cristae. The strain of *Saccharomyces cerevisiae* which they used only showed this inhibition at concentrations of chloramphenicol 4 mg./ml. medium, and as already pointed out this concentration of chloramphenicol acts as a general inhibitor in many organisms. During the screening of a group of fungi for chloramphenicol sensitivity, two were

found which were inhibited by chloramphenicol at 100 μg./ml. or less. The present report concerns the effect of chloramphenicol on the growth and ultrastructure of one of these organisms, *Pythium ultimum*.

METHODS

Organism. The organism used was an isolate of *Pythium ultimum* Trow (provided by Dr A. P. J. Trinci, Queen Elizabeth College, London); stock cultures were maintained on oatmeal agar (Marchant, Peat & Banbury, 1967) and grown at 25°.

Growth experiments. The effect of chloramphenicol on the growth of the organism was measured in liquid culture for dry-weight production, or on a solid medium for rate of linear growth. The liquid culture medium used was that of Hendrix & Lauder (1966) at pH 7·0 without added thiamine. The rate of linear growth of the organism in the presence of chloramphenicol 100 μg./ml. was compared with the control rate on oatmeal agar in 30-cm. growth tubes at 25°.

The sensitivity of *Pythium ultimum* to increasing concentrations of chloramphenicol was ascertained by measuring the dry-weight of duplicate samples grown in stationary liquid culture in 250 ml. conical flasks at 25°. The rate of growth, in terms of dry-weight production, in the presence of chloramphenicol 100 μg./ml. was measured on duplicate samples from stationary liquid cultures over a period of seven days.

Measurement of cytochromes. The absorption spectra of the cytochromes were measured *in vivo* with a Unicam SP800 spectrophotometer. The hyphal material was placed in a cuvette and read against a scattering blank. Cotton-wool was found to give the same result as extracted hyphae as the blank and was therefore used. The extinction of the experimental material was adjusted against a single blank to give a constant extinction. The cytochrome spectra were recorded between 500–650 mμ from normal hyphae and from hyphae grown in chloramphenicol 100 μg./ml. for 6 days in liquid culture at 25°. The effect on the cytochrome spectrum was also noted when normally grown hyphae were treated with chloramphenicol 100 μg./ml. for 24 hr.

Isolation of mitochondria. The mitochondria were isolated from normal hyphae and hyphae grown in chloramphenicol 100 μg./ml. for 6 days at 25° by the following method. After harvesting and washing mycelium, the equivalent of 100–300 mg. dry wt was blended for 15 sec. at 80% maximum speed in a Vertis blendor in 5 ml. isolation medium. The isolation medium comprised 0·65 M-mannitol + 0·1 mM-EDTA in 20 mM-tris buffer (pH 6·5). All the extraction and centrifugation procedures were done at 2–4°. The blended material was centrifuged three times at 2000*g* to remove debris and then at 9500*g* to sediment the mitochondria. The mitochondrial pellet was subsequently washed with 2 ml. fresh medium and centrifuged down before being used for electron microscopy. The respiratory activity of these isolated mitochondria was not examined.

Electron microscopy. The isolated mitochondria were examined by negative-staining and thin-sectioning techniques. Two negative stains were used, 2% potassium phosphotungstate (pH 7·4) and 2% aqueous uranyl acetate. Material from mitochondrial pellets was spread on the surface of drops of negative stain and then picked upon on carbon-formvar coated grids (Parsons, 1963). For sectioning, mitochondria were fixed in 2% osmium tetroxide which was made iso-osmotic with the isolation medium by the addition of mannitol. The pellet of fixed mitochondria was washed, dehydrated in tert.-butanol mixtures (Johansen, 1940) and embedded in Araldite. Sections were

stained on the grids with 2% aqueous uranyl acetate and lead citrate (Reynolds, 1963).

Hyphae of *Pythium ultimum* grown in the presence and absence of chloramphenicol 100 μg./ml. on oatmeal agar for 4 days at 25° were fixed in the following way: acrolein (6%) in 0·1 M-cacodylate buffer (pH 7·2–7·4) for 1 hr at room temperature, followed by thorough washing and post-fixation in 1% buffered osmium tetroxide (pH 7·2–7·4, Hess, 1966). The material was again dehydrated in tert,-butanol mixtures and embedded in Araldite. All sections were cut with glass knives on an LKB ultratome, and after staining with lead citrate were viewed in a Siemens Elmiskop 1 electron microscope.

RESULTS

Growth experiments

The dose response of *Pythium ultimum* to chloramphenicol is shown in Fig. 1. This response was measured in terms of net dry wt. production after 4 days. The organism showed an increasing response up to a concentration of chloramphenicol 100 μg./ml.,

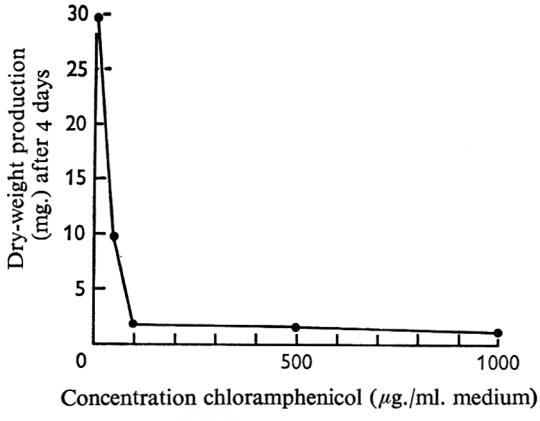


Fig. 1

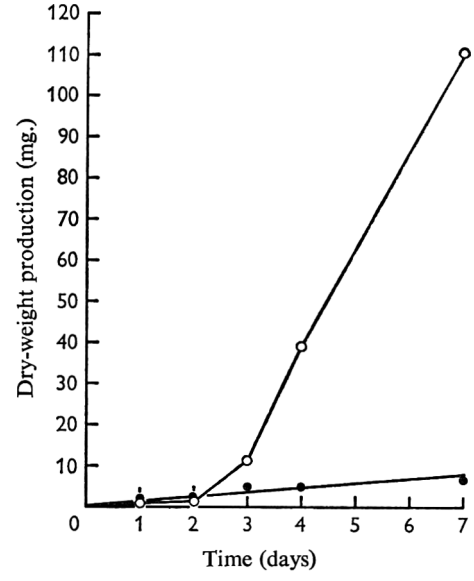


Fig. 2

Fig. 1. The effect of concentration of chloramphenicol on the growth of *Pythium ultimum*, as measured by dry-weight production after 4 days' growth at 25°.

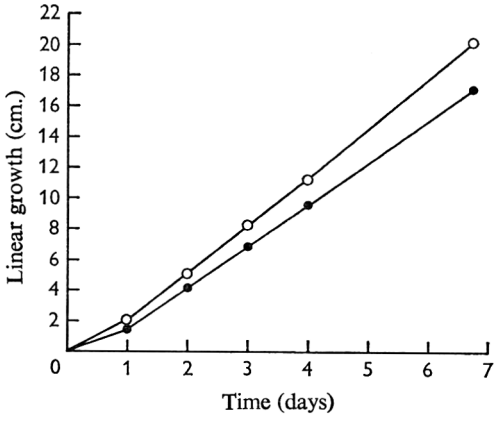
Fig. 2. The dry-weight production of *Pythium ultimum* grown in the presence and absence of chloramphenicol at 25°. O—O, control with no chloramphenicol; ●—●, with chloramphenicol 100 μg./ml.

after which further increases in dose produced little more inhibition. Because of this sharp effect at chloramphenicol 100 μg./ml. this concentration was adopted for further studies.

The results of experiments on the effect of chloramphenicol on the rate of growth of the organism are shown in Fig. 2. Duplicate dry wt determinations were done, over a period of 7 days, on mycelium grown in the presence and absence of chloramphenicol.

The control material, after a lag period, reached an exponential phase of growth and produced about 110·0 mg. dry wt mycelium in 7 days. The growth of the mycelium in the presence of chloramphenicol 100 μg./ml. remained linear over the period of the experiment and the total production of the organism after 7 days was only 6·3 mg dry wt.

The effect of chloramphenicol on the morphology and growth of *Pythium ultimum* on a solid medium was investigated in growth tubes; the distance grown along the tube was plotted against time (Fig. 3). The effect of chloramphenicol on the rate of linear growth can be seen to be far less than the effect on dry wt production. The chloramphenicol-treated mycelium on the solid medium, however, had a far lower density of hyphae and less aerial mycelium than the control. These results are comparable with the well known effect obtained when fungi are grown on low carbon source levels. Transfer of chloramphenicol-grown mycelium on to fresh chloramphenicol medium indicated that there was no adaptive mechanism in the hyphae permitting improved growth after prolonged exposure to the drug.



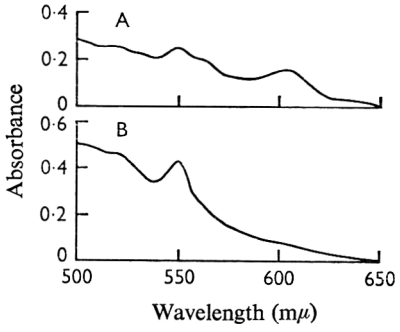


Fig. 3. Linear growth rate of *Pythium ultimum* across a solid medium at 25°. O—O, control with no chloramphenicol; ●—●, with chloramphenicol 100 μg./ml.

Fig. 4. Absorption spectra of whole mycelia of *Pythium ultimum* grown (*a*) in the absence of chloramphenicol, (*b*) in the presence of chloramphenicol 100 μg./ml.

Measurement of cytochromes

The absorption spectra of the cytochromes in normal and chloramphenicol-grown mycelium are shown in Fig. 4. The normal hyphae showed three peaks similar to those in yeast (Huang *et al.* 1966); the peak at 605 mμ represents the absorption by cytochrome aa_3, and those at 562 mμ and 550 mμ the absorption by cytochromes *b* and *c* respectively. The mycelium grown in chloramphenicol showed a complete absence of absorption peaks at 605 and 562 mμ, indicating inhibition of cytochromes aa_3 and *b*. There also appeared to be an increase in the cytochrome *c* concentration in the presence of chloramphenicol. When normally grown mycelium was treated with chloramphenicol for 24 hr there was again a decrease in cytochromes aa_3 and *b* and some evidence for the appearance of a peak at 587 mμ which Huang *et al.* (1966) ascribed to cytochrome a_1, a derivative of cytochrome aa_3. These results agree closely with those

of Huang *et al.* (1966), the only difference being the increase in cytochrome *c* in *Pythium ultimum* which was not observed in yeast.

Electron microscopy

Sections of mature but still active hyphae, from normal mycelium (Pl. 1, fig. 1) showed few differences from the hyphal apices (Marchant, Peat & Banbury, 1967). The major difference in organelle structure was in the dense mitochondrial matrix observable in mature regions of the hyphae. Such a difference may be explainable in terms of observations on isolated animal mitochondria (Hackenbrock, 1966), where it was found that freshly isolated mitochondria had a dense contracted matrix but returned to the normal *in vivo* appearance when allowed to phosphorylate. The structure of hyphae grown in chloramphenicol (Pl. 1, fig. 3) shows no difference from the normal hyphae, and even the mitochondrial membranes appear normal, despite the observation of Huang *et al.* (1966) that chloramphenicol caused the breakdown of the inner mitochondrial membrane in yeast. Sections of isolated mitochondria (Pl. 1, fig. 2), although the membranes are not well stained, show that the isolation procedure does not cause a significant alteration in the structure of the mitochondria.

A large proportion of the isolated mitochondria can also be seen to be relatively entire using the negative-staining technique (Pl. 3, fig. 6). When mitochondria isolated from normal hyphae are spread and negatively stained by the method of Parsons (1963) the inner surfaces of the cristae can be seen (Pl. 2, fig. 4) to be covered with the elementary particles reported by Fernández-Morán *et al.* (1964). When observed more closely (Pl. 2, fig. 5) these elementary particles can be seen to consist of the typical headpiece on a stalk, which is attached to a base-piece in the membrane. We were unable to show this type of elementary particle on the cristae of mitochondria from hyphae grown in chloramphenicol, despite the fact that these mitochondria appeared normal in sections. In an effort to determine whether these elementary particles were in fact absent in the presence of chloramphenicol, mitochondria were subjected to brief (10 sec.) ultra-sonic treatment before being negatively stained. It has already been established (Stasny & Crane, 1964; Green & Perdue, 1966) that prolonged severe ultra-sonic treatment is necessary to remove the particles from the membrane. The elementary particles were still visible in normal mitochondria after ultra-sonic treatment, but we were again unable to show them in mitochondria produced in the presence of chloramphenicol.

DISCUSSION

It now seems fairly certain (Green & Perdue, 1966) that the electron transport system resides in the base-pieces of the tripartite elementary particles and that some other systems are located in the stalked headpieces (Racker, Chance & Parsons, 1964). The complete inhibition of competent cytochromes aa_3 and *b* by chloramphenicol in the mitochondria is indicated by the absorption spectra obtained. If the disappearance of the stalked particles from the membrane is linked to the absence of the cytochromes it is likely that there has either been a reorganization of the whole membrane structure, absorbing the particles into the main structure, or they have become detached. It is not possible to exclude the possibility that the disappearance of the stalked particles is correlated with some other effect of chloramphenicol in the mitochondria.

The growth data suggest that a low supply of energy is available for the growth of the organism in the presence of chloramphenicol, and coupled with the structural evidence indicates a specific effect of chloramphenicol on the mitochondria. This is further borne out by the selective inhibition of cytochromes aa_3 and *b* and adds further weight to the hypothesis that the protein synthesizing system in the mitochondrion may be analagous to the system in prokaryotic cells. It is interesting to note that there is a low rate of growth which can not be inhibited even by high concentrations of chloramphenicol. *Pythium ultimum* is an obligate aerobe (Marchant & Smith, unpublished) and presumably therefore has no complete anaerobic fermentative metabolism. If this is so the residual uninhibited growth may either be supported by reorganized mitochondrial metabolism or by extra-mitochondrial processes.

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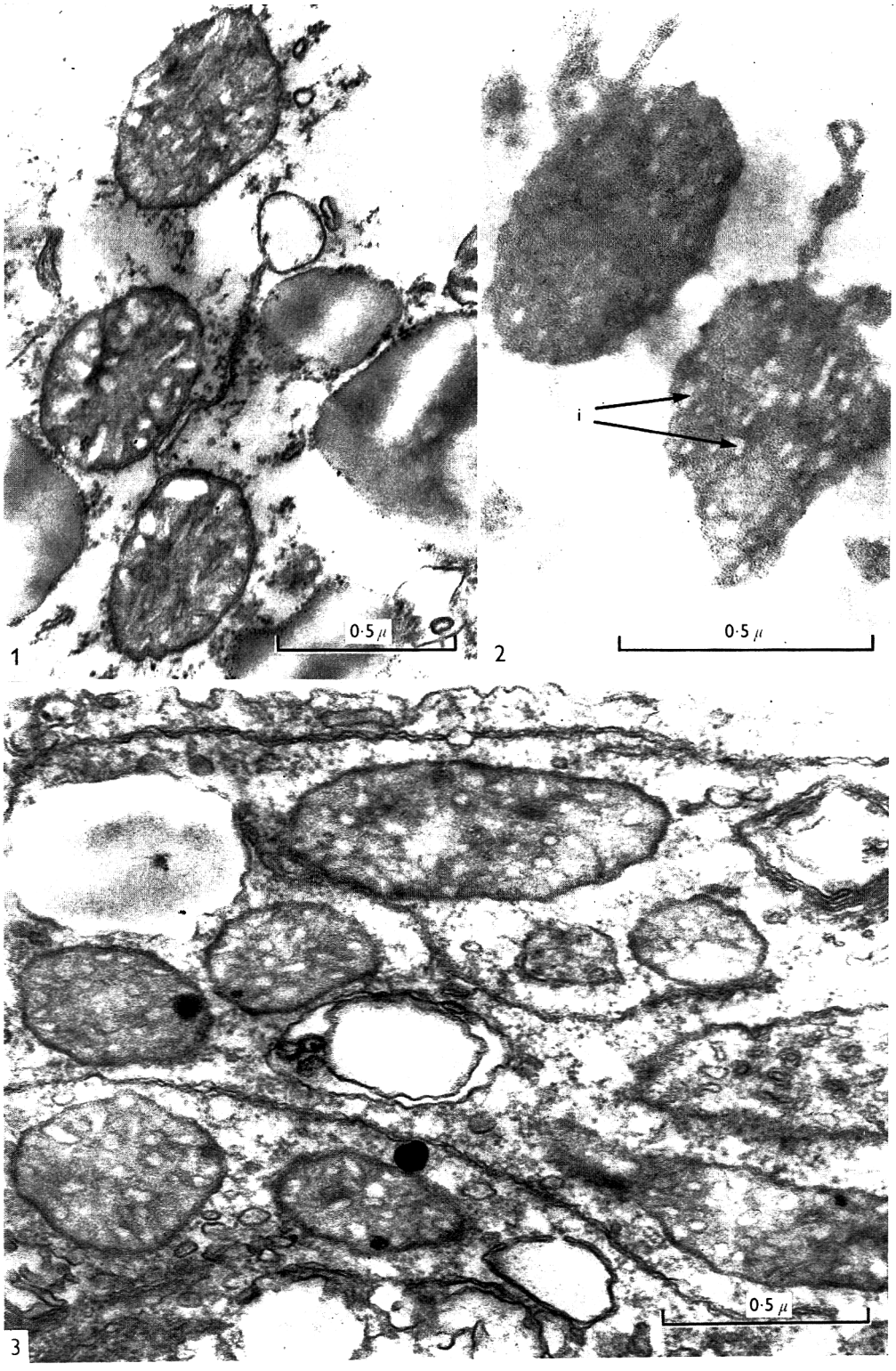

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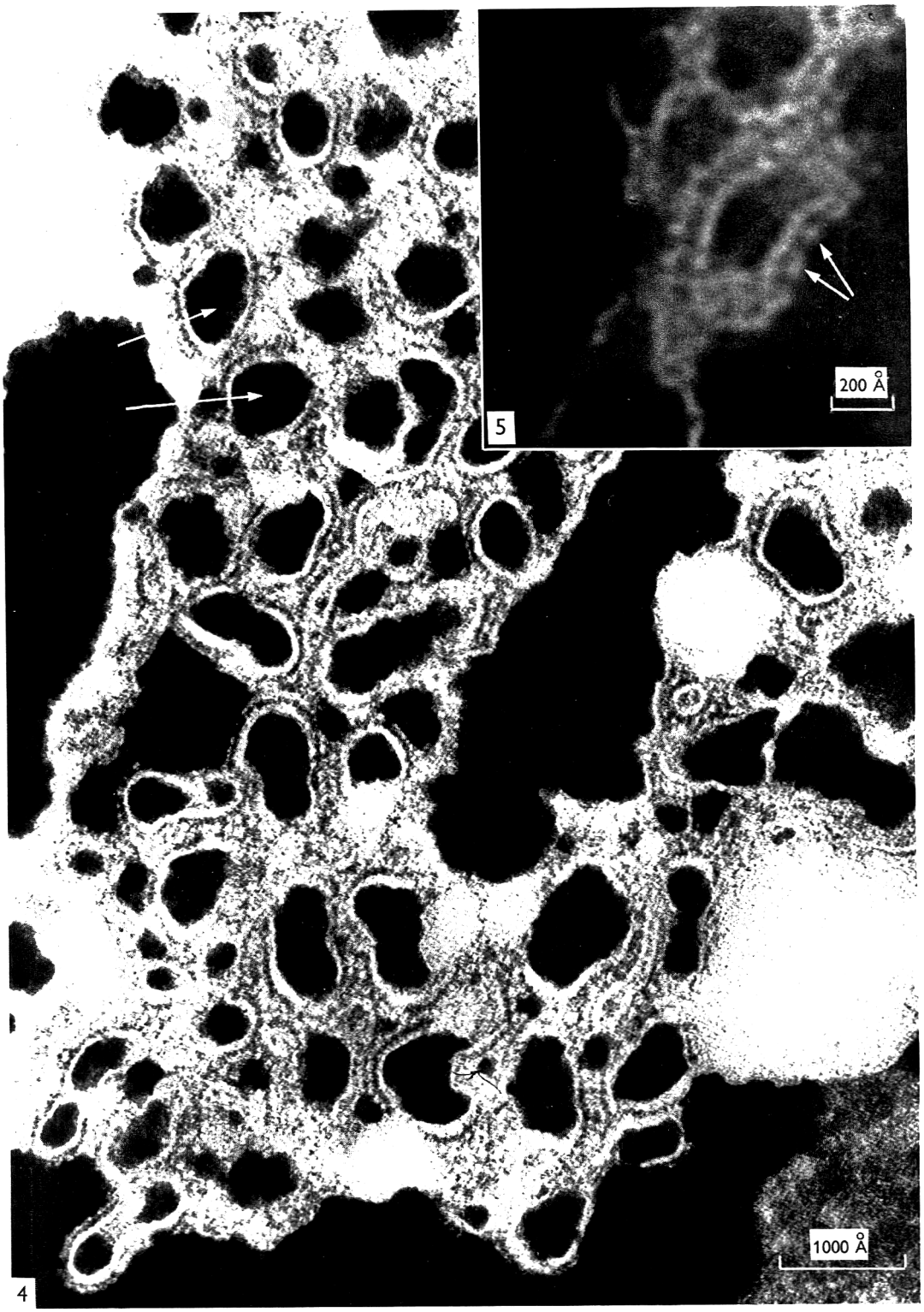
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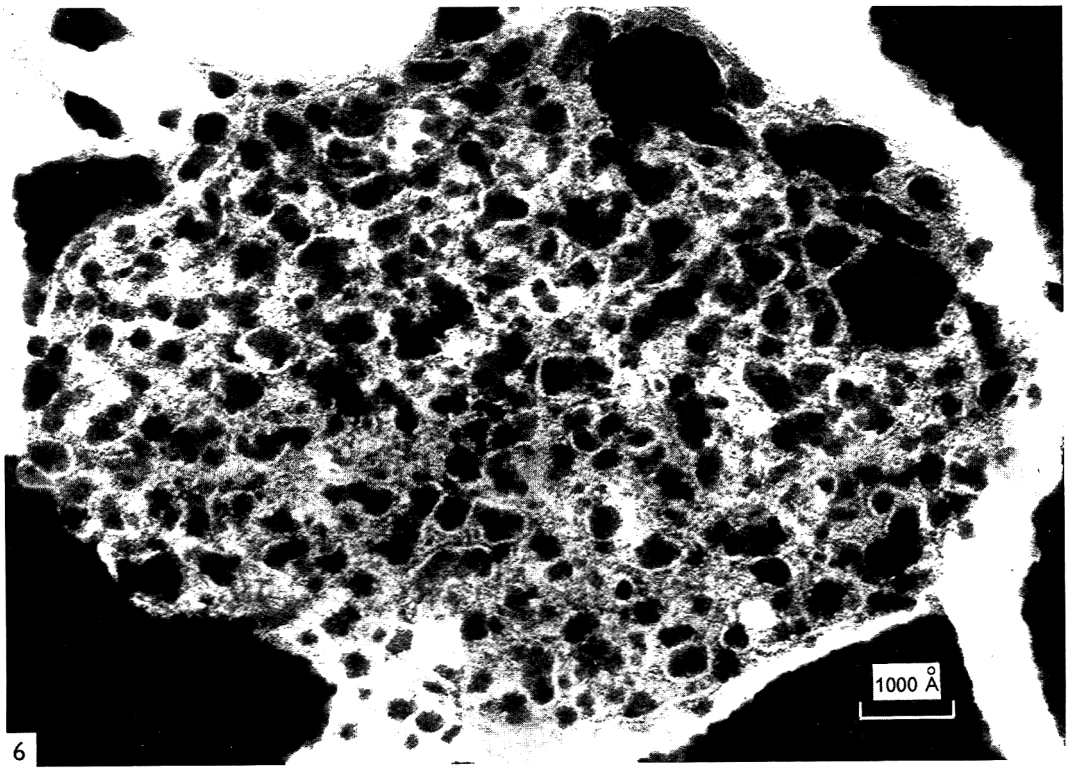
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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Thin section of a normal hypha, showing mitochondria with dense matrices. Acrolein fixation × 52,000.

Fig. 2. Thin section of isolated mitochondria from normal hyphae. Note the numerous small intracristal spaces (i). Osmium tetroxide fixation. × 75,000.

Fig. 3. A longitudinal section of a hypha grown in the presence of chloramphenicol 100 μg./ml. There is no apparent difference in mitochondrial structure from those in normal hyphae. Acrolein fixation. × 61,000.

PLATE 2

Fig. 4. Negatively stained (phosphotungstate) isolated mitochondria from normal hyphae, showing regular distribution of elementary particles and dense intracristal spaces (arrows). × 195,000.

Fig. 5. Higher magnification photograph of stalked particles (arrows) in normal mitochondrion. Negatively stained in phosphotungstate. × 480,000.

PLATE 3

Fig. 6. Negatively stained (phosphotungstate) intact normal mitochondrion. × 122,000.

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The Influence of Maintenance Energy and Growth Rate on the Metabolic Activity, Morphology and Conidiation of *Penicillium chrysogenum*

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(*Accepted for publication* 17 *August* 1967)

SUMMARY

The rates of utilization of energy-yielding substrates (glucose and oxygen) by *Penicillium chrysogenum* in glucose-limited chemostat cultures were resolved into requirements or 'rations' for growth and maintenance. The maintenance ration of glucose was almost all oxidized to carbon dioxide. Over the growth rate range 0·023–0·075 hr^{-1} only vegetative growth occurred; although the filamentous growth form predominated, the occurrence of pellets and swollen organisms increased with growth rate. At growth rates of 0·014 hr^{-1} and below, conidiation occurred and was maximal at a specific growth rate of 0·009 hr^{-1} (average doubling time 78 hr). After growth in chemostat culture the organism could be maintained in a non-growing state by supplying only the maintenance ration of glucose (0·022 g. glucose/g. mycelial dry wt/hr). When growth in the chemostat was suddenly stopped by stopping the glucose feed, the mould autolyzed; autolysis was prevented by supplying the maintenance ration of glucose. When the glucose feed rate in chemostat cultures was decreased to the maintenance ration, mycelial differentiation occurred. Differentiation involved increased hyphal vacuolation, a decreased degree of oxidation of glucose, breakdown and resynthesis of nucleic acids and conidiation. The rates at which these changes occurred were inversely related to the growth rate prior to stopping growth. For maximum conidia formation there was an optimal glucose feed rate about 0·038 g. glucose/g. mycelial dry wt/hr, that is 1·7 × maintenance ration. The maintenance ration of glucose was shown to play a role in preventing autolysis and facilitating conidiation. Electron microscope studies showed that there was no change in the observed ultrastructure of cells (except degree of vacuolation) with change in specific growth rate from near the maximum to zero. The septa of the hyphae were found to be plugged.

INTRODUCTION

The relatively slow growth rates and inconvenient growth form of filamentous fungi in submerged culture has led to their growth kinetics being neglected by comparison with unicellular organisms. Smith (1924) showed the growth of individual hyphal systems to be exponential, but it has only recently been established that the growth of fungi in submerged cultures can follow the exponential law (reviewed by Pirt, 1966).

It has also been shown that the chemostat continuous culture principle can be applied to the growth of a filamentous mould (Pirt & Callow, 1960). In the present work the chemostat method has been applied to determine the influence of growth rate on the carbon and energy requirement of *Penicillium chrysogenum*. At the same time the influence of growth rate on the morphology of the mould was investigated; there appears to be no previous study of cell morphology in relation to growth rate.

Maintenance energy. The concept of maintenance energy in bacterial cultures was reviewed by Pirt (1965). In general, metabolic activities may be resolved into growth-rate dependent and growth-rate independent components. If q = metabolic quotient (g. material metabolized/g. dry wt organism/hr), we can write

$$q = \alpha\mu + \beta, \tag{1}$$

where β = growth rate independent component and $\alpha\mu$ = growth rate dependent component (μ = specific growth rate, α is a constant).

For substrate (s) utilization in small time interval dt we can write

$$-ds = \frac{dx}{Y_g} + mx\,dt, \tag{2}$$

where Y_g = true growth yield, x = organism concentration and m = maintenance coefficient.

Hence

$$\frac{-ds}{dt} = \frac{1}{Y_g}\frac{dx}{dt} + mx. \tag{3}$$

Substitute in (2)

$$\frac{-ds}{dt} = qx, \quad \text{and} \quad \frac{dx}{dt} = \mu x$$

and we have

$$q = \frac{\mu}{Y_g} + m. \tag{4}$$

Relation (4) is a special case of (1).

In the chemostat we can vary μ, measure q and thus estimate m and Y_g by means of equation (4). So-called maintenance functions probably include turnover of macromolecules, osmotic regulation, cellular organization and special activities such as motility or production of secondary metabolites such as penicillin. In the present study we determined the specific rates of metabolism of the main substrates and products of carbon and energy metabolism (glucose, oxygen, mycelium, carbon dioxide) of a *Penicillium chrysogenum* strain at different growth rates in glucose-limited chemostats.

Morphology. A variety of environmental factors have been shown to affect gross morphology, fine structure and morphogenesis in fungi. Hydrogen ion concentration is an important factor in determining the predominance of the filamentous or pellet growth form in *Penicillium chrysogenum* (Pirt & Callow, 1959), whilst the atmosphere of incubation has been shown to play a role in the dimorphism of *Mucor rouxii* (Bartnicki-Garcia & Nickerson, 1962). Nutrition is another factor of prime importance. For instance, nitrogen exhaustion caused conidiation in *Penicillium griseofulvum* and related species (Morton, 1961) and Hawker (1939) has shown the importance of carbohydrate availability to fungal morphogenesis. The present work with *P. chrysogenum* adds growth rate to the list of parameters which can affect morphology. In morphological studies. therefore, careful consideration must be given to the conditions of growth of the organism. The practise of using fungi grown on defined media in shake flasks for morphological studies gives reproducible results, but their interpretation

is complicated by the ill-defined and transient nature of the environmental conditions in the sequential growth phases. The use of stirred culture vessels improves the homogeneity of cultures and allows precise control of factors such as pH value, aeration and nutrient supply. The chemostat type of continuous culture in addition enables control of growth rate. We are aware of only one study of mould morphology in which a chemostat was used (Pirt & Callow, 1959).

METHODS

Growth rate. Specific growth rate (μ) is used to refer to the growth rate per unit mass of organism. If x is the mass of organism and t is time (hr) then

$$\mu = (1/x)\,(dx/dt)\,(\text{hr}^{-1}).$$

The specific growth rate is related to the doubling time (t_d) by the expression,

$$\mu = (\log_e 2)/t_d.$$

Apparatus. Two-litre cultures were made in a vessel adapted for continuous cultivation of filamentous micro-organisms (Righelato & Pirt, 1967).

Cultural conditions. Medium (g./l.): glucose, 2·3 × dry wt organism required; K_2HPO_4, 6·00; NaH_2PO_4, 1·86; $MgSO_4.7H_2O$, 0·25; $CaCl_2$, 0·05; $ZnSO_4.7H_2O$, 0·02; $MnSO_4.4H_2O$, 0·02; $CuSO_4.5H_2O$, 0·005; $FeSO_4.7H_2O$, 0·10; Na_2SO_4, 1·00; disodium ethylene-diaminotetracetic acid (EDTA), 0·60; $(NH_4)_2SO_4$, 4·72; phenylacetic acid (neutralized with NaOH) 1·0. Glucose and phenylacetate, phosphates and salts solution were each autoclaved separately. Glucose was the growth-limiting substrate in this medium up to a mycelium dry wt of at least 20 g./l. The dry wt of organism did not exceed 14 g./l. medium in the work described here. The residual glucose concentration was always < 0·1 g./l. Glucose and phenylacetate were fed separately from the rest of the medium; in some batch cultures the specific glucose supply rate (g. glucose/g. mycelium dry wt/hr) was kept constant by frequent manual adjustment of the metering pumps. The pH value was controlled at 7·0 ± 0·1 by automatic addition of 2 N-NH_4OH or 2 N-H_2SO_4 and temperature was controlled at 25°. Foaming was controlled by automatic addition of polypropyleneglycol (P2000 Dow Chemical Co. (U.K.) Ltd., 105 Wigmore St, London, W. 1) at 4-hr intervals, to give a concentration of about 0·1 ml./l. culture. The air flow through the culture was adjusted to give 1 to 3 % (v/v) carbon dioxide in the effluent air. The dissolved oxygen concentration as recorded by an oxygen electrode was 70–100 % of the saturation value.

Organism. Penicillium chrysogenum WIS 54–1255 was used; it was maintained on slopes of the sporulation medium of Moyer & Coghill (1946). Spore inocula were produced on bran cultures (Whiffen & Savage, 1947) and inoculated into the germination medium of Jarvis & Johnson (1947). After germination the inoculum was transferred to the fermentor.

Analytical methods. The organism concentration was measured as equiv. mycelium dry wt after filtration of 10 ml. culture, twice washing with 15 ml. distilled water, and drying at 105–110° to constant weight. Glucose concentrations were measured by a glucose oxidase method (Blood sugar kit, Boehringer Corporation (London) Ltd., Bilton House, 54/58 Uxbridge Road, Ealing, London, W. 5). Ammonia was estimated by the microdiffusion method of Conway (1957). Mycelial nitrogen was converted to

ammonia by Kjeldahl digestion and estimated by Conway's method. Cell carbon and hydrogen analyses were kindly done by the analytical department of the Chemical Defence Experimental Establishment, Porton, Wiltshire. Total mycelial carbohydrate was estimated by an anthrone method (Trevelyan & Harrison, 1952) and mycelial protein by a biuret method (Stickland, 1951). Nucleic acids were extracted with 0·5 N-$HClO_4$ at 70° after removal of the ice-cold 0·2 N-$HClO_4$ soluble fraction. Ribonucleic acid was calculated from the ribose content of the extract estimated by the orcinol method (Umbreit, Burris & Stauffer, 1947) and deoxyribonucleic acid from the deoxyribose estimated by the diphenylamine method (Burton, 1956). Penicillin was estimated by a cup method with *Bacillus subtilis* as the assay organism.

The oxygen content of the effluent air was measured by a Paramagnetic Oxygen Analyser, Mark II (Servomex Controls Ltd., Crowborough, Sussex). Carbon dioxide output was measured by washing the effluent air with KOH in an Orsat apparatus. Acid production was measured by the accumulation of ammonia, the pH control agent, in the culture filtrate.

Light and electron microscopy. The culture samples for light microscopy were fixed in equal volumes of formal-acet-alcohol (formalin (HCHO 40 %, w/v) 13 ml. + glacial acetic acid, 5 ml. + 50 % (v/v) ethanol in water, 200 ml.). Conidia were counted by using the fixed material in a Helber chamber. The hyphae were stained with trypan blue in lactophenol (lactophenol, 100 ml. and trypan blue, 0·25 g.). The photographs were taken with a Zeiss photomicroscope, measurements of hyphal dimensions were taken from the photographs.

For observation with the electron microscope culture samples were fixed in 2 % (w/v) $KMnO_4$ in veronal acetate buffer (pH 6·5) for 1 hr at about 18°. The hyphae were dehydrated in ethanol–water mixtures and embedded in araldite. Sections were cut with an LKB ultramicrotome with glass knives and viewed in an AEI EM6B microscope.

RESULTS

The influence of growth rate on metabolic quotients

The specific rates of utilization of oxygen and glucose by *Penicillium chrysogenum* WIS 54-1255 in steady state glucose-limited chemostat cultures were measured over the growth rate range 0·014–0·075 hr^{-1} (maximum specific growth rate = 0·09 hr^{-1}). Straight line relationships of the type described by equation (4) were obtained (Fig. 1). These, together with the constant respiratory quotient (CO_2/O_2 = 0·97 ± 0·03), indicate that the glucose metabolism remained qualitatively the same over the range of growth rates investigated. Similar linear relationships between Q_{O_2} and dilution rates were described by Herbert (1958) for glycerol-limited growth of *Aerobacter aerogenes* and by Tempest & Herbert (1965) for *Torula utilis* with growth limited either by carbon or by nitrogen source.

The intercepts on the ordinates of Fig. 1 are the maintenance coefficients for glucose and oxygen, 0·12 m-moles (0·022 g.) glucose/g. mycelium dry wt/hr and 0·74 m-moles O_2/g. dry wt/hr. The only previous report of a maintenance coefficient for a filamentous mould is by Terroine & Wurmser (1922) who calculated a value of 0·11 g. glucose/g. dry wt/hr. However, their experimental method depended on decreasing the growth rate by lowering the pH value to a growth-inhibitory value. Qualitative differences in metabolism may have occurred at the different pH values,

altering both growth yield and maintenance coefficients and thus invalidating their calculations. The value for the maintenance coefficient found here for *Penicillium chrysogenum* growing aerobically on glucose at 25° is considerably lower than those found for bacteria. Pirt (1965) observed a maintenance coefficient of 0·094 g. glucose/g. dry wt/hr with *Aerobacter cloacae* under aerobic conditions in a glucose-limited chemostat at 37°. The higher coefficient of *A. cloacae* may have resulted from the higher growth temperature.

Elementary analysis of the dried Penicillium mycelium showed that carbon and hydrogen contents were unaffected by the growth rate (Fig. 2) but the nitrogen con-

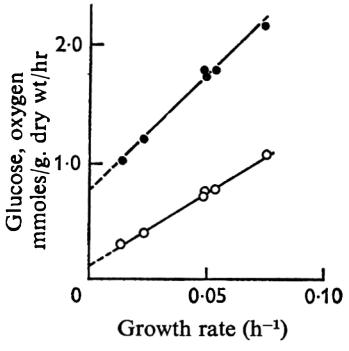

Fig. 1

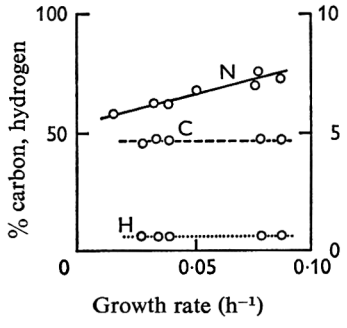

Fig. 2

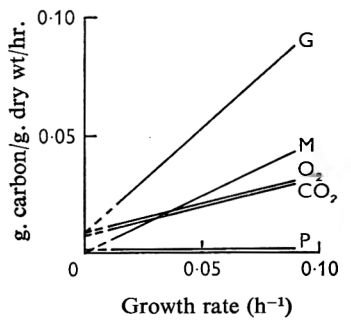

Fig. 3

Fig. 1. The relationship between specific growth rate and the specific utilization rates of glucose (○), and oxygen (●) of *Penicillium chrysogenum* WIS 54–1255 in steady state glucose-limited chemostat culture. Each point represents the mean of one steady state. 1/slope of glucose line = growth yield = 0·081 g. dry wt/m-mole glucose consumed. Intercept of glucose line on ordinate = maintenance coefficient = 0·12 m-moles glucose/g. dry wt/hr. 1/slope of oxygen line = growth yield = 0·050 g. dry wt/m-mole O_2. Intercept of oxygen line on ordinate = maintenance coefficient = 0·74 m-moles O_2/g. dry wt/hr.

Fig. 2. The relationship between specific growth rate and carbon, hydrogen and nitrogen content of *Pencillium chrysogenum* WIS 54–1255 in steady-state glucose-limited chemostat cultures.

Fig. 3. The recovery of glucose, carbon and oxygen as carbon dioxide, mycelial carbon and penicillin carbon in glucose-limited chemostat cultures of *Penicillium chrysogenum* WIS 54–1255 at growth rates from 0·014 to 0·075 hr^{-1}. G, glucose carbon; M, mycelial carbon; CO_2, CO_2-carbon; O_2, O_2 utilized as equivalent CO_2; P, penicillin-carbon.

tent increased with the growth rate. The increase in nitrogen content probably reflected an increase in ribonucleic acid (RNA) content of cells with increasing growth rate; Herbert (1958) and Schaechter, Maaløe & Kjeldgaard (1958) have observed that the RNA content of bacteria is directly related to growth rate. The true growth yield, Y_g, of *P. chrysogenum* for glucose, which was 0·081 g. dry wt/m-mole glucose and for oxygen 0·050 g. dry wt/m-mole O_2, did not vary with growth rate.

The fate of the maintenance glucose and oxygen can be seen by comparing the maintenance coefficients with the specific rates of formation of the products of carbon and energy metabolism extrapolated to zero growth rate (Fig. 3). Of the maintenance glucose-carbon 8% was recovered as penicillin, the remainder as carbon dioxide. Of the maintenance oxygen, 90% was equivalent to the carbon dioxide formed. The maintenance requirement for glucose and oxygen would thus appear to be primarily used as a source of energy.

Effect of decreasing glucose supply to the maintenance ration

If extrapolation of Fig. 3 to zero growth rate describes the glucose metabolism of non-growing mould, it would be expected that a glucose supply rate of 0·12 m-moles glucose/g. dry wt/hr would maintain the mycelium concentration constant, most of the glucose would be oxidized to carbon dioxide and penicillin would be produced at the same rate as in growing cultures. Non-growing cultures were obtained experimentally by stopping the flow of medium to steady-state chemostat cultures and supplying only glucose at the maintenance rate. Glucose supplied in this way was termed the 'main-

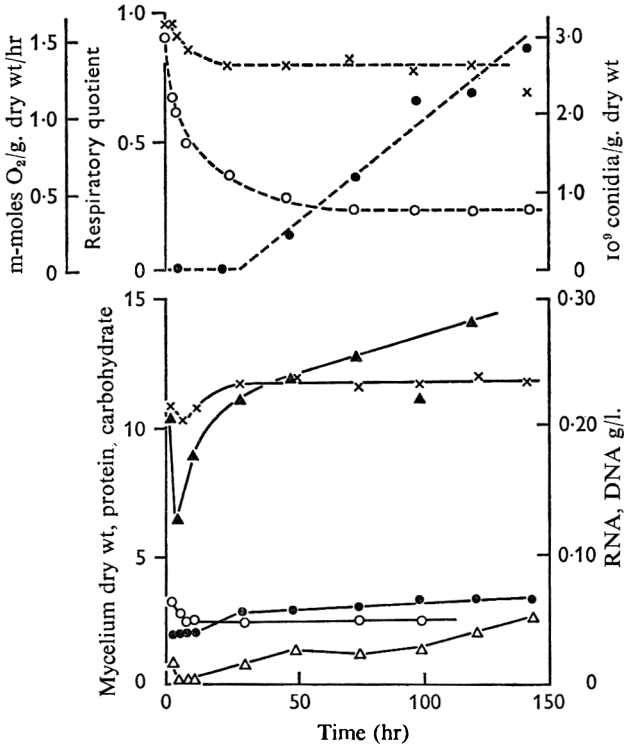

Fig. 4. Conidia production and changes in respiration and macromolecular composition of *Penicillium chrysogenum* WIS 54–1255 supplied the maintenance ration of glucose (0·022 g. glucose/g. dry wt organism/hr) after steady-state growth in glucose-limited chemostat culture at a specific growth rate of 0·051 hr^{-1}. Zero time points are the steady-state chemostat observations. Broken lines: × × ×, respiratory quotient (CO_2/O_2); ○, specific oxygen uptake rate; ●, conidia. Continuous lines: × × ×, mycelium dry weight; ○, mycelium protein; ●, mycelium carbohydrate; △, DNA; ▲, RNA.

tenance ration'. All other substrates, including oxygen, were present in excess. The maintenance ration maintained constant the mycelial dry weight and, after a slight initial adjustment, the cellular protein and carbohydrate; but the nucleic acid composition was not maintained (Fig. 4). The data of Fig. 4 indicate that there was a considerable turnover of both RNA and DNA during the ageing of non-growing maintained cultures. When the glucose supply was decreased to the maintenance ration the respiration rate fell to a lower value than that predicted (0·74 m-moles O_2/g. dry

wt/hr) for zero growth rate. The decrease in the respiration rate was accompanied by a change in the respiratory quotient (CO_2/O_2) which decreased from 0·97 to 0·72 (Table 1). Only half the glucose carbon was then recovered as CO_2 and acid began to accumulate in the medium at the rate of 0·1–0·2 m-moles H^+/g. dry wt/hr. Thus there appeared to be a qualitative change in the glucose metabolism in the maintained state. The time taken for this change in glucose metabolism to occur, as measured by the onset of acid production, was proportional to the previous growth rate of the mould. The time of the change was 10 hr after growth stopped when the previous growth rate was 0·023 hr^{-1} and 49 hr when the previous growth rate was 0·053 hr^{-1}. The penicillin synthetic activity of the mould decreased to zero after growth was stopped at a rate related inversely to the previous growth rate (Pirt & Righelato, 1967).

Glucose supplied in excess of the maintenance ration inhibited the change in glucose metabolism and at a supply rate 2·5 × maintenance ration, the metabolic quotients for oxygen, carbon dioxide, penicillin and cell material remained constant. At this glucose supply rate steady-state growth homologous with that of the chemostat culture was observed.

Conidiation, like the time of onset of acid production and the decay in penicillin synthetic activity, was related to the previous growth rate of the mould. In a culture supplied the glucose maintenance ration after growth at a specific growth rate of 0·023 hr^{-1}, $2·6 \times 10^9$ conidia/g. dry wt were counted 45 hr after cessation of growth. When the previous growth rate was 0·053 hr^{-1}, only $1·0 \times 10^9$ conidia/g. dry wt were present at 45 hr. Once the differentiation described above, involving the glucose metabolism, hyphal vacuolation, conidiation and cessation of penicillin production was complete, the culture appeared not to change further. In one culture grown initially at a specific growth rate of 0·023 hr^{-1} (residence time = 43 hr) the adaptation to the non-growing state was complete within 50 hr and the only difference observed 170 hr later was an increase in the number of conidia present. Another culture grown at 0·086 hr^{-1} (residence time = 11·6 hr) took about 150 hr to complete the ageing process. The total age of the organism at the completion of the ageing process in the former culture was about 90 hr and in the latter about 160 hr. Thus the time taken for the mould to complete the differentiation was not determined by its overall age.

The influence of the glucose metabolic rate on the rate of formation of conidia is shown in Table 1. There was an optimum glucose metabolic rate for conidia production (0·21 m-moles glucose/g. dry wt/hr), that is, about 1·7 × maintenance ration. In the absence of any glucose feed autolysis occurred without conidiation. The conidia were not observed to germinate in the culture but were subsequently found to be viable.

It is difficult to assess the viability of the hyphae of a filamentous organism such as *Penicillium chrysogenum*. Since large amounts of empty hyphal walls did not accumulate in the cultures supplied glucose at the maintenance ration and above, and the protein content of the mycelium remained constant, it is thought that most of the hyphae were actively metabolizing.

Light microscopy

Chemostat cultures. The cultures were composed of filamentous hyphae together with a few mycelial pellets; the diameter of the pellets never exceeded 1 mm. and the mean length of the hyphal filaments at a specific growth rate of 0·053 hr^{-1} was 189 ± 80 μ. The occurrence of the pellet growth form increased with growth rate, though with

Penicillium chrysogenum WIS 54-1255 it always formed only a small proportion of the total mycelial mass. At the maximum growth rate (0·09 hr^{-1}) there were about three times as many pellets as at 0·075 hr^{-1} and ten times as many as at 0·053 hr^{-1}. Pellets were not observed at growth rates below 0·04 hr^{-1}. Associated with the pellet growth habit were aberrant mycelial forms, large swollen cells up to three or four times the diameter of the normal hyphae and very closely branched hyphal systems (Pl. 1, fig. 1). The closely branched systems may be early stages in the growth of pellets. Pirt & Callow (1959) observed similar swollen cells, close branching and stromatic pellets

Table 1. *The relationship between glucose supply rate, specific growth rate, oxidation of glucose and conidiation by Penicillium chrysogenum WIS 54-1255*

Growth rate hr^{-1}	Glucose supply rate g. glucose/g. dry wt/hr	% glucose C utilized (excluding glucose recovered as cell and penicillin C) recovered as CO_2	Respiratory quotient	10^9 conidia/g. dry wt
0*	0·022	47	0·72	2·9
0·009*	0·038	71	0 90	6 5
0·014*†	0·056	72	0·94	0·6
0·023‡	0·068	82	0·95	0
0·051‡	0·133	72	0·97	0
0·075‡	0·189	63	0·98	0

* Readings taken 90 hr after cessation of chemostat growth phase at a specific growth rate of 0·051 ± 0·002 hr^{-1}.

† Steady state, exponential growth.

‡ Glucose-limited chemostat culture.

in glucose-limited chemostat cultures of *P. chrysogenum* WIS 54-1255 growing at 0·05 hr^{-1}. They found that pellets were the predominant growth form at pH values above those optimal for the production of long thin sparsely-branched hyphae. Bent & Morton (1963), also with *P. chrysogenum*, observed similar aberrant hyphae to those described above when the pH value was very near to the minimum at which mycelial growth would occur (pH = 2·15–2·35).

The apical 5–15 μ of normal filaments contained non-vacuolated protoplasm but vacuoles of various sizes were found further from the tips (Pl. 1, fig. 1, 2). A similar morphology was observed when the fungus was grown on solid media. A few hyphae in the chemostat cultures were more vacuolated than the rest; they may have represented filaments which had been in the culture longest.

Non-growing organism and conidiation. The most marked morphological change in the culture after its growth had been stopped and then supplied the glucose maintenance ration, was an increase in hyphal vacuolation; even the apical hyphal 'cells' were usually vacuolated (Pl. 1, fig. 4). The vacuolation of hyphal tips was related to the growth rate of the mould. In the non-growing cultures supplied glucose at the maintenance ration most tips were vacuolated; at growth rates of 0·014 and above most were non-vacuolated (Pl. 1, fig. 2; Pl. 2, fig. 8). Vacuole formation may play an important part in hyphal growth. Translocation of cell material from the vacuolating regions to the growing tips may allow higher rates of hyphal extension than could obtain solely by synthesis at the hyphal tips.

Another major morphological change in the maintained culture was the appearance

of phialides and conidia. Throughout the period in which glucose was supplied at the maintenance ration there was an increase in the number of these structures present in the culture (Fig. 4). The phialides were usually formed singly at the tips of hyphae (Pl. 1, fig. 3) but pairs of phialides were also common (Pl. 1, fig. 5) and occasionally groups of three were observed. Penicillia consisting of more than one phialide were more common and conidiation was more rapid when glucose was supplied at $1{\cdot}7\times$ maintenance ration. The species *Penicillium chrysogenum* forms a typical asymmetrical penicillus when cultured on solid media (Pl. 1, fig. 6, 7). The free spores (Pl. 1, fig. 3) in the culture were spherical in shape and had a mean diameter of $2{\cdot}0 \pm 0{\cdot}5\ \mu$. They were non-vacuolated and appeared to be dormant, since none were observed in the process of germination. Never more than one spore was attached to a phialide; the formation of spore chains, which is a characteristic feature of asexual reproduction under aerial conditions (Pl. 1, fig. 6, 7) was presumably prevented by the vigorous agitation of the culture. As the non-growing mould aged, the branched hyphal systems broke into smaller units and the viscosity of the culture decreased. In all cultures a few empty hyphae and a little protoplasmic debris were observed, due presumably to the shearing action of the impeller.

Electron microscopy of growing and non-growing mould

In some sections the hyphal walls appeared to be composed of two faintly divided layers of approximately equal thickness; in other sections, however, the walls appeared to consist of a single layer. An ill-defined aggregation of electron-dense material was present around the outside of some hyphae. Similar layers have been observed around the hyphae of *Neurospora crassa* (Shatkin & Tatum, 1959) and *Aspergillus niger* (Tanaka & Yanagita, 1963).

There was little difference in the thickness of the walls of hyphae cultured at the growth rates examined (Table 2). The presence of small cytoplasmic invaginations in the inner layer of the wall appeared to be a characteristic feature of the hyphae (Pl. 2, fig. 9). Tangential sections of the walls revealed that this inner layer had a reticulate appearance which indicates that the invaginations take the form of papillae rather than grooves (Pl. 3, fig. 11). The only detectable morphological difference between the walls of the maintained hyphae and of the growing hyphae was the somewhat greater prominence of the cytoplasmic invaginations of the inner layer in the maintained hyphae.

Table 2. *Penicillium chrysogenum WIS 54-1255: thickness of the walls of hyphae grown under various conditions*

			Wall thickness (mμ)	
Cultural conditions	Growth rate	Doubling time (hr)	Extreme values observed	Mean value
Chemostat culture	$0{\cdot}075\ hr^{-1}$	13	86–240	144
Chemostat culture	$0{\cdot}053\ hr^{-1}$	19	120–280	159
Culture maintained for 2 days	0	—	128–194	154
Culture maintained for 4 days	0	—	62–156	124

Several septa of hyphae from growing and non-growing cultures were sectioned through the region of their pores. Each septum was perforated by a more or less centrally placed pore which was plugged with electron-dense material. In most cases

membrane-bound electron-dense structures were present in the cytoplasm on either side of the pore (Pl. 3, fig. 12). These structures seem to be identical to the so-called Woronin bodies which Reichle & Alexander (1965) found associated with the septal pores of Fusarium spores and hyphae. Reichle & Alexander suggested that the function of these structures is to plug the pores when hyphae become damaged. If the septal pores were to become occluded soon after their formation it would most certainly decrease the amount of protoplasm lost as a consequence of shear. The manner in which the septal pores are plugged and the conditions which influence the process have not yet been investigated. Whether or not mechanical agitation enhances the process of plugging remains to be determined.

The nuclei present in the hyphae were of the usual eucaryotic type and the hyphal 'cells' were multinucleate. The mitochondria possessed few cristae and a relatively sparse endoplasmic reticulum ramified throughout the hyphae. These features appear to be typical of the cellular organization of fungal hyphae. Although a large number of sections was examined, no structures resembling dictyosomes or lomasomes were observed.

Organisms supplied glucose at the maintenance ration showed none of the morphological signs of mitochondrial and nuclear degeneration observed by Bracker (1966) in developing sporangia of *Gilbertella persicaria*. The structural features of those organelles of *Penicillium chrysogenum* appearing in electron micrographs were maintained by the maintenance ration of glucose (Pl. 3, fig. 11) However, the extensive vacuolation observed in the non-growing cultures might mean that the total number of these organelles had decreased.

Vacuoles with flocculent contents were present in the cytoplasm of some sections of growing organisms but not in others. Each vacuole was bounded by a unit membrane, the tonoplast, which had the same appearance as the protoplasmic membrane. There was a variation in the size of the vacuoles and in some cases there was more than one vacuole present per 'cell' (Pl. 2, fig. 10). In some sections diffuse, electron transparent areas were present in the cytoplasm adjacent to the hyphal walls (Pl. 3, fig. 12). These regions were never bounded by a membrane, which indicates that they were probably not associated with vacuole formation.

A considerably greater proportion of the hyphae in samples taken from the maintained culture contained vacuoles. In some cases a single vacuole occupied almost the entire area of the hyphal lumen, leaving only a narrow 200–300Å rim of cytoplasm between the tonoplast and the protoplasmic membrane (Pl. 3, fig. 13). Examination of a large number of sections failed to reveal the presence in the cytoplasm of glycogen, which usually appears as small rosettes of electron-dense particles (Revel, Napolitano & Fawcett, 1960). The absence of a carbohydrate reserve is to be expected when growth is carbohydrate-limited. A few small lipid inclusions were detected in sections of both growing and maintained hyphae, and the presence of small amounts of this reserve was also confirmed by staining fresh material with Sudan III.

DISCUSSION

The specific utilization rates (utilization rate/g. mycelium dry wt) of glucose and oxygen during glucose-limited growth of *Penicillium chrysogenum* WIS 54-1255 were related linearly to specific growth rate. They have been resolved into growth rate dependent and growth rate independent components, the latter being the substrate

requirement at zero growth rate or the substrate requirement for maintenance energy. The catabolism of glucose in the growing cultures differed from that of the non-growing mould, a greater proportion of the catabolized glucose was converted to carbon dioxide in growing cultures. From 0·014 hr^{-1} to nearly the maximum specific growth rate (0·09 hr^{-1}) steady states with homologous carbon metabolism were obtained.

Over nearly the whole of its growth rate range the mould was essentially filamentous and its morphology varied little. However, growth rate did determine the degree of vacuolation and the proportion of swollen cells and pellets in the cultures. The most important conclusion to be drawn from the electron microscope studies is that the observed ultrastructure of the cytoplasm (excluding vacuoles) did not change with the specific growth rate even down to the zero growth rate of the maintained cultures. The ribosome content of the cell would be expected to vary with the growth rate; however, ribosomes were not shown up by the permanganate fixation.

The maintenance ration of glucose was necessary to maintain mycelial dry weight, protein content and ultrastructure of the non-growing mould, but the maintenance ration did not prevent cell reorganization followed by conidiation. Glucose was essential to permit conidiation and maximum conidiation occurred at a growth rate between zero (i.e. supplied the maintenance ration of glucose) and 0·014 hr^{-1} (doubling time = 50 hr), the critical growth rate above which steady state vegetative growth occurred.

The reorganization of cell material necessary to bring about the transition from the vegetative to the conidiating state probably depends to a large extent on turnover of macromolecules. The rapid changes of cellular RNA and DNA concentrations which occurred soon after stopping the growth of chemostat cultures and supplying the glucose maintenance ration probably indicated breakdown of macromolecules and reorganization, perhaps elsewhere in the hyphae, e.g. the phialides. The glucose maintenance ration supplies energy for this process and in its absence autolysis occurs without conidiation.

Conidiation of *Penicillium griseofulvum* can be induced by transfer to nitrogen-free media with high concentrations of calcium and sugar (Morton, 1961). Morton reported that *P. chrysogenum* (ARL no. 167) remained obstinately vegetative in submerged conditions after transfer to nitrogen-free media. However, the strain WIS 54-1255 used in the present study sporulated in glucose-limited cultures and in shaken flasks in which nitrogen was the first substrate to become exhausted. In both nitrogen-exhausted media and in cultures supplied glucose at or near the maintenance ration, with ammonia and all other substrates in excess, growth was restricted to a rate at or near zero.

Our results reinforce the generally held opinion that conidiation occurs in response to conditions which severely restrict vegetative growth. The restrictions may be imposed by the exhaustion of the nitrogen sources and perhaps some other substrates whilst the energy source is still present, or by the limited availability of the energy source, other essential substrates being in excess. The biochemistry of sporulation in fungi is little understood. There is some evidence from other workers (Hawker & Hepden, 1962; Bu'Lock, 1965; Turian, 1966) and from the non-growing mould in the present study that sporulation is preceded by a qualitative change in the energy metabolism of the organism, but in no case have the biochemical changes been clearly

characterized. Our observations show that although conidiation in non-growing cultures was accompanied by a change in glucose catabolism, maximum conidiation occurred when the catabolism was like that of growing cultures.

The mechanism relating the rate of conidiation in a non-growing mould to its previous growth rate is not understood. The age of the mycelium does not appear to control the ageing processes. Unidentified metabolites have been postulated as sporulation inducers (Hadley & Harrold, 1958) and as vacuolation inducers (Park & Robinson, 1964). It is possible that such substances are produced by *Penicillium chrysogenum* in the cultures described here. Another possibility is that the time taken to break down the RNA present in the cells at the cessation of growth, and its resynthesis as a species appropriate to the new conditions, is a function of the amount of RNA in the cells, which probably increases with increasing growth rate. Whatever the cellular mechanisms involved, it is clear that the differentiation of *P. chrysogenum* during glucose-limited growth is controlled by its history and the rate of supply of glucose. Above a critical glucose supply rate giving a specific growth rate of 0·014 hr^{-1} only vegetative mould was produced. Below that value, conidiation, penicillin production and the oxidative metabolism were controlled by the rate at which glucose was supplied.

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EXPLANATION OF PLATES

Abbreviations used

c = septum; cw = hyphal wall; ct = cytoplasm; e = hyphae empty of contents; er = endoplasmic reticulum; et = electron transparent area of cytoplasm; i = wall invagination; m = mitochondrion; n = nucleus; nv = non-vacuolated hyphal tip; p = phialide; pm = protoplasmic membrane; s = spore; sp = septal plug; t = tonoplast; ts = tangential section of hyphal wall; v = vacuole; vt = vacuolated hyphal tip; w = Woronin body.

PLATE 1

Light micrographs of *Penicillium chrysogenum* WIS 54-1255

Fig. 1. Mould taken from a glucose-limited chemostat culture at a specific growth rate of 0·053 hr^{-1}. Showing separately branched filaments with few vacuoles and some swollen abberrant cells.

Fig. 2. Mould taken from a glucose-limited culture growing at a specific rate of 0·053 hr^{-1}. Showing the distribution of vacuoles in a hypha.

Figs. 3–5. Mould from a non-growing culture supplied only the maintenance ration of glucose for 45 hr. The hyphae are considerably more vacuolated than those in figs. 1–2. The filaments have a typically banded appearance after they have been stained with trypan blue. In each 'cell' there is a large central vacuole. Some hyphae have formed phialides at their tips and free spores may be detected in the culture.

Figs. 6–7. Sporing heads of *P. chrysogenum* taken from a culture grown on 0·1 % Difco malt extract, 2 % agar.

PLATE 2

Light and electron micrographs of *Penicillium chrysogenum* WIS 54–1255

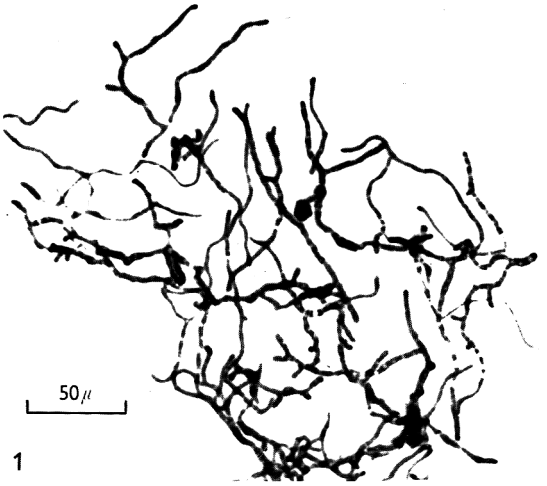
Fig. 8. Light micrograph of mould taken from a glucose-limited culture growing at a specific rate of 0·014 hr^{-1}. Showing hyphae less vacuolated than those of the non-growing mould.

Figs. 9–10. Electron micrographs of hyphae from a glucose-limited chemostat culture growing at a specific rate of 0·053 hr^{-1}. Showing a section through a plugged septum, mitochondria, nuclei, vacuoles and endoplasmic reticulum.

PLATE 3

Electron micrographs of *Penicillium chrysogenum* WIS 54–1255

Figs. 11–13. Electron micrographs of hyphae from a non-growing culture supplied only the maintenance ration of glucose for 96 hr. Showing sections through a plugged septum with the associated Woronin bodies (fig. 12) and through a highly vacuolated part of a hypha (fig. 13).



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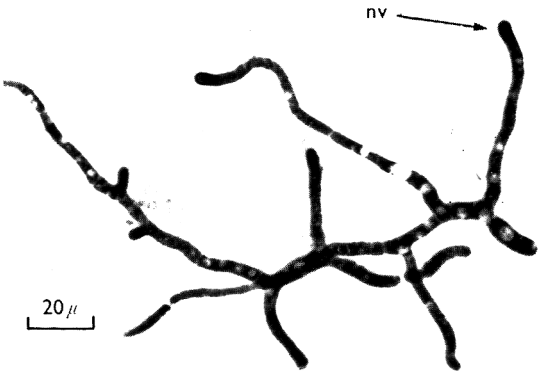

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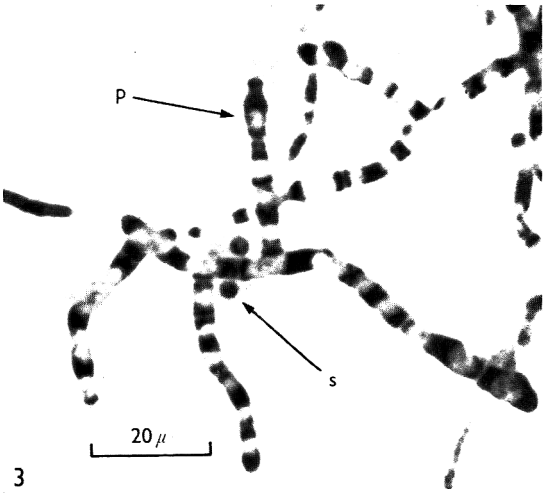

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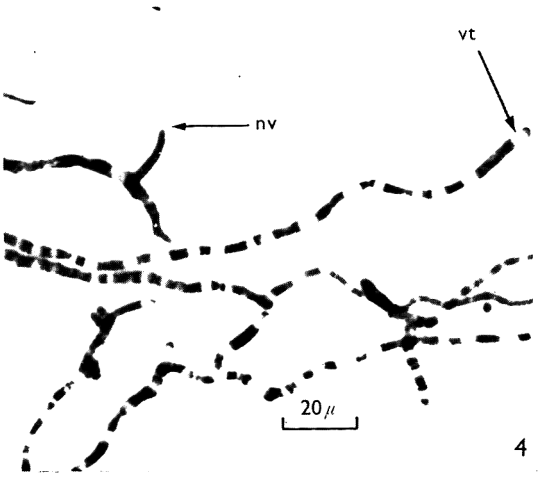

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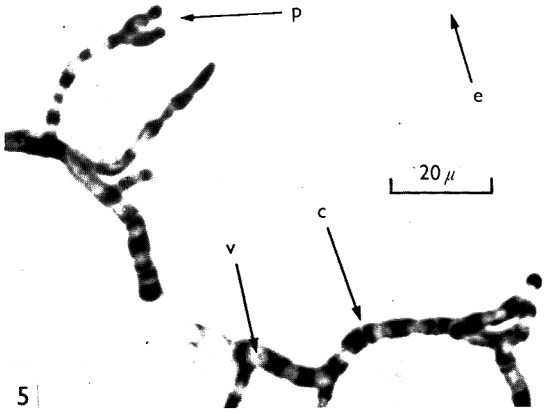

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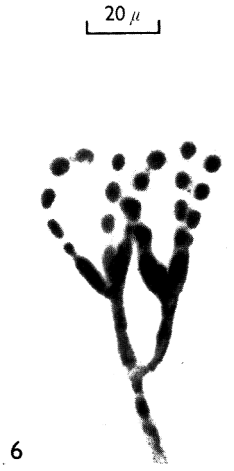

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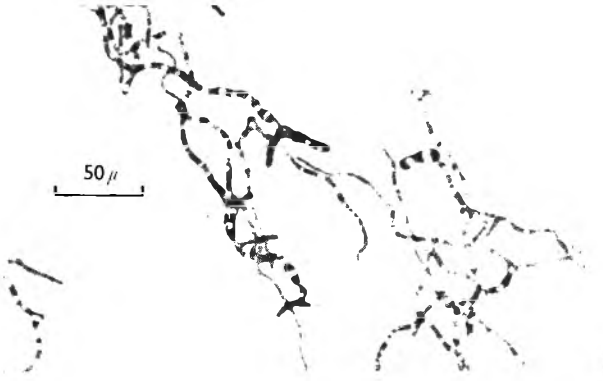

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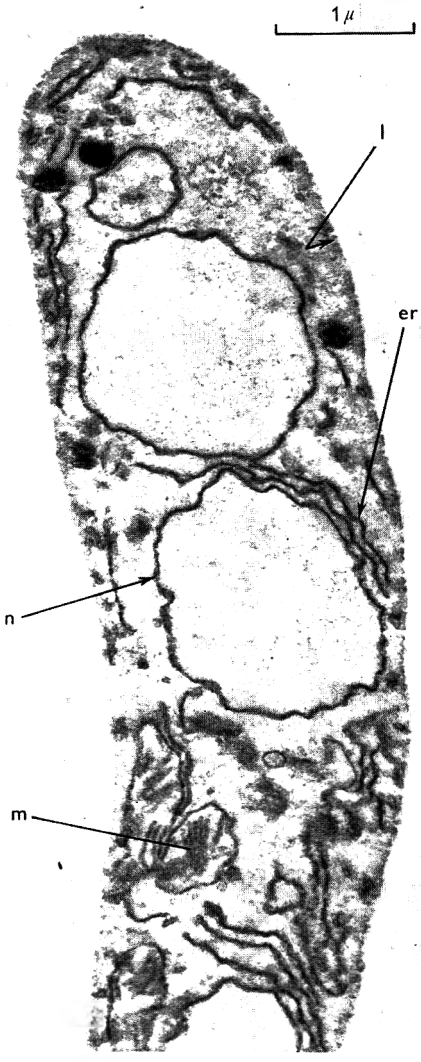

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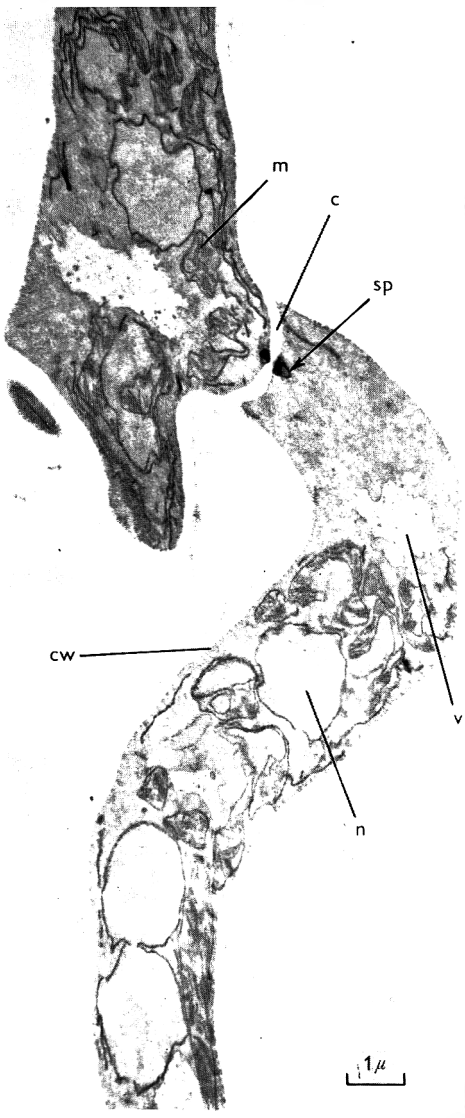

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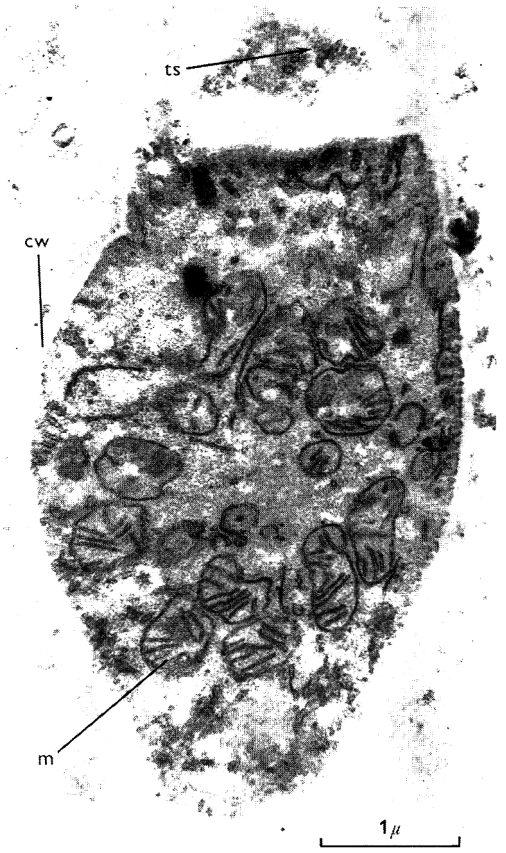

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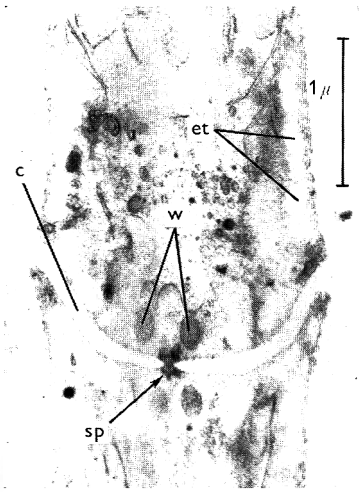

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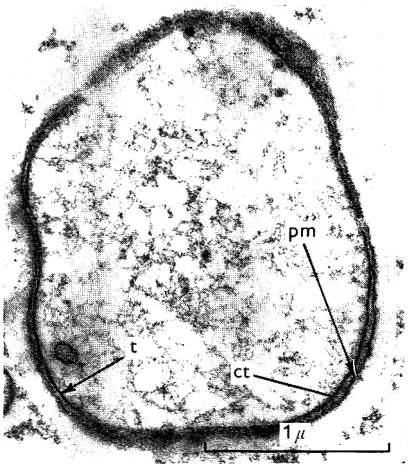

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Biological Studies on Free Endotoxin and a Non-toxic Material from Culture Supernatant fluids of *Escherichia coli* 078 K 80

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SUMMARY

A description is given of the biological properties of two substances produced extracellularly by a strain of *Escherichia coli* 078 K 80, grown in aerated liquid culture. One of the materials was of high molecular weight and possessed the properties of endotoxin extracted from whole organisms by chemical methods. In mice this material was toxic, induced non-specific resistance to infection with *Salmonella typhi*, necrotized Sacroma 180, and elicited the local and general Shwartzman reactions. In rabbits it was strongly pyrogenic, dermonecrotic, and induced formation of precipitating antibodies. Heat, chemicals and enzymes did not affect its toxicity, but acid hydrolysis rendered it non-toxic. We have called this substance 'free endotoxin'. The other material was of lower molecular weight and apart from inducing non-specific resistance to infection with *S. typhi* in mice, it had none of the properties of the endotoxin.

INTRODUCTION

Endotoxin can be extracted from Gram-negative organisms by simple but biologically drastic procedures. These include extraction with trichloroacetic acid at 4° (Boivin & Mesrobeanu, 1933), with aqueous phenol at 65–68° (Westphal, Lüderitz, Eichenberger & Keiderling, 1952) with aqueous ether at 6–12° (Ribi, Haskins, Landy & Milner, 1961) and with water at 80° (Roberts, 1966). Such chemically-extracted endotoxins are toxic, pyrogenic, provoke non-specific resistance to infection, cause dermal and tumour necrosis and elicit the local and general Shwartzman reactions.

The term 'free endotoxin' (Crutchley, Marsh & Cameron, 1967), was introduced to describe material found free in aerated liquid cultures of several species of Gram-negative bacteria and possessing the properties of chemically-extracted endotoxin. Treatment of the supernatant fluids of such cultures with ammonium sulphate yielded a precipitate which, after purification on diethylaminoethyl cellulose and Sephadex G-100 was found to contain two main fractions, one indistinguishable from endotoxin and the other a lower molecular weight non-toxic material which lacked most of the biological properties of endotoxin. This latter fraction we have tentatively identified (Marsh & Crutchley, 1967) as native hapten, or as it is now called, native protoplasmic polysaccharide (Rudbach *et al.* 1967). The present paper describes the biological properties and serological relationships of these two fractions: free endotoxin and the non-toxic material from culture supernatants of *Escherichia coli* 078 K 80.

METHODS

Organisms. Most of the experiments were done with a strain of *Escherichia coli* serotype 078 K 80. Other serotypes of *E. coli* and strains of *Pasteurella multocida*, *Serratia marcescens*, *Vibrio cholerae*, and *Salmonella typhi* were also used. All these organisms are kept as freeze dried cultures in the Culture Collection of the Wellcome Research Laboratories. For everyday use strains were maintained on Dorset's egg medium. Concentrations of suspensions of organisms were measured by using Brown's Opacity Tubes (Burroughs Wellcome & Co.) or a Hilger spectrophotometer (model H810.301).

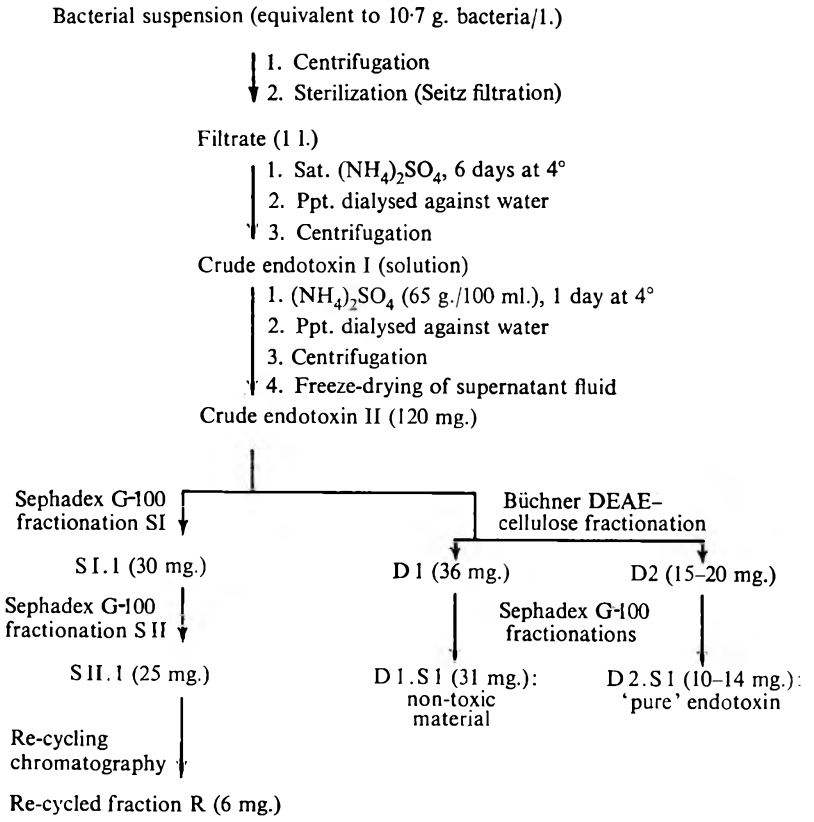

Fig. 1. The preparation and purification of endotoxin and non-toxic fractions from *Escherichia coli* 078 K 80. Yields of freeze-dried salt-free fractions per litre of sterile culture fluid are given in brackets after the appropriate fractions (from Marsh & Crutchley, 1967).

Medium. The casein sucrose yeast-extract medium used (D. C. Edwards, unpublished) was described previously (Marsh & Crutchley, 1967); it will be referred to as casein sucrose.

Growth of Escherichia coli 078 K80 and isolation of material from culture fluids. This was described previously (Marsh & Crutchley, 1967). A flow diagram for the isolation of free endotoxin and non-toxic material is shown in Fig. 1.

Growth of organisms. Escherichia coli serotypes other than 078 K 80 and other

Gram-negative bacteria were grown in casein sucrose medium for 24 hr at 37° in 250 ml conical flasks shaken rapidly to provide aeration. Seitz-filtered cell-free culture fluids were tested as such, or as crude ammonium sulphate precipitates prepared as in the first stages of Fig. 1.

Preparation of endotoxin from organisms. Chemically-extracted endotoxin was prepared from bacteria grown on casein sucrose medium solidified with agar. The extraction methods of Westphal *et al.* (1952), and of Ribi *et al.* (1961) were used.

Biological tests

W-Swiss mice were used throughout. The rabbits were of miscellaneous breeds.

Mouse lethality. This was determined in mice (20 g.) by the method of Haskins, Landy, Milner & Ribi (1961). LD50 values were calculated by the method of Reed & Muench (1938).

Non-specific resistance to infection. Groups of mice (13–15 g.) were injected intraperitoneally with increasing doses of the different preparations under test. Twenty-four hr later they were challenged by the same route with approximately 3LD 50 doses of *Salmonella typhi* TY 2 in saline (Rowley, 1955). ED 50 values were calculated by the method of Haskins *et al.* (1961).

Pyrogenicity in rabbits. The method of Haskins *et al.* (1961) was used.

Tumour necrosis. The method of Haskins *et al.* (1961) was used, but with Sarcoma 180 instead of Sarcoma 37. Tumours were sectioned and examined visually for necrosis. TD 50 values were not measured.

Shwartzman reactions

(a) *Local.* Groups of five mice were injected subcutaneously with endotoxin, 18 hr later an intravenous challenge of endotoxin was given and the skin reactions read after 8 hr (Homma, 1952).

(b) *General.* Groups of four mice were given intravenous injections of increasing different amounts of endotoxin; 6-8 hr later the members of each group were challenged intravenously with the same amount of endotoxin as they had received initially. Shock, the presence of kidney damage, and deaths were recorded.

Serological relationships of fractions

These were examined in 1% agar plates (Oxoid, Ionagar No. 2), containing 0·5% phenol as a preservative, by Ouchterlony (1949) double-diffusion technique. Antisera were prepared in horses and rabbits by the intravenous injection of formalin-killed cells of *Escherichia coli* 078K80. In addition, antisera against both the crude endotoxin II and the non-toxic material were prepared in rabbits by injecting them subcutaneously with the substances incorporated in an oil emulsion. Precipitin lines were allowed to develop for 72 hr at room temperature and the diffusion patterns photographed, with dark field illumination.

RESULTS

The injection of endotoxin into guinea pigs causes hypothermia and diarrhoea (Olitzki, Avinery & Koch, 1942). Similar effects were noted when sterile culture fluids of Gram-negative bacteria, grown in aerated casein sucrose medium, were injected parenterally into mice. Rectal temperatures of these animals fell from about 101 °F

(38.5 °C) to below 88 °F (31 °C). Severe diarrhoea started 10–15 min. after injection. The body temperatures remained low for as long as 12 hr, whereas the diarrhoea lasted for only 2 hr. These findings suggested that culture fluids from aerated cultures contained endotoxin which we propose to call 'free endotoxin', to distinguish it from material extracted from whole bacteria by chemical methods (Crutchley *et al.* 1967).

Free endotoxin was found in culture fluids of *Vibrio cholerae*, *Vibrio el Tor*, *Salmonella typhi*, *Escherichia coli*, and *Serratia marcescens*. These all grew to a population density of about 10^{11} organisms/ml. in aerated casein sucrose medium. *Pasteurella multicoda* also produced free endotoxin but had special growth requirements and only grew to 10^{10} organisms/ml.; little free endotoxin was found in static cultures of these organisms grown in the same media, where growth reached about 10^{9} organisms/ml.

In addition to free endotoxin, non-toxic material of lower molecular weight was present in culture fluids of *Escherichia coli* serotype 078 K 80 (Marsh & Crutchley, 1967). This substance was similar to native hapten described by Anacker *et al.* (1964) or as it is now called, native protoplasmic polysaccharide (Rudbach *et al.* 1967). This had none of the properties of endotoxin except its ability to induce non-specific resistance to *Salmonella typhi* infection in mice.

Origin of free endotoxin and non-toxic material. The presence of these materials in aerated culture fluids suggested that they were released by the lysis of the organisms since it is widely held that endotoxin is a cell-wall constituent of Gram-negative bacteria. We have prepared phenol + water extracts from *Escherichia coli* 078 K 80 organisms grown in aerated casein sucrose medium and on the same medium solidified with agar, and found the same amounts of endotoxin in both. However, the supernatant fluid from the liquid aerated culture contained, in addition, as much endotoxin again as could be extracted from the bacteria. This result suggested that free endotoxin might arise from metabolic overproduction during aerated growth rather than by the lysis of the bacteria.

Biological properties of endotoxin and the non-toxic material

All the materials tested were from culture fluids of *Escherichia coli* serotype 078 K 80 grown in aerated casein sucrose medium. Preparation of free endotoxin and the non-toxic fraction was described by Marsh & Crutchley (1967) and the nomenclature is that used in Fig. 1.

Lethality of free endotoxin, chemically-extracted endotoxin and non-toxic material. LD50 values for the mouse are shown in Table 1. Purified free endotoxin (D2S1) was as toxic as chemically-extracted endotoxin, and the fraction D1S1 was non-toxic. The latter was, moreover, non-toxic for guinea-pigs even at a dose of 10 mg./animal. The toxicities of fractions S 1.1 and R indicated that free endotoxin was removed during chromatography of crude material on Sephadex G-100 and that the non-toxic material had accumulated.

Non-specific resistance to infection. All endotoxin samples elicited this phenomenon so characteristic of endotoxin. The non-toxic material was also effective. ED50 values are shown in Table 1.

Pyrogenicity of rabbits. The FI40 values (amounts of material giving an area of 40 cm.^{2} under a fever curve) are shown in Table 1.

Dermal and tumour necrosis. Severe lesions were caused in rabbits injected subcutaneously with either free or chemically-extracted endotoxin. They arose 2–3 days

after the injection of 10 μg. endotoxin. No reaction followed repeated injections of a 1 mg. dose of the non-toxic material.

All endotoxin preparations caused necrosis of Sarcoma 180 in mice. A dose of 1 μg. free endotoxin/mouse induced necrosis; on the other hand, 600 μg. of the non-toxic material was inactive. Shear endotoxic lipopolysaccharide prepared from culture fluids of *Serratia marcescens* and known to cause tumour necrosis, was used as a control preparation (Shear & Turner, 1943).

Table 1. *Some biological properties of chemically extracted endotoxin, free endotoxin, and non-toxic material from Escherichia coli 078K80*

LD 50, ED 50 and FI 40 (the amount of material giving an area of 40 cm^2 under a fever curve) values were determined by the methods of Haskins *et al.* (1961).

Stage of purification	Fraction*	LD 50 (μg.) per mouse	ED 50 (μg.) per mouse‡	FI 40 (μg.) per kg. (rabbit)
Ammonium sulphate precipitation	Crude endotoxin II	370	0·016	17
Preliminary Sephadex G-100 chromatography	SI.1	484·2	0·150	15·6
	R	> 1000	N.D.†	N.D.
DEAE cellulose chromatography	D1	> 1000	0·070	> 600
	D2	N.D.	0·014	N.D.
Followed by				
Sephadex G-100 chromatography	D1S1	> 600	0·007	$> 6·25 \times 10^3$
	D2S1	241	0·004	9·5
Purified phenol extracts	P1	595	0·100	11
	P2	N.D.	0·320	20·7
Purified ether extract	E	550	0·040	19

* All fractions are as in Fig. 1, other than P 1 and P 2 which were purified fractions of a phenol + water extract and E which was a pure aqueous ether extract from *Escherichia coli* 078K80.

† Not determined.

‡ ED 50 is the amount of material protecting 50% of mice in a group.

Table 2. *Local Shwartzman reactions produced by purified free endotoxin (fraction D2S1)*

Groups of five mice were injected intradermally with the sensitizing dose and challenged intravenously 18 hr later with 50 μg. of fraction D2S1. Results were read on the inner skin surface 8 hr later.

Sensitizing dose (μg.)	Reaction to challenge* (50 μg. of D2S1 intravenously)
10	2±, 3−
20	2+, 3±
40	5+

* No. of mice reacting and extent of reaction: + = positive; ± = weak positive; − = negative.

Shwartzman reactions. Fraction D2S1 (pure free endotoxin) was tested. (*a*) *Local.* A haemorrhagic lesion developed at the site of the primary injection. Results are shown in Table 2. (*b*) *General.* Death occurred almost immediately on challenge when both the primary and challenge injections were greater than 4 mg./mouse. Smaller doses resulted in a severe spasm followed by death within 12 hr; the least amount producing this effect was 2 mg./mouse for both primary and challenge doses. In shocked animals,

kidney damage and exudation of blood into the peritoneum was observed. Far larger amounts of endotoxin were required to elicit these reactions than were required to produce other effects of endotoxin, such as pyrogenicity or non-specific protection against infection.

Serological relationships between endotoxins and the non-toxic material. Hyperimmunization of rabbits with repeated subcutaneous injections of crude endotoxin II fraction (Fig. 1) yielded an antiserum giving precipitin lines with all samples of endotoxin prepared from *Escherichia coli* 078 K80 whether extracted from bacteria or isolated from culture fluids. This antiserum, however, did not react with the non-toxic material. Nor did this latter material stimulate the production of precipitating antibodies in rabbits and in this respect it resembled the hapten described by Anacker *et al.* (1964) and Rudbach *et al.* (1967). All the endotoxin fractions, and the non-toxic material gave a common precipitin line with antisera prepared by hyperimmunization of rabbits and horses with whole bacterial cells. A multiplicity of precipitin lines was produced against rabbit antiserum, but against antiserum prepared in horses only a single line was observed. This single precipitin line between hyperimmune horse antiserum and endotoxin is almost type specific for *E. coli* strains. Horse antiserum prepared against whole bacteria of five common animal enteropathogens gave a clear preciptin line against endotoxin derived from the immunising serotype. At low concentration the reaction was fully specific and has been used as a basis for the assay of endotoxin from a known serotype for which horse antiserum was available.

Stability of free endotoxin. Endotoxins are so toxic that it is not possible to inject them in other than very small amounts to invoke immunity either against themselves or against the organism from which they were derived. Attempts were made to detoxify free endotoxin, the extent of detoxification being assessed by the loss of ability to cause hypothermia and diarrhoea in mice. Endotoxin proved resistant to exposure at 100° for 60 min. at pH 7·5 and it also resisted 0·5% neutral formalin for 30 min, at 100 ° and 8 weeks at 37°. It was unaffected by freezing and thawing from −70° to 50° fifty times. Hydrolysis by N-hydrochloric acid at 100° abolished toxicity and antigenicity.

Kim & Watson (1964), and Badakhsh & Foster (1966) reported the detoxification of chemically-extracted endotoxin with papain and lysoyzme, respectively. We were unable to detoxify free endotoxin with lysozyme. Equally inactive were: β-glucosidase, β-galactosidase, cellulase, β-amylase, Mylase-P (a mixture of carbohydrases from *Aspergillus oryzae*), chymotrypsin, trypsin, pepsin and chitinase. Free endotoxin was subjected to high voltage paper electrophoresis (Gross, 1961) after treatment with lysozyme and chitinase. No free carbohydrates could be found after this treatment.

DISCUSSION

Although endotoxin extracted chemically from whole bacteria has been extensively studied, little is known about endotoxins found free in culture fluids. Shear & Turner (1943) studied the tumour-necrotizing ability of their cell-free endotoxic lipopolysaccharide. Todd, Shaw, Blain & Boyle (1958), showed that bacteria grown in inorganic media liberated endotoxin into the culture fluid and, Taylor, Knox & Work (1966) showed that a lipopolysaccharide endotoxin was released into cultures by a lysine-requiring mutant of *Escherichia coli*. Our own results showed that in aerated

liquid cultures of *E. coli* 078 K 80, large amounts of endotoxin were liberated into the culture fluid, together with a non-toxic entity which could be separated on DEAE cellulose and purified using Sephadex G-100 (Marsh & Crutchley, 1967). The origin of these substances in culture fluids is uncertain, but it seems unlikely that they result from leaching or lysis of the bacteria during growth. Taylor *et al.* (1966) showed that their lipopolysaccharide was excreted by a rough lysine-dependent mutant when lysine was deficient in the medium, whereas the *E. coli* 078 K 80 (used in the present work) was a smooth ovine enteropathogen without any unusual requirements. As Taylor *et al.* (1966) found, the endotoxin in the culture fluid appeared to be derived by excretion from the bacteria rather than by lysis or leaching. The non-toxic fraction probably arose similarly, although its relation to endotoxin is not yet established (Marsh & Crutchley, 1967). The purified free endotoxin separated from culture fluids of *E. coli* 078 K 80 possess all the properties of endotoxin extracted from the bacteria by chemical procedures.

The non-toxic low molecular weight material (Fraction DIS1) has some properties in common with the hapten (native protoplasmic polysaccharide) described by Anacker *et al.* (1964) and Rudbach *et al.* (1967). Apart from its ability to induce resistance to *Salmonella typhi* infection in mice (and to act as an adjuvant for diptheria and tetanus toxoids; P. Knight, personal communication) properties not shared by native hapten (native protoplasmic polysaccharide) it has none of the biological properties of endotoxin.

While neither the non-toxic preparation nor native hapten (Anacker *et al.* 1964) stimulated the formation of precipitating antibodies in rabbits, the non-toxic material, unlike native hapten, gave precipitin lines against horse and rabbit antisera prepared by immunisation of animals with whole bacteria. This suggests that the non-toxic material is combined in the organism with substances which may allow it to act as an antigenic determinant. These differences may be a reflection of the mild methods used in preparing the non-toxic material as compared with the vigorous methods employed by Anacker *et al.* (1964) and Rudbach *et al.* (1967). There may be a relationship between our non-toxic material, native hapten, and other low molecular weight isolates such as the acidic polysaccharides described by Westphal *et al.* (1964). These seem to possess both some of the properties of endotoxin and of native hapten.

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The Cell Wall of *Prototheca zopfii*

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SUMMARY

Cell walls of *Prototheca zopfii* KRUGER 236/65 were prepared by differential centrifugation after disruption of the organisms in a French press. The isolated walls were free from whole organisms and subcellular particles as shown by examination by light and electron microscopy. A quantitative analysis of the walls was made with respect to total nitrogen, phosphorus, ash and lipid. Paper chromatography of hydrolysates of walls showed glucose, mannose, glucosamine and twelve amino acids. A quantitative amino acid analysis was made by ion-exchange chromatography; trace amounts of several other amino acids were detected. The structure of the walls was studied before and after treatment with NaOH and a variety of hydrolytic enzymes. The results are compared with those obtained by other workers with various species of Chlorella, Aspergillus and yeasts. The results are discussed with reference to the taxonomic position of *Prototheca zopfii*.

INTRODUCTION

Prototheca zopfii is a unicellular colourless organism which lives heterotrophically and has been classified as an achlorotic relation of the genus *Chlorella* on the basis of morphology and life cycle (Beijerinck, 1904; Chodat, 1913; Printz, 1927). A comparison between the nutrition and metabolism of artificially produced colourless mutants of Chlorella and Prototheca was made by Ciferri; while Prototheca required added thiamine for growth, the green forms and their colourless mutants were able to synthesize thiamine (Ciferri, 1957). The colourless mutants of Chlorella, like Prototheca no longer have the autotrophic mechanism of CO_2 fixation and incorporate CO_2 by carboxylation of phosphoenolpyruvate (Ciferri & Sala, 1962; Lloyd & Callely, 1965). Ciferri concluded that such differences as exist between the organisms studied might be explained if some changes had occurred during the evolution of Prototheca from the colourless forms of Chlorella. An alternative explanation which he considered was that Prototheca was not an alga but an ascomycete with ascospores and without vegetative stages of reproduction, as was originally proposed by Saccardo & Traverso (1911).

Since cell-wall composition has been used as a taxonomic criterion in recent years it was decided to examine the cell wall of Prototheca in the light of recent investigations on walls from Chlorella (Northcote, Goulding & Horne, 1958; Punnett & Derrenbacker, 1966), Aspergillus (Johnston, 1965) and yeast (Korn & Northcote, 1960). It was also hoped that such a study might provide information for the preparation of protoplasts of this organism. A preliminary report of the results obtained has been published (Turner & Lloyd, 1966).

METHODS

The organism used in this work was a culture of *Prototheca zopfii* KRUGER 263/65 isolated by E. F. Pringsheim, obtained from the Culture Collection of Algae and Protozoa at Cambridge University. The organism was maintained and grown with acetate as sole carbon source as previously described (Callely & Lloyd, 1964). The cultures were harvested by centrifugation at 1500*g* for 10 min. and washed twice with distilled water. Cell breakage was usually done by two passages through a French press (Milner, Lawrence & French, 1950) at 2° under a pressure of 37,000 lb./in.^{2}; this method gave virtually complete disruption of the organisms. Large pieces of wall for electron microscope examination were prepared after breakage in a French press at 20,000 lb./in.^{2}.

Cell wall preparation. The broken cell suspension was diluted with distilled water and centrifuged at 800*g* for 10 min. in an MSE '17' centrifuge (6 × 250 ml. head). The supernatant fluid, consisting of all particles and soluble fraction, was discarded and the residue (cell walls and contaminating particles) was suspended in 200 ml. tris buffer (2 g./l.; pH 7·0) and 0·1 ml. Tween 80 added. The suspension was thoroughly dispersed with a hand homogenizer and centrifuged at 800*g*. These operations were repeated until the cell walls had been washed, dispersed and centrifuged six times. Finally, the wall material was centrifuged at 1500*g* 10 min. and washed twice with distilled water.

Examination of the walls at this stage of purification by light and electron microscopy indicated that the broken and torn fragments were not visibly contaminated by whole organisms or adhering subcellular particules. The possibility that absorbed soluble protein was still present could not be discounted. The wet walls were heated at 55° until most of the water had been removed, and the drying completed in a vacuum desiccator over silica gel at room temperature; storage was at −15° over the desiccant.

Analysis of walls. Ash was determined on a sample of material that had been maintained at red heat for 1 hr in a silica combustion tube. Lipid was estimated gravimetrically after separate extractions with boiling methanol and ether. Total nitrogen was determined by the micro-Kjeldhal method of Chibnall, Rees & Williams (1943); digestion of the wall material was followed by distillation of the NH_3 and titration. Total phosphorus was determined by the method of Fiske & SubbaRow as modified by Leloir (1957); organic phosphorus was first converted to phosphate by digesting walls with concentrated H_2SO_4.

For amino acid analysis 5–10 mg. samples of wall were hydrolysed in 5 ml. of 6 N-HCl for 48 hr in sealed tubes at 110°. The hydrolysates were evaporated to dryness at 60° and stored in vacuum over NaOH at −15°. Qualitative analysis of the amino acids by two-dimensional paper chromatography was made with the solvent systems of Calvin & Benson (1949) or of Stepka (1957). A ninhydrin spray (0·3 % in *n*-butanol) was used to detect the amino acids on the chromatograms. Quantitative amino acid analysis was done by ion-exchange chromatography according to Moore & Stein (1951) by using a Technicon Amino Acid Autoanalyzer.

Paper chromatography of sugar components was done on HCl hydrolysates and on $BaCO_3$-neutralized H_2SO_4-hydrolysates (2 N; 100°; 12 hr). The solvent system used was that of Jermyn & Isherwood (1949) and the sprays were *p*-anisidine HCl (0·1 %

in acetone) or resorcinol. The same solvents and sprays, also the ninhydrin spray, were used to detect amino sugars.

The effects of various enzymes (1–2 mg./ml.) were tested by incubation with whole organisms in tris buffer (pH 6·0) for 3 hr at 30°. The sources of enzymes used were as follows: cellulase (from *Aspergillus niger*), chitinase, hyaluronidase, glucuronidase, mycozyme (from *A. oryzae*), papain, all from Koch-Light, Colnbrook, Bucks; trypsin, papain, pancreatin, all from British Drug Houses, Poole, Dorset; snail digestive enzyme Glusulase was from Endo Laboratories Inc. Richmond Hill, New York, and from Industries Biologique Francaise, 35 à 49 Quai de Moulin de Cage, Gennevilliers (Seine), France. The suspensions of organisms were examined, after enzyme treatment, by the light microscope; the gross integrity of the walls was easily assessed by this method.

Whole organisms for sectioning were fixed in 2% (w/v) osmium tetroxide solution in 0·02 M-phosphate buffer (pH 7·2). Dehydration in an alcohol series was followed by embedding in a 9 + 1 (v/v) mixture of *n*-butyl methacrylate + methyl methacrylate and the blocks were sectioned at 60 mμ on a Huxley Ultramicrotome (Cambridge Instrument Co., Cambridge). The sections were stained on the grids with lead, (Lever, 1960).

Fragments of cell wall were fixed as for whole cells, spread on formvar-coated grids, and shadowed at 45° with platinum. Grids were examined in an Akashi T.S. 50 or an A.E.I. EM. 6 operating at 75 kV.

RESULTS

Yield of and composition of cell-wall material

From 20 l. of culture giving 50 g. wet wt organisms about 400 mg. dry light-yellow wall material were obtained. This contained the following constituents: ash 6·5%; lipid 11·6%; total nitrogen 1·6%; total phosphorus 0·09%.

Amino acid analysis. The amino acids detected in the wall material by paper chromatography were: leucine, phenylalanine, alanine, glycine, serine, glutamic acid, histidine, arginine, aspartic acid, lysine, cysteine, proline. No diaminopimelic acid was detected. Glucosamine was present. The quantitative amino acid analysis obtained by ion-exchange chromatography is presented in Table 1.

Sugar analysis. The only sugar components detected were glucose and mannose in approximately equimolar amounts. The solvents and sprays used clearly distinguish between these sugars and other sugars used as references (galactose, arabinose, xylose, rhamnose).

Treatment of whole organisms with enzymes

The following enzyme preparations and mixtures were incubated with suspensions of whole organisms: cellulase, chitinase, hyaluronidase, glucuronidase, mycozyme, papain, trypsin, pepsin, pancreatin, culture filtrate of *Trichoderma viride* (from Dr M. Davies), snail digestive enzymes + 2-aminoethanethiol, snail enzymes + papain. Examination in the light microscope of organisms incubated up to 24 hr revealed that none of the enzymes produced any great changes in the appearance of the walls and in no case was there any significant lysis of the organisms. Wall preparations or whole organisms added to mixed cultures of soil micro-organisms showed no signs of disruption even after several months. The Prototheca organisms were found to pass through the alimentary canal of pond snails without being digested.

Electron microscopy

Sections of whole organisms, prepared after OsO_4 fixation, showed that the wall was about 1000 Å thick (mean diam. of log phase organisms, 14 μ) and consisted of a thin (200 Å) outer osmiophilic layer and a much thicker (800 Å) inner electron-translucent layer (Pl. 1, fig. 1). The outer surface appeared to be highly corrugated and bore hemispherical projections (nodules) of various sizes. Surface views of the walls in shadowed preparations showed the nodules as electron-dense protrusions; these

Table 1. *Amino acid, sugar and amino sugar composition of hydrolysates of walls of Prototheca zopfii* KRUGER *236/65; comparison with results for Chlorella species (Punnett & Derrenbacker, 1966), Aspergillus niger (Johnston, 1965) and Saccharomyces cerevisiae (Korn & Northcote, 1960)*

	Prototheca zopfii (mμmoles/mg. cell wall)	Chlorella species	*Aspergillus niger*	*Saccharomyces cerevisiae*
Aspartic acid	17·2	++	+	+
Threonine	14·8	−	+	+
Serine	23·0	++	+	+
Glutamic acid	16·8	++	+	+
Glycine	19·9	++	+	+
Alanine	23·2	++	+	+
Isoleucine	8·9	++	+	+
Leucine	13·7	++	+	+
Phenylalanine	6·3	+*	+	+
Lysine	8·5	−	+	+
Arginine	++	−	+	−
Cysteine	2·8	−	+	+
Proline	Trace	++†	+	−
Valine	Trace (1·64)	++	+	+
Methionine	Trace	+‡	−	+
Tyrosine	Trace	+	+	+
Histidine	Trace (1·75)	−	+	+
Glucose	+	+	+	+
Galactose	—	+	+	−
Mannose	+	+	+	+
Glucosamine	13·4	+	+	+
Galactosamine	−	+§	+	+

In the results for Chlorella, ++ indicates major component, + minor component. * minor component in *C. pyrenoidosa*, only. † major component in *C. pyrenoidosa*, hydroxyproline in *C. vulgaris*. ‡ minor component in *C. vulgaris* and *C. ellipsoidea*. § detected in *C. vulgaris* and *C. ellipsoidea* only.

structures provided an indication of the orientation of the wall, since the nodules were shadowed when presented uppermost but appeared as diffuse dark areas when viewed through the wall (Pl. 1, fig. 2, 3, 4). The outer surface of the walls also bore parallel striations while the inner surfaces had a smoother fine-grained structure (Pl. 1, fig. 2, 3, 4).

Mild treatment with NaOH (N for 30 min.) had little visible effect on wall structure. Treatment with 5 N-NaOH for 13 hr resulted in a disintegration of the walls into irregularly shaped pieces, and saponified lipid products dried on the grids to patches of solid of characteristic vesicular appearance (Pl. 2, fig. 5). No microfibrils were seen

after NaOH treatment but certain surface corrugations became very much more apparent (Pl. 2, fig. 6). Examination of walls after treatment with papain or hyaluronidase revealed little visible change of structure, but the striated appearance of the outer surfaces of the wall seemed to become less obvious after incubation with papain.

DISCUSSION

The cell wall of *Prototheca zopfii* is a rigid structure of high tensile strength and the forces required to produce efficient breakage of organisms are very considerable. Previous work has shown that many of the usual methods for the mechanical disruption of micro-organisms are only of limited success when applied to this organism. For example, ultrasonic treatment for periods up to 1 hr at the full output of the MSE 500 W sonicator, disruption in a Hughes (1951) press, and methods involving shaking or grinding with glass beads, all produce only a small proportion of broken organisms (Lloyd, 1967).

Marked differences can be seen between the amino acid composition of the wall of Prototheca and that reported for Chlorella species by Punnett & Derrenbacker (1966). These Chlorella walls were prepared after washing with M-NaCl and detergent and the authors claimed that the simplicity of the amino acid patterns detected in six different algae suggested that the wall preparations contained peptides rather than proteins. The quantitative data for Prototheca might suggest that this is indeed the case, as there are twelve major and five minor components, the major amino acid constituents being ten times the concentration of the minor components.

Diaminopimelic acid (DAPA) was not detected in the wall of Prototheca, although Ciferri *et al.* (1961) showed that extracts of this organism possessed the DAPA activating enzyme. It seems likely that this acid is an intermediate in lysine biosynthesis rather than a wall constituent (Vogel, 1959). With Chlorella species, there have been two reports of the existence of DAPA (Fujiwara & Akabori, 1954; Hoare & Work, 1957), but it is not present as a wall component (Punnett & Derrenbacker, 1966).

The marked resistance of the wall of Prototheca to attack by a variety of hydrolytic enzymes is another indication of differences between this organism and Chlorella, since the digestive juice of *Helix pomatia* partially digests walls of *Chlorella pyrenoidosa* (Northcote *et al.* 1958).

Sections of the walls of Prototheca showed the presence of only two layers, whereas *Chlorella pyrenoidosa* walls have an electron translucent layer sandwiched between osmiophilic inner and outer lamellae (Northcote *et al.* 1958). The presence of nodules on the surface of the walls also distinguishes Prototheca from Chlorella. The origin and nature of these structures is unknown.

Microfibrils such as those observed in *Candida tropicalis* (Houwink & Kreger, 1953), bakers' yeast (Nickerson, 1959) and *Chlorella pyrenoidosa* (Northcote *et al.* 1958) were not detected in Prototheca, even after treatment with NaOH.

It thus seems that, although Prototheca has long been considered to be a colourless alga closely related to Chlorella, differences in cell wall composition and ultrastructure cast doubt on this relationship.

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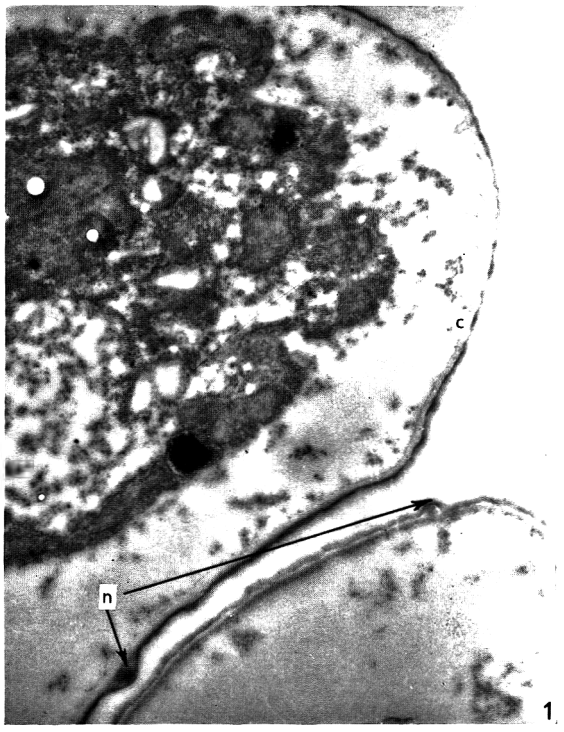

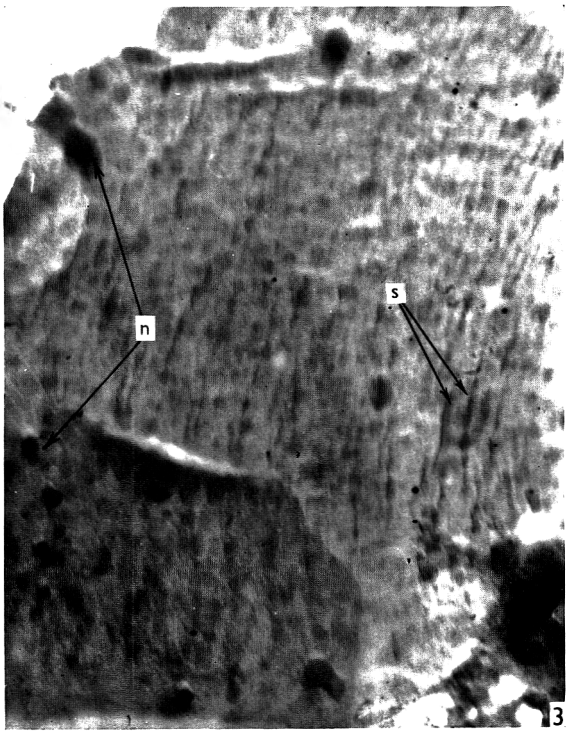

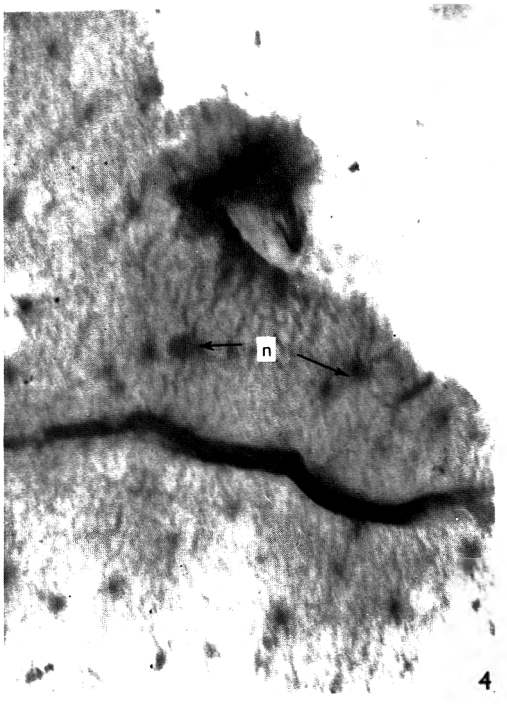

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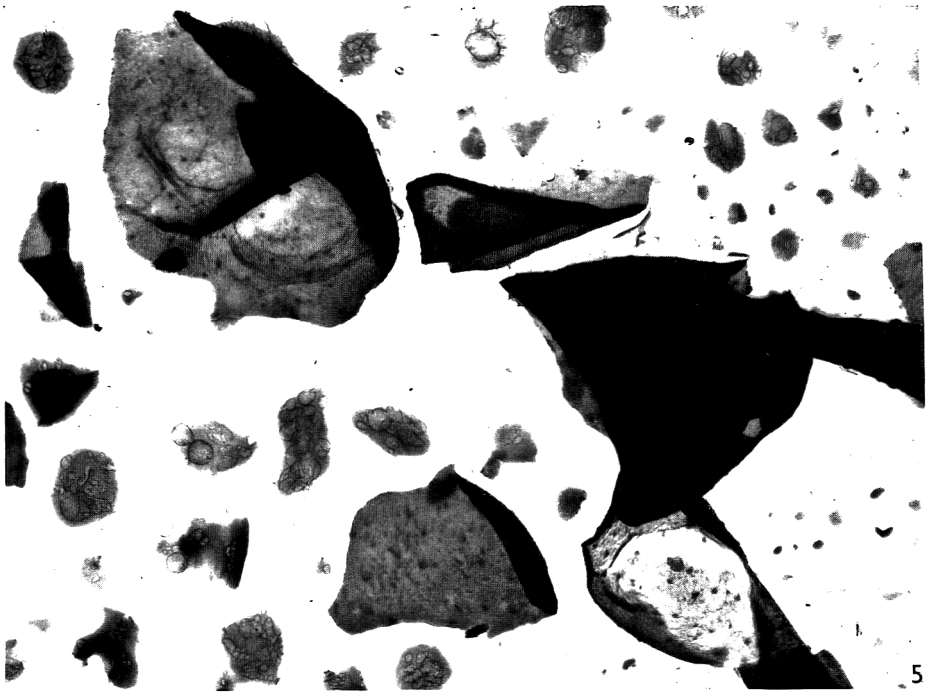

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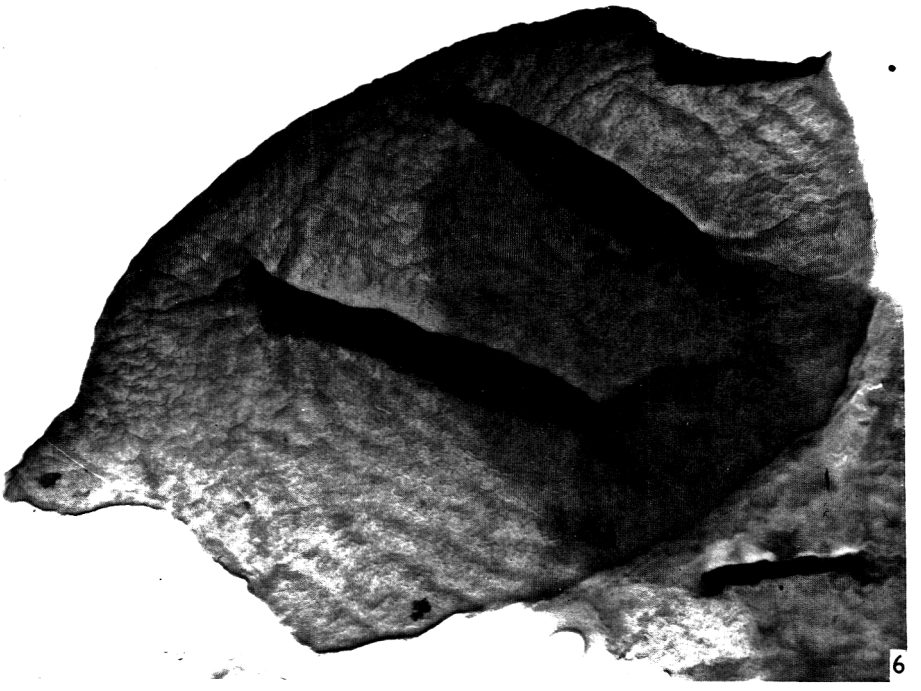

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *Prototheca zopfii:* section showing cell wall (c) and nodules. (n) × 35,000.

Fig. 2. Cell wall after isolation. × 4600.

Fig. 3. Striations (s) and nodules (n) on outer surface of cell wall. Shadowed. × 20,000.

Fig. 4. Inner surface of cell wall with the nodules (electron dense areas) seen through the cell wall. Shadowed. × 20,000.

PLATE 2

Fig. 5. Cell walls after treatment with 5 N-NaOH for 12 hr. showing disintegration and saponification of the cell wall lipid. Shadowed. × 2,500.

Fig. 6. Surface corrugations after NaOH treatment. Shadowed, × 15,000. In the shadowed preparations pictures are positives made from actual negatives obtained (i.e. shadows are white).

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Cold Shock in a Mesophilic and a Psychrophilic Pseudomonad

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SUMMARY

A mesophilic strain of *Pseudomonas aeruginosa*, grown at 30° and suspended to a concentration of equiv. 0·02 mg. dry wt/ml. in 30 mM-NaCl, showed rapid loss of viability when chilled rapidly from 30° to 0° or −2°. The viability of a psychrophilic pseudomonad, grown at 30°, did not decrease to the same extent when a dilute suspension (equiv. 0·02 mg. dry wt/ml.) was rapidly chilled from 30° to 0° or −2°. Concentrated suspensions (equiv. 3·0 mg. dry wt/ml.) of both the mesophile and the psychrophile released about the same proportion of total endogenous ultraviolet-absorbing compounds when rapidly chilled from 30° to 5°, 0° or −2°. Loss of viability following rapid chilling of a dilute suspension of the mesophile or the psychrophile was partly or completely prevented by 5 mM-Mg^{2+} and by bacteria-free filtrates from chilled concentrated suspensions of either bacterium. The viability of the bacteria grown at 10° did not decrease when dilute suspensions were rapidly chilled from 10° to −2°. Bacteria grown at 10° contained a greater proportion of unsaturated fatty acids than bacteria grown at 30°. Fatty acid analyses showed that susceptibility of the mesophile and psychrophile to cold shock could be correlated with the contents of unsaturated fatty acids in their lipids.

INTRODUCTION

Sherman & Albus (1923) reported that sudden chilling of a dilute suspension of *Escherichia coli* in buffer caused the death of many of the organisms. This phenomenon, which has been termed 'cold shock', has been demonstrated with other strains of *E. coli* (Hegarty & Weeks, 1940; Meynell, 1958) and with strains of *Pseudomonas pyocyanea* (*aeruginosa*) (Gorrill & McNeil, 1960), *Aerobacter aerogenes* (Strange & Dark, 1962), *Salmonella typhimurium* (Gorrill & McNeil, 1960), and *Serratia marcescens* (Strange & Ness, 1963). Gram-positive bacteria are thought to be insensitive to cold shock, although Ring (1965*a*, *b*) described a similar phenomenon in *Streptomyces hydrogenans*. Susceptibility to cold shock is usually found only in bacteria from exponential-phase cultures (Gorrill & McNeil, 1960; Houghtby & Liston, 1965) and is greater with bacteria grown in chemically defined media than in complex media (Strange & Ness, 1963). The decrease in viability of a population of pseudomonads is usually greater the more simple the diluent (Gorrill & McNeil, 1960).

Very little has been reported on the physiological basis of cold shock. Strange & Dark (1962) showed that endogenous solutes, including u.v.-absorbing compounds, amino acids and ATP, were released from *Aerobacter aerogenes* when thick suspen-

sions were subjected to cold shock, and they suggested that the major effect may be damage to the cytoplasmic membrane. Recent work in this laboratory (Farrell & Rose, 1967*a*, *b*) has shown that certain physiological properties of the cytoplasmic membrane are differently affected by near-zero temperatures in mesophilic and psychrophilic bacteria and yeasts. We have therefore investigated cold shock in a mesophilic and a psychrophilic Pseudomonas strain to discover whether the differences in the properties of the cytoplasmic membrane affect the susceptibility of the bacteria to cold shock.

METHODS

Organisms. The bacteria used in this study were a strain of *Pseudomonas aeruginosa* (NCTC 8506), one of the organisms used by Gorrill & McNeil (1960), and a psychrophilic pseudomonad isolated from a Norwegian lake by Dr S. O. Stanley. The bacteria were maintained on nutrient-agar slopes. Stock cultures were stored in the lyophilized state.

Experimental cultures. The bacteria were grown in the medium described by Rose, Ilahi & Kelemen (1965). Portions of glucose-free medium (900 or 1350 ml.) were dispensed into 2-l. round, flat-bottomed flasks which were plugged with cotton wool and sterilized by autoclaving at 15 lb./in.^{2} for 15 min. On cooling, the medium was supplemented with 100 ml. or 150 ml. of glucose solution (20 mg./ml.) which had been sterilized by autoclaving at 10 lb./in.^{2} for 10 min. Inocula were prepared by suspending bacteria from a 24-hr. slope culture in 6 ml. saline (0·85%, w/v). In the majority of experiments, the inoculum contained equiv. 0·1 mg. bacterial dry wt/ml., but for cultures that were to be incubated at 15° or below, it contained equiv. 4·0 mg. dry wt/ml. One-litre portions of medium were inoculated with 4 ml. of the light or heavy inoculum; 1·5-l. portions received 6 ml. inoculum. Cultures were incubated in individual Perspex baths through which was circulated water at the specified temperature. The cultures were stirred with a polytetrafluoroethylene-covered magnet (4 cm. long) that revolved at 1250 rev./min. Growth was measured turbidimetrically in the Hilger 'Spekker' absorptiometer (model H760) with neutral green-grey H508 filters and a water blank. Turbidity readings were related to dry weight by a calibration curve. Maximum specific growth rates ($\mu_{max.}$) of the bacteria were calculated from measurements of the turbidity of cultures by using the formula:

$$\mu_{max.} = \frac{2{\cdot}303\ (\log x_2 - \log x_1)}{t_2 - t_1}$$

in which x_2 and x_1 are the turbidity readings at times t_2 and t_1 during the period of maximum growth rate.

Experiments on cold shock. Bacteria from mid exponential-phase cultures (equiv. 0·26–0·34 mg. bacterial dry wt/ml.) were harvested by centrifuging at the growth temperature (30° or 10°) at 1300 *g* in an International centrifuge (model PR-2; head no. 209) or an M.S.E. High Speed '18' centrifuge (rotor no. 69179). In some experiments the temperature in the centrifuge bowl was continuously recorded with a thermistor probe (Grant Instruments Ltd., Toft, Cambridge) fitted to a Rustrak miniaturized temperature recorder (Grant Instruments Ltd., Toft, Cambridge); the maximum variation of temperature in the bowl was about ±2°. The bacterial pellet was resuspended in 100 ml. 30 mM-NaCl at 30° or 10° and washed by centrifuging at 30° or 10° to the required concentration. Duplicate portions (3 ml.) of the culture

supernatant fluid and of the saline washings were filtered through membrane filters (Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.; 25 mm. diam.; 0·45 μ pore size). Portions (1·0 ml.) of these filtrates were made up to 3·0 ml. with 30 mM-NaCl and the extinctions of the solutions were measured at 260 mμ in a Unicam SP500 spectrophotometer using cuvettes of 1 cm. light path.

In experiments on the effect of chilling on the viability of the bacteria, the washed suspensions were diluted with 30 mM-NaCl at 30° or 10° to a concentration of equiv. 0·02 mg. dry wt/ml. Portions (1–3 ml.) of these suspensions were dispensed into Samco tubes (Northam & Norris, 1951) which had been incubated for 1–3 hr in baths at the test temperature. Samples of suspension took less than 1 min. to reach the test temperature. Immediately after dispensing and at intervals thereafter, duplicate tubes were removed from the bath and the suspensions diluted 100,000-fold with 30 mM-NaCl at 30° or 10°. Samples (0·1 ml.) of the diluted suspension were spread on plates of nutrient agar (Oxo Ltd., London); triplicate plates were used with each sample. The plates were incubated at 30° for 48 hr and the numbers of colonies on the plates were counted with an electric colony counter (Sintacell Ltd., London, E.C.4); all colonies were counted. Attempts were made to measure the viability of suspensions of bacteria using slide cultures (Postgate, Crumpton & Hunter, 1961). However, the small size of the bacteria made it difficult to count the numbers of dead organisms on slide cultures. The method was therefore abandoned.

In order to study the release of endogenous solutes after chilling, portions (2 ml.) of dense washed suspensions (equiv. 3 mg. bacterial dry wt/ml.) were dispensed into precooled Samco tubes as already described. At intervals, duplicate tubes were removed from the bath, and the suspensions rapidly filtered through a Millipore membrane filter (25 mm. diam.; 1·5 μ pore size) placed on another Millipore filter (0·45 μ pore size). Filtration usually took less than 1 min. Occasional samples filtered more slowly; these filtrations were stopped after 1 min. and the unfiltered portion of the suspension rejected. Portions (1·0 ml.) of filtrate were diluted to 3·0 ml. with 30 mM-NaCl, and the extinctions of these solutions measured at 260 mμ in the Unicam SP500 spectrophotometer with cuvettes of 1 cm. light path and a blank of 30 mM-NaCl. These diluted filtrates were stored at $-20°$ and later assayed for ninhydrin-positive compounds by a modification of the method of Smith & Agiza (1957) as described by Hagen & Rose (1962) with glycine as a standard.

Total u.v.-absorbing compounds in the bacteria were determined by centrifuging portions of washed suspensions containing equiv. 9 mg. dry wt and extracting the organisms with 3 ml. ice-cold 5% (w/v) trichloroacetic acid. The suspensions were immediately centrifuged at 0°. Portions (1·0 ml.) of the supernatant were made up to 3·0 ml. with 5% (w/v) trichloroacetic acid, and the extinctions of the solutions measured at 260 mμ in cuvettes of 1 cm. light path with a blank of 5% (w/v) trichloroacetic acid. Total ninhydrin-positive compounds in the bacteria were determined by placing suspensions (3·0 ml.) containing equiv. 3·0 mg. dry wt/ml. in a bath of boiling water for 15 min. After cooling, the cell debris was removed by centrifuging. The supernatant solution was supplemented with washings (2 × 0·5 ml.) of the debris and made up to 3·0 ml. with water. Portions (1·0 ml.) of these extracts were made up to 3·0 ml. with water, and analysed for ninhydrin-positive compounds as already described.

Fatty acid analyses. Bacteria from mid exponential-phase cultures were harvested as already described. The organisms were washed once with 30 mM-NaCl and portions

containing equiv. 250–400 mg. dry wt were suspended in 1 ml. water. The suspension was transferred to a 50 ml. round-bottomed flask and methanol (5 ml.), chloroform (5 ml.) and glass beads (no. 3; 1·2–1·4 mm. diam.; Jencons Ltd., Hemel-Hempstead, Herts.; 5 g.) added to the flask. The stoppers were secured with Sellotape, and the flasks placed on a wrist-action shaker (Griffin and George Ltd., London; model 536-682) for 30 min. (Trevelyan, 1966). The supernatant liquid from the suspension of disrupted organisms was decanted into a stoppered tube and the beads and debris washed with portions (5 ml.) of methanol + chloroform mixture (1 + 1, by vol.). The supernatant solution and washings were pooled and centrifuged for 30 min. at 0° at 1300 *g*. The clear supernatant solution was carefully transferred to a stoppered tube, and taken to dryness on a rotary evaporator (Buchler Instruments Ltd., Fort Lee, New Jersey, U.S.A.) at 40°. The residue was extracted with methanol (2·5 ml.), and the extract transferred to a clean tube and taken to dryness. This procedure was repeated until no insoluble material remained in the tube. Methyl esters of the fatty acids in the extracted lipids were prepared by direct methanolysis by the method of Stoffel, Chu & Ahrens (1959) as modified by Kates (1964*a*). The esters were separated by gas-liquid chromatography with a Pye Panchromatograph (W. G. Pye & Co. Ltd., Cambridge) with a 231 cm. × 0·4 cm. glass column containing polyethylene glycol succinate (10%, w/w) on Chromosorb-W (60–80 mesh). The column temperature was 170° and the rate of nitrogen flow 50 ml./min. Methyl esters were detected with a flame-ionization detector (W. G. Pye & Co. Ltd., Cambridge; cat. no. 12210) using a mixture of hydrogen (30 ml./min.) and oxygen (100 ml./min.). The analyser unit amplification was 3×10^{-8}. The signal from the detector was recorded on a continuous-balance potentiometer recorder (Honeywell Controls Ltd., Newhouse, Motherwell, Lanarkshire) with a chart speed of 15 in./hr. The Panchromatograph was allowed to stabilize for at least 24 hr. before use. Portions (1 μl.) of extract were applied to the column with a microsyringe (Hamilton Co. Ltd., Whittier, California, U.S.A.). Methyl esters were identified by comparing their retention times with those of known fatty acid methyl esters. The areas of peaks corresponding to the separated esters were calculated by multiplying the height of the peak by the width at half the height. The retention times of methyl esters of oleic, palmitoleic and stearic acids were checked before each experiment.

Chemicals. All chemicals used were Analar or of the highest purity available commercially. Calcium-D-pantothenate, pyridoxin-HCl and thiamine-HCl were purchased from Koch-Light Laboratories, Colnbrook, Buckinghamshire. Other vitamins and amino acids were obtained from British Drug Houses Ltd., Poole, Dorset. Fatty acid methyl esters (99% pure) were supplied by Sigma Chemical Co. Ltd., London.

RESULTS

Effect of chilling temperature on the viability of, and release of endogenous solutes from, the bacteria

Arrhenius plots of the maximum specific growth rates of the mesophilic and the psychrophilic pseudomonad (Fig. 1) show that the minimum temperature for growth of the mesophile is around 5–6° and that of the psychrophile around −1°. Previous workers demonstrated cold shock only at temperatures well below the minimum for growth of mesophilic bacteria. A comparison was therefore made of the effect of

temperatures between 5° and −2·5° (the suspending fluid froze at approx. −3·0°) on the viability of, and release of endogenous u.v.-absorbing compounds from, the mesophilic and the psychrophilic pseudomonads. The data (Fig. 2) show that rapid chilling from 30° to 5° had little effect on the mortality of the mesophile, but that chilling to 0° or −2° led to a rapid decrease in viability. Similar data were reported for cold shock on this bacterium by Gorrill & McNeil (1960). The viability of the psychrophile did not diminish to the same extent as that of the mesophile, even when the chill temperature was −2° (Fig. 2).

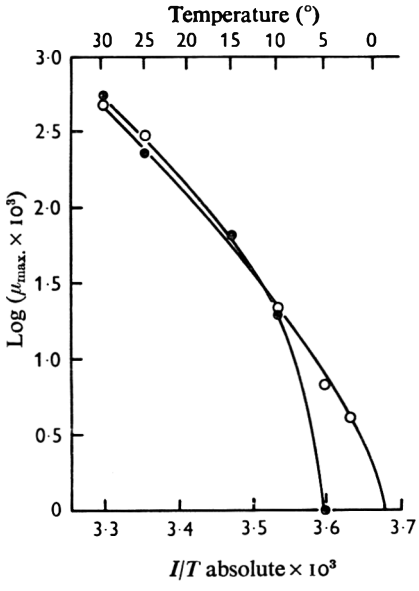

Fig. 1

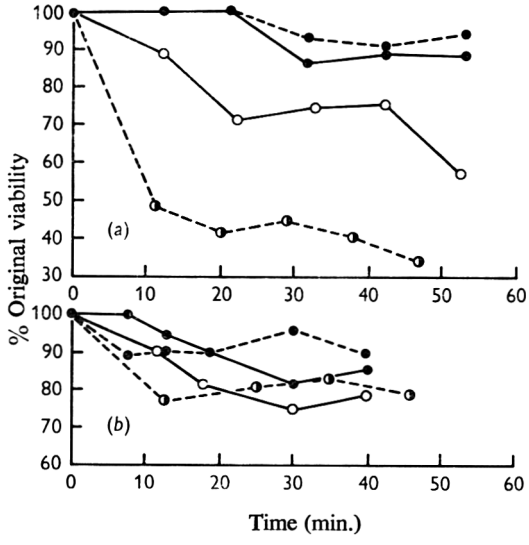

Fig. 2

Fig. 1. Arrhenius plots of the maximum specific growth rates of the mesophilic *Pseudomonas aeruginosa* NCTC 8506 (●) and of the psychrophilic pseudomonad (○).

Fig. 2. Effect of time of exposure on the viability of (*a*) the mesophilic, and (*b*) the psychrophilic pseudomonad grown at 30° in suspensions maintained at 30° (●- - -●), or chilled rapidly from 30° to 5° (●—●); 4·5° with the psychrophile), 0° (○—○), or −2° (◑ - - - ◑). Suspensions of washed bacteria contained equiv. 0·02 mg. dry wt/ml. 30 mM-NaCl.

The effects of different chilling temperatures on the release of u.v.-absorbing compounds from the mesophile and the psychrophile are shown in Fig. 3. There was a rapid release of compounds from the mesophile when a concentrated (equiv. 3·0 mg. dry wt/ml.) suspension was rapidly chilled from 30° to 5°, although the total amounts released were greater from bacteria chilled to 0° or −2°. The amounts of u.v.-absorbing compounds released from the psychrophile at 0° and 5° were similar to those released from the mesophile. When the temperature was lowered to −2°, filtrates from suspensions of the psychrophile had a smaller extinction at 260 mμ than filtrates from suspensions chilled to 5°. Filtrates from cultures of both the mesophile and the psychrophile had extinctions of 0·1–0·2 when measured at 260 mμ in cuvettes of 1 cm. light path using a blank of uninoculated medium ($E^{1cm.}_{260 m\mu}$ (values). Moreover, despite the precautions taken to ensure that the bacteria did not experience a temperature

stress during harvesting and washing, there was a further release of u.v.-absorbing compounds from both organisms during washing in 30 mM-NaCl. The extinction readings of supernatant solutions obtained during washing of the bacteria in 30 mM-NaCl at 30° were slightly greater for the mesophile than for the psychrophile. The amounts of u.v.-absorbing compounds released during washing amounted to about 50% of those that remained in the psychrophile and about 75% of the amounts retained in the mesophile.

Chilling temperature had a similar effect on the release of ninhydrin-positive compounds by the mesophile and the psychrophile. In particular, the amounts of these compounds released by the psychrophile were greater in suspensions chilled to 0° than in those chilled to −2°.

Table 1. *Effect of magnesium sulphate on the viability of the mesophilic and psychrophilic pseudomonads in suspensions rapidly chilled to −2°*

Bacteria were grown at 30°. Details of the harvesting of organisms and the preparation of suspensions are given in the text. Magnesium sulphate (5 mM) was incorporated in the suspending liquid (30 mM-NaCl) and the concentration of bacteria was equiv. 0·02 mg. dry wt/ml.

	% Original viability			
	Mesophile		Psychrophile	
Period of incubation at −2° (min.)	30 mM-NaCl	30 mM-NaCl + 5 mM-$MgSO_4$	30 mM-NaCl	30 mM-NaCl + 5 mM-$MgSO_4$
0	100	100	100	100
20	44	92	70	100
50	40	97	56	100

Table 2. *Effect of chilling on the viability of the mesophilic and psychrophilic pseudomonads suspended in saline or filtrate*

Bacteria were grown at 30°, harvested, and washed and suspended in 30 mM-NaCl at 30°. Portions (3 ml.) containing equiv. 3·0 mg. dry wt/ml. were rapidly chilled from 30° to −2° and, after 1 hr, were rapidly filtered through a double layer of membrane filters (see Methods for details) and the filtrates pooled. Portions of filtrate were warmed to 30° and used for suspending the test bacteria at equiv. 0·02 mg. dry wt/ml. Portions of the dilute suspension were then either held at 30° or rapidly chilled to −2°. Control suspensions were made in 30 mM-NaCl.

		% Viability							
		Temperature of suspension (°)							
		−2				30			
	Incubation	Psychrophile		Mesophile		Psychrophile		Mesophile	
Bacteria suspended in filtrate from	period (min.)	Saline	Filtrate	Saline	Filtrate	Saline	Filtrate	Saline	Filtrate
Psychrophile	0	100	100	100	100	100	100	100	100
	50	71	59	51	54	100	57	100	53
Mesophile	0	100	100	100	100	100	100	100	100
	50	71	100	39	80	86	64	100	88

Protective effect of exogenous compounds

Strange & Dark (1962) reported that sucrose (0·3 M), Mg^{2+} or Ca^{2+} (5 mM) and bacteria-free filtrates from chilled concentrated suspensions of exponential-phase

Aerobacter aerogenes substantially protected a dilute suspension of their strain of *A. aerogenes* from the lethal effects of chilling. Incorporation of magnesium sulphate (5 mM) in the suspending fluid gave complete, or almost complete, protection from loss of viability with both pseudomonads when dilute (equiv. 0·02 mg. dry wt/ml.) suspensions were rapidly chilled to −2° (Table 1). Filtrates from concentrated suspensions of the mesophile that had been chilled from 30° to −2°, when used as the diluent, gave a considerable measure of protection against loss of viability of this bacterium when dilute suspensions (equiv. 0·02 mg. dry wt/ml.) were rapidly chilled from 30° to −2° (Table 2). Filtrates from chilled (−2°) concentrated suspensions of the psychrophile gave some protection against loss of viability of the psychrophile

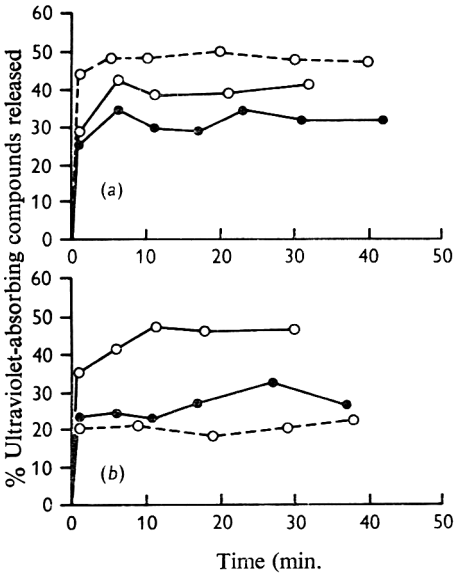

Fig. 3

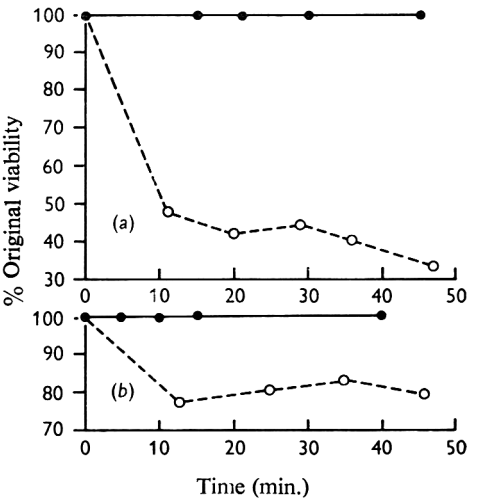

Fig. 4

Fig. 3. Effect of time of exposure on the release of ultraviolet-absorbing compounds from (*a*) the mesophilic, and (*b*) the psychrophilic pseudomonad in suspensions chilled rapidly from 30° to 5° (●—●), 0° (○—○) or −2° (○ - - - ○). Suspensions of washed bacteria contained equiv. 3·0 mg. dry wt/ml. 30 mM-NaCl.

Fig. 4. Effect of time of exposure on the viability of (*a*) the mesophilic, and (*b*) the psychrophilic pseudomonad in suspensions chilled rapidly from the temperature at which the organisms had been grown to −2°. ●—● indicates the response of bacteria grown at 10°, and ○ - - - ○ that of bacteria grown at 30°. Suspensions of washed bacteria contained equiv. 0·02 mg. dry wt/ml. 30 mM-NaCl.

when dilute suspensions were rapidly chilled from 30° to −2°. The non-specific nature of this protective effect was shown by the ability of filtrates from chilled concentrated suspensions of the psychrophile to protect the mesophile against loss of viability, and of filtrates from suspensions of the mesophile to protect the psychrophile (Table 2). When filtrates from chilled concentrated suspensions of either organism were used as diluent, there was an appreciable decrease in the viability of both the psychrophile and the mesophile maintained at 30°. Thus, suspensions of the mesophile in saline, held at 30°, had a viability of 100%, and of 51% when the suspension

was chilled to $-2°$. When the mesophile was suspended in filtrate from the psychrophile, and the suspension held at 30°, toxic compounds in the filtrate decreased the viability to 53%. However, when a suspension of the mesophile in psychrophile filtrate was chilled to $-2°$, there was no further decrease in viability of the mesophile. This toxic effect, which was greater with filtrates from suspensions of the psychrophile than of the mesophile, might explain the incomplete protection of viability by these filtrates with suspensions chilled to $-2°$.

Table 3. *Effect of growth temperature on the fatty acid composition of the mesophilic and psychrophilic pseudomonads*

Experimental methods are described in the text. Fatty acids are indicated as $x:y$ in which x equals the number of carbon atoms and y the number of double bonds/molecule; — indicates a fatty acid methyl ester content of less than 0·1%. Δ/mole values were calculated using the formula of Kates & Baxter (1962).

	Fatty acid composition (%) Growth temperature (°)			
	30		10	
Fatty acid	Mesophile	Psychrophile	Mesophile	Psychrophile
10:0	—	—	—	—
11:1	—	—	—	—
12:0	—	—	0·18	0·10
13:1	—	—	0·42	0·49
14:0	1·0	0·95	0·65	0·92
15:0	—	—	—	—
16:0	29·6	28·1	20·6	18·6
16:1	18·8	23·1	27·6	28·3
17:1	—	—	—	—
18:0	—	—	—	—
18:1	50·9	48·0	50·7	51·5
18:2	—	—	—	—
Δ/mole value	0·69	0·71	0·79	0·80

Effect of growth temperature on the fatty acid composition of, and the susceptibility to cold shock in, the bacteria

At temperatures below the optimum for growth, many and probably all microorganisms synthesize increased amounts of unsaturated fatty acids (Kates, 1964*b*; Farrell & Rose, 1967*a*, *b*). The principal fatty acids in both the mesophilic and the psychrophilic pseudomonads, grown at 30°, were octadecenoic acids, with palmitic acid and hexadecenoic acids accounting for most of the remaining acids (Table 3). The fatty acids in the lipids extracted from the psychrophile contained a slightly greater proportion of double bonds (expressed as the Δ/mole value; Kates & Baxter, 1962) compared with the fatty acids in lipids from the mesophile. Similar results were reported by James & Martin (1956) for the fatty acid composition of another mesophilic strain of *Pseudomonas aeruginosa*. These workers also detected small amounts of a branched-chain saturated or unsaturated C_{19} fatty acid in their pseudomonad. However, neither of the pseudomonads examined in the present study contained detectable amounts of fatty acids containing more than 18 carbon atoms. When the pseudomonads were grown at 10°, the main changes in the fatty acid composition were among the C_{16} acids. In both the mesophile and the psychrophile, the proportion of

hexadecenoic acids increased largely at the expense of palmitic acid. The proportion of octadecenoic acids increased slightly on lowering the temperature for growth of the psychrophile, but remained almost contant in the mesophile.

Neither the mesophile nor the psychrophile, when grown at 10°, showed a measurable decrease in viability when dilute suspensions (equiv. 0·02 mg. dry wt/ml.) were rapidly chilled from 10° to −2° (Fig. 4). The amounts of u.v.-absorbing compounds released during washing of the bacteria grown at 10° were about the same as those released from the organisms grown at 30°. No measurements were made of the release of u.v.-absorbing compounds from bacteria, grown at 10°, and chilled to −2°.

DISCUSSION

Previous studies on cold shock have been confined to mesophilic Gram-negative bacteria, and have demonstrated a loss in viability when suspensions of the bacteria were rapidly chilled to temperatures below the minimum for growth. The data reported in the present paper show that cold shock also occurs in a psychrophilic bacterium and at temperatures just above the minimum for growth. Under the conditions used in the present study, the decrease in viability in suspensions of the psychrophile chilled to −2° was much less than in suspensions of the mesophile chilled to the same temperature. Nevertheless, cold shock in the psychrophile appears to be similar in many respects to cold shock in the mesophile, particularly with regard to the kinetics of the process and the ability of Mg^{2+} to protect the bacteria against loss of viability. The ability of bacteria-free filtrates from concentrated chilled suspensions of the mesophile to protect the psychrophile against loss of viability, and of filtrates from suspensions of the psychrophile to protect the mesophile, supports this contention.

Probably the most significant finding reported in the present paper is that neither the psychrophile nor the mesophile, when grown at 10°, showed a measurable decrease in viability when dilute suspensions were rapidly chilled to −2°. This indicates that growth of the bacteria at this temperature caused certain changes in composition of the organisms such that they became insensitive to cold shock. Houghtby & Liston (1965) reported briefly that *Escherichia coli* K-12 became more resistant to cold shock when the growth temperature was lowered from 35° to 22°. The best documented change in composition caused by growing micro-organisms at temperatures below the optimum for growth is the increased synthesis of unsaturated fatty acids (Kates, 1964*b*; Farrell & Rose, 1967*a*, *b*). In view of the suggestion (Meynell, 1958; Strange & Dark, 1962) that cold shock is accompanied by, and indeed may result from, changes in the permeability properties of the bacterial cytoplasmic membrane, it seems likely that insensitivity to cold shock in bacteria grown at 10° may be associated with the increased content of double bonds in the membrane lipids. It is worth noting that the lipids from the psychrophile, grown at 30°, contained fatty acids with a slightly greater proportion of double bonds than fatty acids in lipids from the mesophile grown at 30°, and that the psychrophile grown at 30° was less susceptible to cold shock than the mesophile grown at 30°.

Phospholipids have a liquid-crystalline nature, one result of which is that the fatty acid chains in the molecules can be in the liquid state at a temperature many degrees below the melting point of the phospholipid as determined by conventional chemical means (Byrne & Chapman, 1964). It has also been shown by Byrne & Chapman (1964)

that the presence of unsaturated fatty acid residues in the phospholipid decreases the melting point of the chains. In the 'protein pore' model of membrane structure, developed by Luzzati & Husson (1962) and embellished by ourselves (Farrell & Rose, 1967*a*, *b*), it is suggested that protein molecules are retained within pores in the membrane as a result of the mobility of the fatty acid chains in the membrane lipids. Once these chains are frozen, orientation of the protein molecules in the membrane is presumably changed. We have suggested that this change in orientation may lead to inactivation of permease activity in the membrane (Farrell & Rose, 1967*a*). Another likely result of the freezing of fatty acid chains is that channels are formed in the membrane which allow the exit of intracellular low molecular-weight solutes. The data reported in the present paper show that the release of u.v.-absorbing compounds is not correlated with the loss of viability, and it is possible that the loss of other intracellular solutes is more directly responsible for death of the organisms. The possibility that freezing of the fatty acid chains in membrane lipids also causes a loss of protein from the membrane cannot be ignored. Piperno & Oxender (1966) showed that amino acid-binding protein can be released from *Escherichia coli* by subjecting the organisms to osmotic shock at 0°. When the osmotic shock was done in the presence of Mg^{2+}, there was no loss in viability; but when Mg^{2+}-free diluent was used, there was an appreciable decrease in viability or the shocked bacteria.

The hypothesis that susceptibility to cold shock in Gram-negative bacteria may be a function of the content of unsaturated fatty acid residues in the membrane lipids could explain why susceptibility to cold shock in some bacteria decreases when the organisms are grown in nutritionally complex media rather than in chemically defined media (Strange & Ness, 1963). Growth in nutritionally complex media in general leads to an increased synthesis of unsaturated fatty acids by bacteria (Kates, 1964*b*).

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The Mechanism of the Bacteriostatic Action of Tetrachlorosalicylanilide: a Membrane-active Antibacterial Compound

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SUMMARY

The antibacterial action of the skin germicides tetrachlorosalicylanilide, tribromosalicylanilide, trichlorocarbanilide and monochlorophenoxysalicylanilide against *Staphylococcus aureus*, depends on the reversible adsorption of the germicides on the cell membrane. Bacteriostasis results from the adsorption of, respectively, $0{\cdot}75 \times 10^5$, $2{\cdot}0 \times 10^5$, $5{\cdot}1 \times 10^5$ and $7{\cdot}2 \times 10^5$ molecules of germicide per bacterium. The resistance of *Escherichia coli* to these compounds results from a decreased adsorption, which is a property of the cell wall. It is proposed that the membrane-active antibacterial compounds, detergents, phenols, quaternary ammonium compounds, polypeptide antibiotics and the germicides under study in this paper, share a common mechanism of action, in which the adsorption of the compound on the cell membrane is a critical step. Resistance to these compounds results from the inhibition of the penetration through the cell wall to the combining sites on the membrane. At the cellular bacteriostatic concentration, the effect of tetrachlorosalicylanilide on the biochemical activities of *Staphylococcus aureus* has been studied. The energy-dependent transport of phosphate and amino acids into the bacteria is inhibited, whereas the energy-independent entry of phosphate, amino acids and glucose is unaffected. The energy-dependent incorporation of lysine and glucose into cellular material is also inhibited. The release of amino acids from the cell pool into the medium results from the inhibition of the energy-dependent processes involved in the maintenance of the amino acid pool. This inhibition of energy metabolism by the germicide at its bacteriostatic concentration is sufficient to cause the inhibition of growth.

INTRODUCTION

A common feature of the antibacterial action of detergents, phenols, quaternary ammonium compounds and polypeptide antibiotics, e.g. polymyxin and tyrocidin, is the ability of these compounds to cause cell leakage and disturbances of normal membrane function (Baker, Harrison & Miller, 1941; Salton 1951; Gale & Taylor, 1947; Hotchkiss, 1944; Newton, 1956; Stedman, Kravitz & King, 1957). Studies with the skin germicides hexachlorophene (Joswick, 1961; Silvernale, 1966) and 3,5,3′,4′-tetrachlorosalicylanilide (Woodroffe & Wilkinson, 1966*a*, *b*) have shown that these compounds can also cause leakage and membrane damage. The antibacterial action of all these compounds has been stated to result from their binding to the cell membrane with the disruption of its function as the semi-permeable barrier between the cell and its environment. However, several other effects have also been noted, e.g. protein

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denaturation (Cooper, 1912; Fogg & Lodge, 1945), enzyme inhibition and loss of oxidative capacity (Gould, Bosniak, Needleman & Gatt, 1953; Newton, 1956) and the chelation of certain metals (Adams & Hobbs, 1958). The generalization has been made for polymyxin (Newton, 1956) and for surface-active compounds (Sykes, 1939) that cell viability is affected by concentrations of the antibacterial compound lower than those which inhibit metabolism, However, it is a common feature of many of the studies quoted here that no clear distinction is drawn between bacteriostatic and bactericidal effects, and that little or no attempt is made to relate the concentrations of the antibacterial substance on the cells, which on the one hand can cause bacteriostasis or cell death, and on the other give rise to cell leakage, enzyme inhibition, etc.

Membrane active compounds tend to be adsorbed in fairly large amounts by sensitive cells, and the antibacterial activity is directly dependent on the amount which is adsorbed (Salton, 1951; Few & Schulman, 1953). Most enzymic or biochemical activities are assayed using cell suspensions containing at least 5×10^9 cells/ml., whereas bacteriostatic activities are assayed with inocula of normally from 10^4 to 10^7 cells/ml. Under these two conditions, the same concentration of germicide per ml. of suspension might well result in very different concentrations per cell, and consequently very different degrees of inhibition of cellular function. In seeking to explain bacteriostasis or cell death in terms of the loss of a particular cellular function, it is extremely important therefore to do so only by comparison of the activities of cell populations with germicide at the same cellular concentration.

In continuing the study of 3,5,3′,4′-tetrachlorosalicylanilide (TCS) in this laboratory, we have first examined its adsorption by sensitive and resistant cells and put forward the hypothesis that all of the membrane active antibacterials have a common mode of action and mechanism of resistance. We have shown that a concentration of $0{\cdot}75 \times 10^5$ molecules TCS/bacterium is sufficient to inhibit the growth of *Staphylococcus aureus*. We have studied the effects of TCS on metabolism at this cellular concentration of the germicide and demonstrated a disruption of energy metabolism which is sufficient to explain the inhibition of growth.

METHODS

Organisms used. Unless otherwise stated, the test organism used was a laboratory isolate of a bovine *Staphylococcus aureus*. Other sensitive organisms examined in this study were *S. aureus* (OXFORD) NCTC 6571 and two laboratory isolates of coagulase-negative staphylococci from human skin. The resistant organism studied was a type 1 *Escherichia coli* NCTC 8196.

Media and growth conditions. For the routine growth of all organisms, two complex media were used. The special medium of Collins & Lascelles (1963) contained (g./l.): tryptone, 10·0; Lab Lemco, 5·0; yeast extract, 1·0; Na_2HPO_4, 5·0; glucose, 20·0; (pH 6·5). Yudkin (1962) medium contained (g./l.): peptone, 20·0; NaCl, 5·0; (pH 7·2). Organisms were grown in Yudkin (1962) medium for studies of the uptake of radioactive germicide and for measurements of the minimum inhibitory concentration (m.i.c.). The special medium was used to grow organisms for the various studies of biochemical activities. In some experiments glucose was omitted from the medium. The organisms were grown in 200 ml. of medium in a Roux bottle which was laid on its side and incubated for 16 hr at 30° on a reciprocal shaker which operated at a rate of 100 strokes/min. with an amplitude of 2 inches.

In experiments to study the effect of increased cellular lipid content on the sensitivity of *Staphylococcus aureus* to germicides, *S. aureus* NCTC 6571 was grown on the glycerol medium of Hugo & Stretton (1966) which contained (g./l.): peptone, 10·0; Lab Lemco, 5·0; NaCl, 5·0; glycerol, 30·0; (pH 7·2). The organism was grown in this medium under semi-anaerobic conditions in 100 ml. volumes in 4 oz. flat bottles without shaking, with incubation for 16 hr at 37°. Organisms were subcultured in this medium ten times before being used for m.i.c. determinations. Control organisms were grown in the same medium, without the glycerol.

For the preparation of spheroblasts, *Escherichia coli* was grown in Fraser & Gerral (1953) medium, which contained (g./l.): Na_2HPO_4, 10·5; KH_2PO_4, 4·5; NH_4Cl, 1·0; casein hydrolysate, 15·0; glycerol, 30·0; gelatin, 0·01; $MgSO_4.7H_2O$, 0·3; $CaCl_2.6H_2O$, 0·3; (pH 7·1). Incubation was at 30° with shaking for 16 hr.

Cultures were maintained at 4° in the appropriate liquid media and subcultured at monthly intervals.

Minimum inhibitory concentration determinations. Series of graded concentrations of germicide in ethanol were prepared by halving dilutions; 0·1 ml. volumes of these concentrations were added to a series of sterile $6 \times \frac{1}{2}$ in test tubes; the control tube contained 0·1 ml. ethanol. To each of these tubes was added 10 ml. of an inoculum culture of the organism under study, either in Yudkin medium, or in Hugo & Stretton medium when the organism was *Staphylococcus aureus* NCTC 6571. Incubation was for 24 hr at 30°, or 37° with NCTC 6571. Growth was determined either by visual examination or total counts and the m.i.c. was taken as the lowest concentration at which no growth of the inoculum was evident after incubation for 24 hr.

In experiments to study the uptake of radioactive germicides by *Staphylococcus aureus* it was necessary to use concentrated inocula of 3×10^8 bacteria/ml. in m.i.c. determinations, as compared to the normal of 10^7 bacteria/ml. in the rest of this study. Under these conditions, incubation was with 10 ml. volumes in 4 oz. bottles at 30° on the reciprocal shaker; this allowed the control culture to grow up to 5×10^9 bacteria/ml. and did not alter the m.i.c. value obtained. These conditions of inoculation and incubation were also used to determine the m.i.c. against *Escherichia coli.*

Uptake of [^{14}*C*]*germicides by bacteria.* Considerable care was necessary in measuring the adsorption of [^{14}C]germicides by bacteria. Apart from TCS, a preliminary study was also made of several other potential skin germicides, hexachlorophene, 3,5,4′-tribromosalicylanilide (TBS), 3,4,4′-trichlorocarbanilide (TCC) and 3′-chloro-6-phenoxysalicylanilide (GL31; Fr. patent, 1966, 1,456,361). All of these compounds have very low water solubility, and due to the necessity of separating the bacteria from supernatant fluid during measurement of the uptake of the germicides by the bacteria, adsorption studies can only be done at concentrations below the saturated solubility of the germicide in the particular medium under study. The measured m.i.c. values for the above compounds were; TCS, 0·15 μg./ml.; hexachlorophene, 0·15 μg./ml.; TBS, 1·0 μg./ml.; TCC, 0·15 μg./ml.; GL31, 0·75 μg./ml. Even at 0·05 μg./ml. hexachlorophene precipitated from solution in Yudkin's medium during 24 hr incubation at 30°, and therefore the adsorption of this germicide by the bacteria at the m.i.c. could not be measured. The other compounds are all soluble under these conditions and their adsorption by the bacteria at the m.i.c. was therefore amenable to study. During short-term experiments in buffer, TCS was not precipitated from solution at concentrations as high as 20 μg./ml.

Membrane filtration could not be used for the separation of bacteria from culture fluid since all of the compounds under study, and crystal violet, phenol, cetyl trimethylammonium bromide (CTAB) and sodium lauryl sulphate, were strongly absorbed by both cellulose acetate and nylon filters. As an example of the magnitude of this adsorption, when 4 ml. of a 2·5 μg./ml. solution of [^{14}C]TCS was passed through a filter which was then washed twice with 4 ml. distilled water, 87% of the [^{14}C]TCS was adsorbed and retained by the filter. It was not found possible to saturate the filters with unlabelled germicide before use. This property of adsorption on to filters was found to extend to polypropylene centrifuge tubes and even, to a small extent, to glass flasks and centrifuge tubes. Provided all-glass vessels were used throughout the experiments, however, the absorption could be decreased to a minimum, although its magnitude had to be determined for each compound. A double centrifugation technique was used, and the whole-suspension counts were obtained from volumes of the suspension which had also been transferred from the treatment flask first to one, and then to a second, centrifuge tube. The loss of radioactivity with [^{14}C]TCS due to the adsorption to glass during such a transfer with an 0·15 μg./ml. solution only amounted to 1%.

As Woodroffe & Wilkinson (1966*b*) showed, about 10% of the germicide adsorbed to bacteria can be removed by suspension in fresh buffer for 75 min. at 30°. We found that the equilibrium between germicide on the bacteria and germicide in solution was established very rapidly, and that radioactivity was lost from a bacterial pellet on suspension in fresh medium followed by centrifugation. A pellet of washed bacteria would therefore have lost radioactivity, while a pellet of unwashed bacteria would be contaminated with supernatant fluid. The amount of radioactivity adsorbed by the bacteria could therefore only be measured using the following technique.

Radioactive germicide was added at the desired concentration to the given bacterial suspension and incubated under specific conditions of time, temperature and aeration. At a chosen time, a 5 ml. sample was removed and 3 ml. transferred to one glass centrifuge tube and 2 ml. to a second tube. The first tube was spun for 5 min. at 9000 ***g*** in a B.T.L. Micro Angle centrifuge and the supernatant fluid drawn off with a Pasteur pipette into a third tube which was centrifuged for a further 5 min. This second centrifugation was necessary in order to obtain a complete separation of the cells at 9000 ***g***, the maximum possible ***g*** value with glass tubes. The suspension in the second tube was transferred after 5 min. to a fourth tube. Duplicate 0·5 ml. volumes were then taken from these third and fourth tubes for assay of the radioactivity of the cell-free supernatant fluid and whole suspension respectively. The radioactivity adsorbed on the bacteria was obtained by difference.

Escherichia coli spheroblasts. Spheroplasts were prepared from *E. coli* by the method of Repaske (1958). The bacteria were harvested from Fraser & Gerral medium and washed and suspended in 30 mM-tris buffer (pH 8·0), containing 10% (w/v) sucrose, to a concentration of 6×10^9 bacteria/ml. To this suspension was added lysozyme 33 μg./ml. and EDTA 133 μg./ml. and a 'pinch of solid DNAase. Incubation was at 30° without shaking; spheroplast formation was complete within 15 min.

Uptake of [^{32}P]inorganic phosphate. Bacteria were grown on special medium minus glucose, harvested and washed twice and suspended in 0·2 mM-phosphate buffer (pH 7·0) at a concentration of 6×10^9 bacteria/ml. The effect of TCS 0·2 μg./ml. on the uptake of 0·002 mM-[^{32}P]inorganic phosphate was examined in the presence and absence of

0·67 mM-glucose. Incubation was at 25° with forced aeration. Samples (4 ml.) were removed at intervals, centrifuged at 37,000 *g* in an MSE High Speed 18 centrifuge at 0° for 15 min. and duplicate 0·5 ml. volumes of the supernatant fluid taken for radioactivity measurement. The bacterial pellet was washed once in ice-cold 0·1 % (w/v) NaCl and then suspended to 4 ml. in distilled water and placed in a boiling water bath for 10 min. The bacteria were again centrifuged down and duplicate 0·5 ml. volumes of the supernatant fluid taken for radioactivity measurement of the pool fraction which is extracted under these conditions.

Uptake of [^{14}C]glutamic acid and [^{14}C]lysine. Bacteria were grown on special medium, harvested and washed twice and resuspended in 33 mM-phosphate buffer (pH 7·0) at a concentration of 6×10^9 bacteria/ml. The effect of TCS 0·5 μg./ml. and 10 mM-dinitrophenol on the uptake of 18 μM-[^{14}C]glutamic acid and 180 μM-[^{14}C]lysine was examined in the presence of 0·5 mM-glucose. Incubation was at 30° with forced aeration. Duplicate 1 ml. samples were removed at intervals, the bacteria collected on membrane filters, washed once with 4 ml. cold 33 mM-phosphate buffer (pH 7·0) and the filters counted for radioactivity.

Release of [^{14}C]glutamic acid and [^{14}C]alanine. By using the technique described above for the uptake of [^{14}C]amino acids, bacteria were preloaded by 30 min. incubation with 18 μM-[^{14}C]glutamic acid in the presence of 0·5 mM-glucose, or by 150 min. incubation with 18 μM-[^{14}C]alanine, again with 0·5 mM-glucose present. Bacteria were then harvested, washed once and suspended in fresh buffer at a concentration of 6×10^9 bacteria/ml. The effect of TCS 0·5 μg./ml., 10 mM-dinitrophenol and 30 mM-sodium azide on the release of the [^{14}C]amino acids from the bacteria was examined in the presence of 0·5 mM-glucose. The sampling technique was the same as that described for the uptake of [^{14}C]amino acids.

Uptake of [^{14}C]glucose and its incorporation into cellular material. Bacteria were grown on special medium, harvested, washed twice and suspended in 33 mM-phosphate buffer (pH 7·0) at a concentration of 6×10^9 bacteria/ml. The effect of TCS 0·5 μg./ml. on the uptake and incorporation into cellular material of 0·57 mM-[^{14}C] glucose was examined. Incubation was at 30° with forced aeration. At intervals, samples were withdrawn and radioactivity measured in the whole suspension, the cell-free supernatant fluid, the washed whole-organism pellet, and in the pool, lipid, nucleic acid and protein fractions obtained from the bacteria. Duplicate 0·5 ml. volumes of the suspension were taken for counting the radioactivity in the suspension; 1·5 ml. volumes of the suspension were passed through a membrane filter and duplicate 0·5 ml. volumes taken for counting the radioactivity in the cell-free supernatant fluid; the filter was washed with 4 ml. cold 33 mM-phosphate buffer (pH 7·0) and counted directly for radioactivity in the whole-cell pellet; 4 ml. volumes of the suspension were taken for cell fractionation.

Oxygen uptake was assayed in a parallel experiment, with volumes $\frac{1}{15}$ those in the incubation flasks.

Fractionation of bacteria. The fractionation technique used was a modification of that of Roberts *et al.* (1963). Four ml. of bacterial suspension were placed in a boiling-water bath for 10 min. to stop metabolic activity and to extract pool material. The extracted bacteria were centrifuged down for 5 min. at 3100 *g* in an MSE super-minor centrifuge fitted with a swing-out head, suspended in 4 ml. of a 1+1 mixture of chloroform+methanol and extracted for 30 min. at 45°. The organisms and extract

were separated by centrifugation, and duplicate 0·1 ml. volumes of the extract counted for radioactivity (because of the quenching by chloroform + methanol in scintillation counting, larger volumes could not be used). The pellet was suspended in 4 ml. 5% trichloroacetic acid and placed in a boiling-water bath for 30 min. The pellet material and extract were separated by membrane filtration, and the acid solution washed three times with 3 ml. ether and duplicate 0·5 ml. volumes of the aqueous layer counted for radioactivity. Care was taken that the volumes of the chloroform + methanol and trichloroacetic acid extracts were still 4 ml. before samples were withdrawn for counting. The precipitate from the hot acid extraction was washed once with 4 ml. cold distilled water and the filter counted directly. The three fractions obtained in the chloroform + methanol extract, the hot acid extract and the insoluble residue were designated the lipid, nucleic acid and protein fractions. The radioactivity of the metabolic intermediates, amino acids, etc., in the pool fraction which was extracted in the hot water treatment, was obtained from the difference between the counts in the whole-organism pellet and the sum of the counts in the lipid, nucleic acid and protein fractions.

Oxygen uptake. Conventional manometric techniques were used to assay the oxygen uptake of bacterial suspensions at 30° in an atmosphere of air with a shaking rate of 100 oscillations/min.

Measurement of radioactivity. Radioactivity was counted on a Nuclear Chicago Liquid Scintillation Counter 720 Series, using 10 ml. volumes of Bruno & Christian's (1961) scintillation solution. All counts were corrected for quench against suitably prepared quench standards; the efficiency of counting was about 76%. Activities have been expressed in terms of amounts of material, using the specific activity of the added compound.

Chemicals. Analytical grade chemicals (British Drug Houses Limited, Poole, Dorset) were used throughout. Uniformly [^{14}C]-labelled L-glutamic acid, L-lysine, glucose and [^{32}P]inorganic phosphate were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. [^{14}C]germidices were synthesized by Dr R. D. Osborne and Mr L. Mindt of the Organic Chemistry Section of this Laboratory. [^{14}C]TCS, TBS and GL31 were prepared from carboxyl-labelled salicylic acid, and [^{14}C]TCC from carboxyl-labelled dichlorobenzoic acid.

RESULTS

Minimum inhibitory concentration (m.i.c.) and uptake of [^{14}C]germicides by bacteria

Molecules/coccus at m.i.c. with Staphylococcus aureus. Determinations of m.i.c. were made with several germicides, using halving dilutions of the [^{14}C]compounds. The radioactivity adsorbed by the cocci of the inocula at the m.i.c. was measured at the end of the 24 hr incubation period, and expressed as molecules germicide/coccus, using the equation:

$$\text{molecules/coccus} = \frac{\text{m.i.c. } (\mu\text{g./ml.}) \times \text{diff. between suspension and supernatant fluid counts} \times \text{Avagadro's number}}{\text{Suspension count} \times \text{mol. wt. germicide} \times \text{total bacterial count} \times 10^6}.$$

The results with the four germicides [^{14}C]TCS, TBS, TCC and GL31 are recorded in Table 1.

To obtain sufficient adsorption of germicide for accurate measurement, it was necessary to increase the inoculum size in these experiments to 3×10^8 cocci/ml. Even so, the amounts of radioactivity adsorbed were extremely low, particularly in the case of [^{14}C]TBS. A measure of the accuracy of the values for the molecules/coccus is also given in Table 1, where the ratio of the actual counts on the cocci to the total counts in the suspension is recorded. The results quoted in Table 1 are average figures from 3 to 6 separate experiments. In the case of [^{14}C]TCC, the figure of $5{\cdot}1 \times 10^5$ molecules/coccus at the m.i.c. was verified in an extended series of experiments to study the adsorption isotherm with a *S. aureus* suspension of 10×10^8 cocci/ml. (A. N. Sharpe & D. C. Kilsby, personal communication).

Table 1. *Adsorption of* [^{14}C]*-labelled germicides by Staphylococcus aureus at the minimum inhibitory concentration*

Germicide	Minimum inhibitory concentration (μg./ml.)	Molecules/coccus	'Accuracy' (ratio of counts on bacterial pellet to counts in whole suspension)
TCS	0·15	$0{\cdot}75 \times 10^6$	15/150, i.e. 10%
TBS	1·0	$2{\cdot}0 \times 10^6$	45/1000, i.e. 4·5%
TCC	0·15	$5{\cdot}1 \times 10^6$	100/260, i.e. 39%
GL31	0·75	$7{\cdot}2 \times 10^6$	40/370, i.e. 11%

Rate and amount of adsorption of [^{14}C]*TCS by Staphylococcus aureus.* The adsorption of [^{14}C]TCS by *Staphylococcus aureus* with concentrations of the germicide between 0·2 and 1·0 μg./ml. at 6×10^9 cocci/ml. was examined. The degree of adsorption after 0 and 60 min. is recorded in Fig. 1. This pattern of maximum adsorption at zero time, followed by an apparent desorption during the first hour of incubation, was quite characteristic, and was noted in every adsorption experiment. The adsorption did not decrease further during incubation up to 24 hr. The adsorption pattern was independent of whether the cocci were suspended in Yudkin growth medium or in 33 mM-phosphate buffer (pH 7·0).

The line in Fig. 1, which represents the adsorption by the cocci at 0 min. in this experiment, is virtually the same line that can be drawn between the average values from five separate experiments for the amounts adsorbed after 60 min. from [^{14}C]TCS 0·2 and 1·0 μg./ml. Using this line, therefore, we can say that with a population of 6×10^9 cocci/ml. the adsorption from [^{14}C]TCS 0·5 μg./ml. will be $0{\cdot}75 \times 10^5$ molecule/coccus. In all our experiments on the effects of TCS on the biochemical activities of *Staphylococcus aureus* populations of 6×10^9 cocci/ml. were used. Therefore, in order to be able to relate our findings in these experiments to the mechanism of bacteriostasis, we studied the effects of TCS at a concentration of 0·5 μg./ml. In both these experiments and at the m.i.c., the effective concentration of TCS was $0{\cdot}75 \times 10^5$ molecules/coccus.

Adsorption of [^{14}C]*TCS by Escherichia coli bacteria and spheroplasts.* In comparison with *Staphylococcus aureus* and other Gram-positive organisms (Woodroffe & Wilkinson, 1966*a*) *Escherichia coli* is very resistant to the action of TCS and the other skin germicides; the m.i.c. with TCS is 30 μg./ml. It would therefore be interesting to know whether this resistance is dependent on the rate or the amount of the adsorption of TCS, or on the site of this adsorption. We studied the adsorption of [^{14}C]TCS

1·0 μg./ml. by 6×10^9 bacteria/ml. in 33 mM-phosphate buffer (pH 7·0) and 30 mM-tris buffer (pH 8·0). The uptake of [^{14}C]TCS by *E. coli* was extremely variable from one experiment to another. Although the initial rate of adsorption was relatively constant at about $0{\cdot}3 \times 10^5$ molecules/bacterium/hr, the maximum amounts adsorbed varied from $0{\cdot}2 \times 10^5$ to $1{\cdot}5 \times 10^5$ molecules/bacterium. In the experiments showing the greatest adsorption of [^{14}C]TCS, 5–10 hr were needed to reach the maximum values. It is clear from the relative rates of adsorption that the affinity of *E. coli* for TCS is markedly less than that of *S. aureus*.

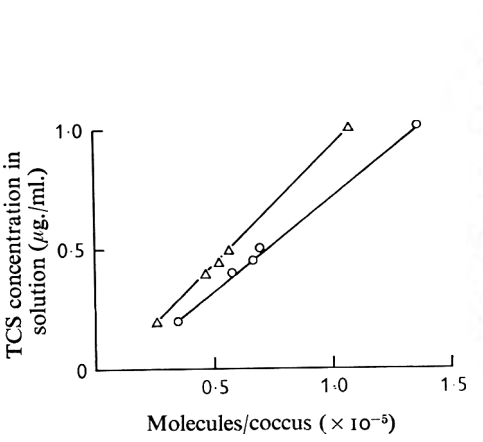

Fig. 1

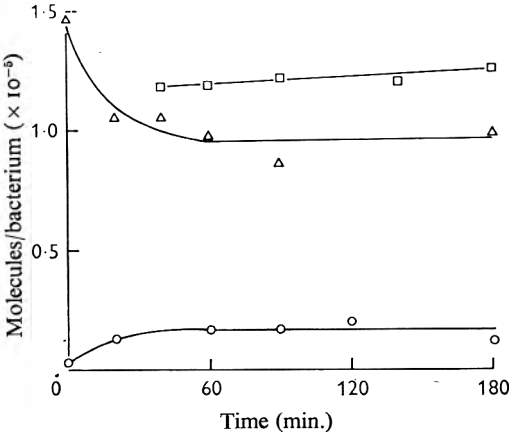

Fig. 2

Fig. 1. Adsorption of [^{14}C]TCS by *Staphylococcus aureus*. o min., ○; 60 min., △.

Fig. 2. Adsorption of 1·0-μg./ml. [^{14}C]TCS by *Escherichia coli*. Whole bacteria, ○; spheroplasts, TCS added at o min., △; spheroplasts, TCS added after 40 min. incubation with lysozyme and EDTA, □.

We examined the uptake of 1·0 μg./ml. [^{14}C]TCS by spheroplasts prepared from *Escherichia coli* by the action of lysozyme and EDTA in tris buffer (Repaske, 1958). The results are recorded in Fig. 2. In this experiment the whole organisms adsorbed very little germicide. [^{14}C]TCS was added to two volumes of spheroplast suspension at different times. In one flask, the germicide was added with the lysozyme and EDTA at zero time; in the second flask the germicide was added after the bacteria had been incubated for 40 min. with lysozyme+EDTA. In both flasks spheroplast formation was complete after 15 min. Throughout the incubation, there was no microscopic evidence of lysis of spheroplasts, and the presence of the germicide did not increase the release into the medium of 260 mμ absorbing material.

When [^{14}C]TCS was added at zero time, the pattern of adsorption was similar to that found with *Staphylococcus aureus*, i.e. maximum adsorption occurred immediately, and during the first hour desorption took place until a steady level was reached. When [^{14}C]TCS was added to the spheroplast suspension after 40 min., this maximum was not found, and the degree of adsorption reached immediately was maintained during the course of the experiment. The amount of [^{14}C]TCS adsorbed by the spheroplasts was $1{\cdot}1 \times 10^5$ molecules/bacterium, taking the average figure from the two flasks. This compares with the maximum figure of $1{\cdot}5 \times 10^5$ molecules/bacterium that was found, but only in some experiments and after prolonged incubation, with whole *Escherichia*

coli organisms, and the figure of $1{\cdot}37 \times 10^5$ molecules/bacterium with *S. aureus*; (Fig. 1).

Minimum inhibition concentration of TCS with Escherichia coli. Attempts were made to decrease the m.i.c. of TCS with *Escherichia coli* by increasing the adsorption of the germicide, or by inhibiting growth during the period of time required for the adsorption by whole organisms. The m.i.c. of TCS with *E. coli* was 30 μg /ml.; this value was not decreased by adding EDTA 200 μg./ml. to the growth tubes. However, when the m.i.c. tubes were incubated overnight at 4° before the growth incubation at 30° for 24 hr, the m.i.c. was decreased to TCS 15 μg./ml. Although the size of the inoculum could be decreased from $2{\cdot}8 \times 10^8$ to $1{\cdot}2 \times 10^8$ viable bacteria/ml. by this pre-incubation at 4°, such a decrease in numbers did not cause any decrease of the m.i.c. below TCS 30 μg./ml.

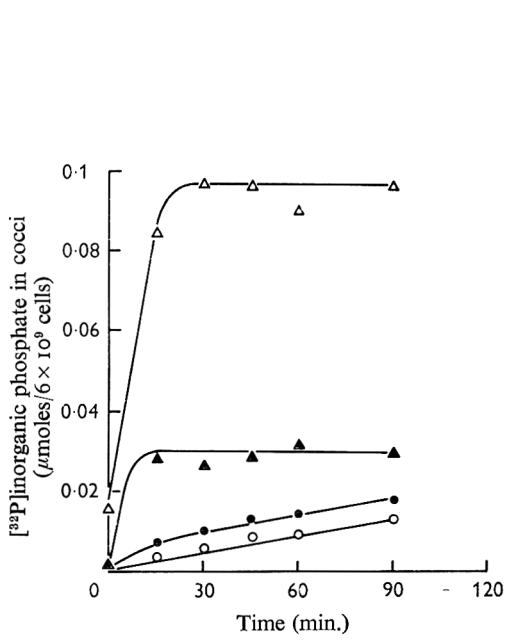

Fig. 3

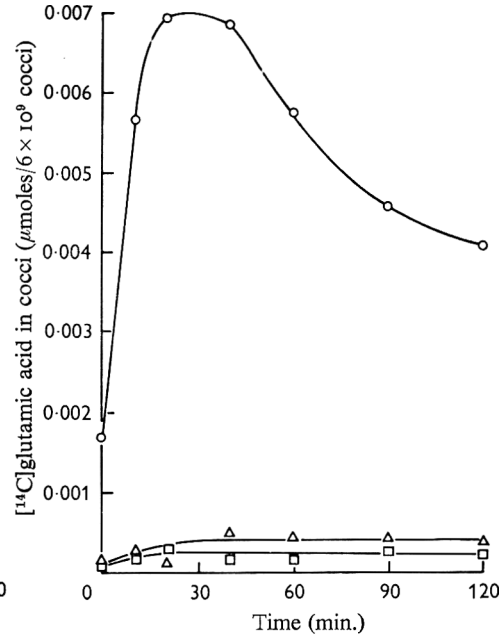

Fig. 4

Fig. 3. Uptake of [^{32}P]inorganic phosphate into the 'pool' fraction of *Staphylococcus aureus*. Control cocci, ○; control cocci plus glucose, △; cocci plus 0·2 μg./ml. TCS, ●; cocci plus 0·2 μg./ml. TCS plus glucose, ▲.

Fig. 4. Uptake of [^{14}C]glutamic acid into *Staphylococcus aureus*. Control cocci, ○; cocci plus 0·5 μg./ml. TCS, △; cocci plus 10 mM-dinitrophenol, □.

Minimum inhibitory concentration with lipid-rich Staphylococcus aureus and two coagulase-negative staphylococci. Hugo & Stretton (1966) showed the increased resistance of *S. aureus* to penicillins when grown in glycerol-containing medium. They claimed that the resistance of these organisms derived from their increased lipid content (from 6·3 to 18·4% bacteria dry wt), and that this lipid was situated at or near the cell surface. We examined the effect of increasing the lipid content of *S. aureus* on its sensitivity to the germicides, TCS, TCC, TBS, GL31 and hexachlorophene. The m.i.c. values were unaltered, although on prolonged incubation very small increases in resistance were sometimes found with the lipid-rich organisms.

We also measured the m.i.c. of TCS for two coagulase-negative skin staphylococci. Ivler (1965) reported many differences between the biochemical activities of coagulase positive and coagulase-negative staphylococci, claiming that the primary endogenous reserve material in coagulase-positive organisms is the amino acid pool, and in coagulase-negative organisms, poly-β-hydroxybutyrate. If bacteriostasis resulted from any specific effect of TCS on the cell's metabolism, one might therefore expect a difference in the sensitivities of coagulase-positive and coagulase-negative organisms. The m.i.c. of TCS for these skin staphylococci was also found to be 0·15 μg./ml. however.

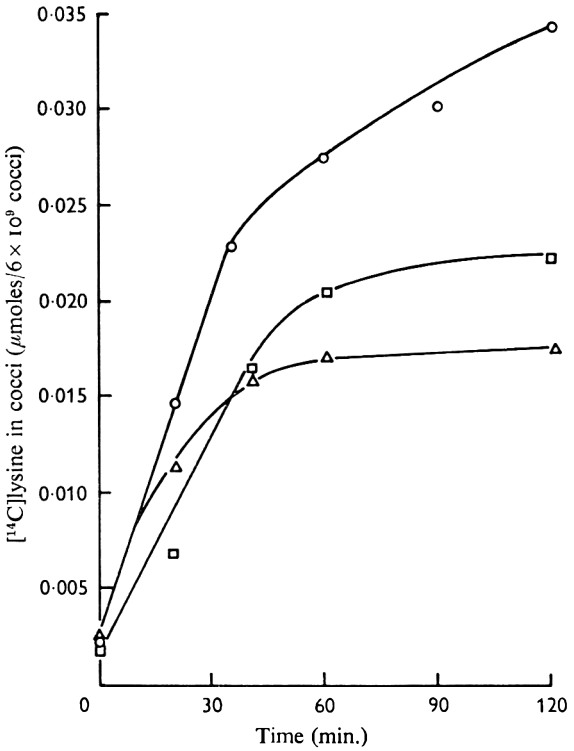

Fig. 5. Uptake of [^{14}C]lysine into *Staphylococcus aureus*. Control cocci, ○; cocci plus 0·5 μg./ml. TCS, △; cocci plus 10 mM dinitrophenol, □.

Biochemical activities

Uptake of [^{32}P]inorganic phosphate by Staphylococcus aureus. The effect of TCS on the uptake of [^{32}P]inorganic phosphate is shown in Fig. 3. [^{32}P] was taken up into the pool fraction of the control organisms at a steady rate throughout the 90 min. incubation period. With glucose as an added energy source the rate of entry of [^{32}P] was greatly increased and a high steady state value was reached after 40 min. TCS did not inhibit the glucose-independent uptake of phosphate (in this particular experiment a slight stimulation was found), but although the rate of entry in the presence of glucose was also not inhibited by TCS, the steady-state value reached was however decreased by the germicide.

Uptake of [^{14}C]amino acids by Staphylococcus aureus. Gale (1954) described the energy-dependent accumulation of glutamic acid by *Staphylococcus aureus* and the

energy-independent accumulation of lysine. We examined the effects of TCS on these two systems. TCS almost completely inhibited the uptake of [^{14}C]glutamic acid (Fig. 4). A similar inhibition was found with the energy uncoupler dinitrophenol.

The results with [^{14}C]lysine are shown in Fig. 5. Although the initial rate of entry of the amino acid was not inhibited by TCS, the amount taken up by the bacteria was, and a similar effect was found with dinitrophenol. In this experiment samples were withdrawn for cell fractionation, and the effect of TCS on the incorporation of [^{14}C]-lysine into the lipid, nucleic acid, and protein fractions examined. In the control flask

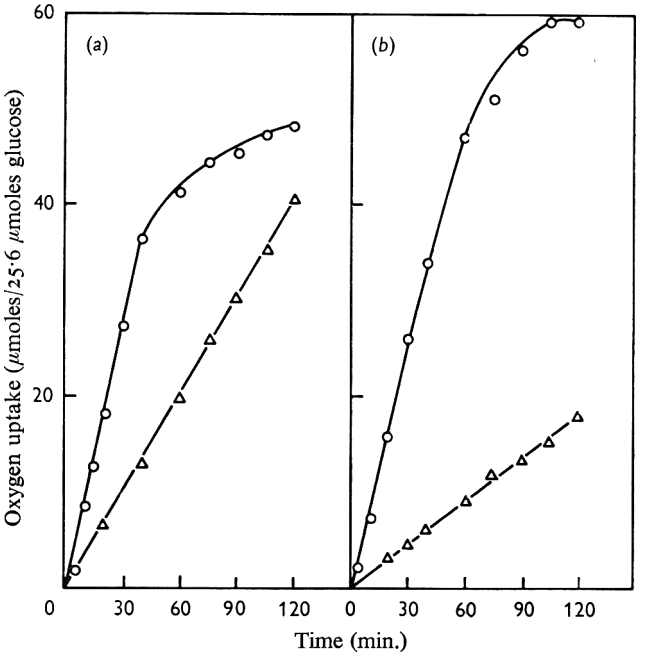

Fig. 6. Oxidation of glucose by *Staphylococcus aureus*. (*a*) Control cocci, and (*b*) cocci plus 0·5 μg./ml. TCS. Glucose oxidation, endogenous subtracted, ○ ; endogenous oxidation, △.

there was a linear rate of incorporation of radioactivity into the lipid and protein fractions. This incorporation was inhibited 75% and 100% respectively by TCS.

Uptake of [^{14}*C]glucose by Staphylococcus aureus and its incorporation into cell fractions.* The effect of TCS on the oxygen uptake of *S. aureus* metabolizing endogenously and with glucose added as exogenous substrate is shown in Fig. 6. From studies of the oxygen uptake with graded amounts of added glucose, it was shown that endogenous oxidation continued in the presence of the oxidation of added substrate, and that this was not affected by TCS. The oxygen uptake figures with glucose in Fig. 6 are corrected for endogenous metabolism. The data are recorded as μmoles O_2/25·6 μmoles glucose, this being the amount of glucose in the 45 ml. volume of the flasks in the experiment illustrated in Fig. 7. The rate of endogenous metabolism is reduced by 56% in the presence of TCS. Although the rate of glucose oxidation was not affected by TCS, in the presence of TCS the oxygen uptake was increased from 48·2 O_2/25·6 μmoles glucose to 59·5 μmoles O_2/25·6 μmoles glucose. This increase in oxygen uptake is typical of the action of the energy uncouplers.

In a parallel experiment, the distribution of the [^{14}C]glucose in the suspension was examined (Fig. 7). Radioactivities have been expressed in terms of μmoles glucose/flask. In the control flask the glucose concentration in the whole suspension was decreased from 25·6 to 15·8 μmoles, and in the cell-free supernatant fluid to 10·0 μmoles after 120 min., i.e. 5·8 μmoles glucose had been incorporated. This figure of 5·8 μmoles was obtained by difference calculation, and the actual figure from the radioactivity of the bacterial pellet was 5·25 μmoles. Of this incorporated glucose, 0·58 μmole was located in the lipid fraction, 0·77 μmole in the protein fraction, and 1·05 μmoles in the nucleic acid fraction. By difference, the glucose in the low molecular weight pool fraction was therefore 2·85 μmoles.

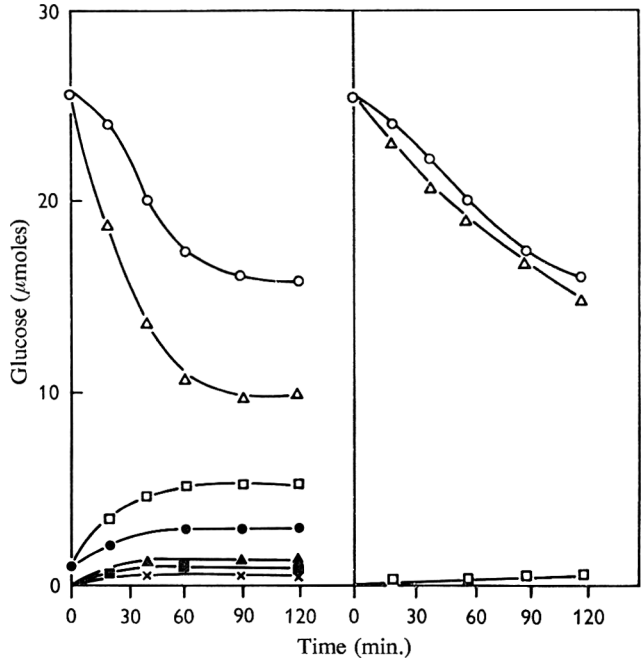

Fig. 7. Assimilation of [^{14}C]glucose into *Staphylococcus aureus*. (*a*) Control cocci, and (*b*) cocci plus 0·5 μg./ml. TCS. Suspension, ○; cell-free supernatant, △; whole pellet, □; 'pool' fraction, ●; nucleic acid; ▲ protein, ■; lipid, ×.

In the presence of TCS, 9·6 μmoles glucose were lost from the suspension during the 120 min. incubation, and 10·6 μmoles from the cell-free supernatant fluid. Therefore by difference the amount of glucose incorporated into the bacteria was 1·0 μmole. The radioactivity of the bacterial pellet however gave a figure of only 0·38 μmole. None of this material was found in the lipid, nucleic acid or protein fractions.

Release of [^{14}*C*]*amino acids by Staphylococcus aureus.* Cocci which had been preloaded with [^{14}C]glutamic acid and contained 0·0064 μmole labelled amino acid/6×10^9 bacteria, leaked about 50% of this material during the first hour of incubation in fresh medium (Fig. 8). In the presence of TCS, all the amino acid was released; this was also found with sodium azide and (not shown on graph) dinitrophenol. A different leakage pattern was found, however, with cocci preloaded with [^{14}C]-alanine (Fig. 9). The amino acid pool which can be extracted with hot water contained

0·0018 μmole [^{14}C]alanine/6 × 10^9 bacteria, and during the incubation for 2 hr the control leaked only 0·00018 μmole, i.e. 10% of the total pool alanine. Even in the presence of TCS or azide, this leakage only increased to 0·00035 μmole/2 hr/6 × 10^9 bacteria.

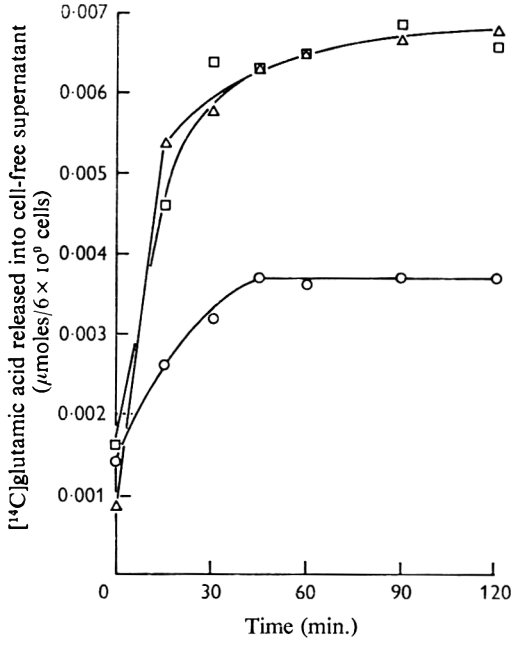

Fig. 8

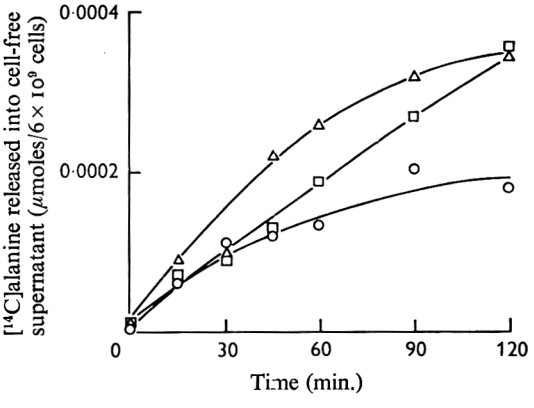

Fig. 9

Fig. 8. Release of [^{14}C]glutamic acid from *Staphylococcus aureus*. Control cocci, ○; cocci plus 0·5 μg./ml. TCS, △; cocci plus 30 mM sodium azide, □.

Fig. 9. Release of [^{14}C]alanine from *Staphylococcus aureus*. Control cocci, ○; cocci plus 0·5 μg./ml. TCS, △; cocci plus 30 mM sodium azide, □.

DISCUSSION

As pointed out in the Introduction, a common feature of the antibacterial action of the detergents, phenols, quaternary ammonium compounds, polypeptide antibiotics, and the skin germicides studied here, is their ability to cause cell leakage and membrane damage. In studies with CTAB and polymyxin, Salton (1951) and Few & Schulman (1953) showed that an important feature of the mechanism of action of these compounds is their adsorption in large amounts on to the sensitive bacterium. From our own studies, and those of Woodroffe & Wilkinson (1966*b*), it appears that TCS, and the other skin germicides, hexachlorophene, TBS, TCC and GL31, function in a like manner through their reversible adsorption on the sensitive cell membrane of the bacterium. It has been suggested (Newton, 1956) that polymyxin combines with ionized phosphate groups of phospholipid components of the membrane. Few & Schulman (1953) and Newton (1954) showed that with organisms resistant to polymyxin, the resistance depended to some extent on the chemical composition and structure of the cell wall. In the resistant organism, the wall itself has only a low

affinity for polymyxin and there is little penetration to the underlying cell membrane. Our results on the adsorption of TCS by whole organisms and spheroplasts of *Escherichia coli* show that a similar mechanism of resistance occurs with TCS. Joswick (1961) has shown that the resistance of *E. coli* to hexachlorophene is a property of the cell wall.

On the basis of these findings, we can propose the following working hypothesis. All of the membrane-active antibacterial compounds share a common mode of action, in which the adsorption of the antibacterial substance on to the cell membrane is a critical step. The amounts of germicide adsorbed, and its rate and ready reversibiity with many of the compounds indicate that this adsorption may be a relatively non-specific physicochemical phenomenon. The antibacterial spectrum of these compounds (cationic detergents, tyrocidin and skin germicides are more active against Gram-positive bacteria, phenol and polymyxin being more active against Gram-negative organisms) is a consequence of the nature of the interaction between the antibacterial substance and the cell wall of the organism under study. As with polymyxin and TCS, resistance results from a greatly decreased penetration of the antibacterial substance to its combining site or sites on the membrane. On the basis of this unifying hypothesis, results gained from the study of TCS have an increased significance for the whole field of membrane-active antibacterial compounds.

The increase in the resistance of Gram-negative organisms to quaternary ammonium compounds is associated with an increase in the cellular lipid content (Chaplin, 1952; MacGregor & Ellicker, 1958). Hugo & Stretton (1966) have increased the resistance of *Staphylococcus aureus* to penicillins by the technique of increasing the cellular lipid content by growing the cells in a glycerol-containing medium. Our results with TCS, however, show that a simple increase in the cellular lipid content of *S. aureus* does not confer resistance to this group of compounds. Under conditions of prolonged incubation, minor increases in the m.i.c. of TCS, TCC and hexachlorophene were found with the lipid-rich bacteria. The significance of these increases is very doubtful, however, and they appear to have little connexion with either the resistance of lipid-rich *S. aureus* to the penicillins, or the resistance of Gram-negative organisms to TCS and related compounds.

With TCS, TBS, TCC and GL31, approximately 10^5 molecules/bacterium are required to inhibit the growth of *Staphylococcus aureus*. This figure has a threefold significance. First, the closeness of the agreement between the figures for molecules/bacterium at the m.i.c. with the four germicides, clearly indicates the similarity in their modes of action. Secondly, the demonstration that such a comparatively large amount of germicide is required to inhibit growth reinforces the conclusion drawn from the results of adsorption studies and the reversible uptake by bacteria and membranes of *Bacillus megaterium* (Woodroffe & Wilkinson, 1966*b*) that the uptake of germicide by cell membrane is a non-specific physicochemical phenomenon. Thirdly, the knowledge of the concentration of the germicide on the bacteria which causes bacteriostasis, allows us to examine alterations and inhibitions in the metabolic activities of the cell at this same cellular concentration of germicide, and to relate the findings to the mechanism of bacteriostasis. The demonstration that succinoxidase, glucose oxidation and glucose fermentation in *S. aureus* are inhibited by TCS, 0·2, 1·0 and 20 μg./ml. respectively (Woodroffe & Wilkinson, 1966*a*), shows that the effects observed with TCS are extremely dependent on concentration. In assessing the relevance of any

findings in such a study, therefore, the importance of working at the same cellular concentration of the germicide as at the m.i.c. cannot be stressed too strongly.

In examining the effects of TCS on washed suspensions of *Staphylococcus aureus* the obvious region to consider first is the cell membrane and the many biochemical activities associated with it. Leakage by itself appears most unlikely to be the primary cause of either bacteriostasis or cell death. Bacteriostasis results from an inhibition which is annulled on the removal of the inhibitor. If leakage plays a part in the bacteriostatic action of TCS, it cannot simply be the loss of cellular material which results in the inhibition of growth, but the continuing inability of the cell to make good this loss in the presence of the germicide. Since maximum leakage from *S. aureus* is found at bacteriostatic rather than bactericidal concentrations of TCS (Woodroffe & Wilkinson 1966*a*), neither can cell death be the direct result of cell leakage. Similar conclusions about the secondary importance of leakage phenomena have been reached in studies of the bactericidal action of hexachlorophene (Silvernale, 1966), chlorhexidine (Hugo & Longworth, 1964, 1966) and the quaternary *p*-diisobutylphenoxyethoxyethyldimethylbenzyl ammonium chloride (Stedman *et al.* 1957). One can, in any case, gain a much better understanding of the functioning of the cell membrane from the study of the uptake of specific nutrients by cells. Danielli (1954) described three mechanisms whereby small molecules can pass through cell membranes. In simple diffusion, molecules move under the driving force of thermal agitation. Facilitated or exchange diffusion again operates under the driving force of thermal agitation, but carrier or transport molecules in the membrane are involved and these facilitate the transport across the membrane at an increased rate. In active transport, metabolic energy is coupled to the process of facilitated diffusion, and the amount of material transported and accumulated within the cells against a concentration gradient is increased. This concept of the mechanisms of membrane transport in bacteria has been extended by the work of Cohen & Monod (1957), Herzenberg (1959) and Egan & Morse (1965).

Our results with the effects of TCS on the uptake of [^{32}P]inorganic phosphate were obtained at a concentration of TCS 0·2 μg./ml. These experiments were made before we had established the necessity of using TCS 0·5 μg./ml. to give the same cellular concentration of the germicide as is required to inhibit growth. In his studies of [^{32}P]inorganic phosphate uptake by *Staphylococcus aureus*, Mitchell (1954) showed that the cells possessed both an energy-independent exchange diffusion mechanism, and in the presence of a metabolizable energy source such as glucose, an energy-dependent active transport. It appears from our results that the energy-independent facilitated diffusion of phosphate is unaffected by TCS, but that the energy-dependent active transport is inhibited by it. That this inhibition of active transport is not complete may be significant in view of the fact that the concentration of TCS on the bacteria in these experiments was less than that necessary to inhibit growth.

Gale (1954) described the uptake of the amino acids glutamic acid and lysine by *Staphylococcus aureus*. The uptake of glutamic acid was entirely energy-dependent, whereas lysine entered and was accumulated by means of an energy-independent facilitated diffusion. Our results show that both the energy uncoupler dinitrophenol and TCS virtually completely inhibited the uptake of glutamic acid, but only partially inhibited the uptake of lysine. With lysine, the initial rate of uptake was not affected, but the total amount accumulated was decreased by both dinitrophenol and TCS. It

appears, therefore, that there are two systems for the uptake of lysine by the staphylococci, one energy-dependent and TCS-sensitive, and the other energy-independent and TCS-resistant. These two systems of lysine transport in *S. aureus* have been verified and further studied by Gale & Folkes (1967).

From our results on the effect of TCS 0·5 μg./ml. on the oxidation of glucose by *Staphylococcus aureus*, it is obvious that the entry of glucose into the organisms was not inhibited by TCS; nor were the glucose degradative enzymes or the electron transport system.

It appears therefore that those systems of entry or accumulation which are energy-dependent (i.e. phosphate uptake in the presence of glucose, glutamic acid uptake, and one of the lysine uptake systems) are inhibited by TCS, whereas those systems which are energy-independent (i.e. facilitated diffusion of phosphate, the other lysine uptake system, the entry and catabolism of glucose) are equally independent of the action of TCS. The lysine and glucose which entered the control bacteria were incorporated in various cell fractions, but no such incorporation was found in the TCS-treated organisms. The common feature of the incorporation of carbon from glucose and lysine into the lipid, nucleic acid and protein fractions is that each of these processes is endergonic and must be coupled to the energy-producing mechanisms within the cell. That is to say, the effect of TCS at 0·5 μg./ml., i.e. $0{\cdot}75 \times 10^5$ molecules/bacterium, is to inhibit either the production of energy or its coupling to the endergonic reactions of the cell's metabolism. This inhibition of the functioning of the energy metabolism in *Staphylococcus aureus* by TCS at its cellular bacteriostatic concentration is sufficient to cause the inhibition of growth.

Our data on the release of amino acids from the cell pool also provide evidence about the mechanism of action of TCS. From these studies, and those of Gale (1954), we know that the uptake of glutamic acid by *Staphylococcus aureus* is an energy-dependent process and the uptake of alanine energy-independent. Our results with the uncoupler sodium azide show that the maintenance of these amino acids within the cell pool are similarly energy-dependent and energy-independent processes, respectively. The release of these amino acids from the cells is the same in the presence of TCS as in the presence of the uncoupler of oxidative phosphorylation. There is no evidence of further membrane damage resulting in the complete release of alanine from the cells. It appears therefore that the leakage of cell contents which has been reported with TCS (Woodroffe & Wilkinson, 1966*a*) is simply the release of those materials whose maintenance within the pool is dependent on an energy-coupled mechanism. Leakage is a secondary effect, and of secondary importance.

TCS at 0·35 μg./ml. has been found to be an uncoupler of oxidative phosphorylation in beef heart heavy mitochondria (R. B. Beechey, personal communication); Hotchkiss (1944) reported that the polypeptide antibiotic gramicidin could act as an uncoupler of oxidative phosphorylation in bacteria. Preliminary data show that the uptake of [^{32}P]inorganic phosphate and [^{14}C]glutamate by *Staphylococcus aureus* are equally sensitive to the action of TCS under anaerobic conditions, but this does not exclude the possibility that TCS acts as an uncoupler of oxidative phosphorylation in *S. aureus* (see Kovač & Kužela, 1966). As yet however we have no data on the mechanism of this inhibition by TCS of the energy metabolism in bacteria.

Nor do we have information about the chemical or physical alterations resulting in the membrane from the adsorption of TCS or any of the other membrane-active

antibacterial compounds, but from the non-specific physicochemical nature of this adsorption one might expect changes in the membrane structure, resulting from the breakage of hydrogen bonds, etc., rather than the loss or inactivation of specific chemical or enzymic groupings. The critical dependence of energy metabolism, and other membrane associated activities, upon the ultrastructure of the membrane has been amply demonstrated, particularly by Green and his colleagues (Green & Perdue, 1966), and the study of the mechanism of action of the membrane-active antibacterial compounds in terms of current theories of membrane structure and function would seem to be a most challenging area of research.

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Electron Microscopy of Salmonella Flagella in Methylcellulose Solution

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SUMMARY

When peritrichously flagellated cells such as normal, curly, paralysed-curly and small-amplitude strains of *Salmonella abortusequi* are suspended in 0·5% methylcellulose solution, flagellar bundles can be clearly seen by electron microscopy. In a bundle, five or more of the component flagella are tightly united in parallel with each other, and the bundle formed into a helix with a shape characteristic for each strain. These figures reveal the structural detail of the flagellar bundle observed under a dark-field microscope. Ten minutes after the cells were suspended in methylcellulose solution, bundled flagella could be seen in approximately 70% of the cells of normal, curly and paralysed-curly strains; the remaining 30% were dispersed. At this time among the normal cells, some were single-bundled and others were multi-bundled. The fraction of single-bundled cells was larger in both motile and paralysed curly-flagellated cells than in normal cells. The fraction of normal cells having single bundles increased with time. Methylcellulose was therefore presumed to enhance aggregation of flagella and/or to inhibit the dispersion of the aggregated flagella.

In the small-amplitude strain, transformation of flagellar shape to curly has been previously observed. In methylcellulose solution, this transformation occurs in bundled flagella but not in dispersed flagella. It is inferred that the tight association of the component flagella in methylcellulose solution enhances the stress among the flagella, thus causing the transformation.

INTRODUCTION

Pijper (1957), using dark-field microscopy, was able to observe bundled flagella in moving bacteria. They can also be observed by electron microscopy (Mitani & Iino, 1965), although with more difficulty, especially in the case of normal flagella. Pijper & Abraham (1954) report that suspension of bacteria in colloidal substances such as agar, gum arabic and methylcellulose facilitates the observation of bundled flagella by dark-field microscopy. Suspension of bacteria in methylcellulose has proved equally useful for electron microscopy. The present paper describes our observations of flagella in normal cells as well as in derived mutant strains. Observations of the transformation of shape of bundled flagella from small-amplitude to curly previously made by dark-field light microscopy (Iino & Mitani, 1966) were repeated by electron microscopy.

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METHODS

The strains used in the present observations were *Salmonella abortusequi* SL23, which has peritrichous normal flagella, and the derived mutants SJ30, SJ706 and SJ712. SJ30 has curly flagella; this is thought to be due to a mutation of the structural gene for flagellin of SL23 (Iino, 1962). SJ706 is a paralysed-curly strain found as a mutation of a motility gene for SJ30 (Mitani & Iino, 1965). SJ712 is a small-amplitude mutant from SJ30, in which transformation of flagella from small-amplitude to curly was observed by dark-field microscopy (Iino & Mitani, 1966).

The medium for the cultivation of bacteria consisted of (w/v) 0·15% beef extract (Difco), 0·15% yeast extract (Difco), 0·5% Kyokuto peptone, 0·1% glucose, 0·35% NaCl, 0·36% K_2HPO_4, and 0·32% KH_2PO_4. Cells cultivated overnight at 37° without shaking, inoculated into .10 ml. of the medium and incubated for 2 hr with aeration at 37°, were used for microscopy unless otherwise stated.

For electron microscopy, cells were harvested by centrifugation at 1200 *g* for 15 min., and resuspended in a solution containing equal volumes of 2% (w/v) potassium phosphotungstate and 0·5% (w/v) methylcellulose, pH 7·0. As a control, centrifuged cells were resuspended in 1% (w/v) solution of potassium phosphotungstate. Motility was retained for at least 180 min. in these suspensions. Small drops of the suspension were then placed on collodion supporting films at the intervals described in each experiment and dried immediately with a vacuum pump. The electron microscope used was a JEM T6S (Japan Electron Optics Laboratory Co., Ltd.), with a single condenser system and accelerating voltage of 60 kV.

Electron micrographs were taken at an initial magnification of × 5000 on Sakura hard process plates. One-micron Bacto-latex particles (Difco Lab., Inc., Detroit, Michigan, U.S.A.) were photographed at the same magnification in order to determine the final magnification. To measure the spiral unit length of waves in flagella, the micrographs were projected and several waves measured in a single flagellum or a flagellar bundle on different organisms; in total 50 to 80 waves were examined per clone.

Dark-field microscopy was done by the procedure of Iino & Mitani (1966).

RESULTS

Bundled flagella in methylcellulose solution

Plates 1 and 2 show electron micrographs of flagellar bundles of normal, curly and paralysed-curly cells suspended in methylcellulose solution. The bundled flagella were more tightly aggregated in the presence of methylcellulose than in its absence (cf. Fig. 3, Mitani & Iino, 1965). The proportion of the cells with bundled flagella in each bacterial culture did not differ significantly among the three strains, even in motile and paralysed ones; approximately 70% of the flagellated cells were bundled and the remaining 30% were dispersed (Table 1). Among the cells with bundled flagella, some were single-bundled, i.e. all flagella of a cell had united to form a bundle, and others were multi-bundled, i.e. more than two flagellar bundles were formed per cell. The fraction of single-bundled cells was substantially larger in both curly (65%) and paralysed-curly cells (57%) than in normal cells (15%).

The observation of normal cells at 10 and 180 min. after the cells had been sus-

pended in methylcellulose solution showed that the fraction of dispersed flagella did not change during this interval, but there was an increase of single-bundled cells at the expense of multi-bundled cells (Table 2). Further observations over shorter intervals indicated that the proportion of single-bundled increased between 10 and 30 min. after suspending of the cells in methylcellulose; after 30 min. the ratio between single- and multi-bundled cells changed little.

Table 1. *The relative frequency of bundled and dispersed flagella observed in normal, curly and paralysed-curly strains of Salmonella abortusequi suspended for 10 min. in 0·5% (w/v) methylcellulose solution.*

Strain	Cells observed	Flagellation (%)	Flagellated cells (%)		Bundle flagellated cells (%)		Average no. of flagella/cell
			Bundled	Dispersed	Single	Multiple	
SL 23 (normal)	100	97	68	32	15	85	9·5 ± 1·2*
SJ 30 (curly)	129	57	70	30	65	35	10·7 ± 2·6
SJ 706 (paralysed-curly)	131	54	69	31	57	43	6·4 ± 1·4

* Ninety-five % confidence level.

Table 2. *Change in the proportion of multi- and single-bundled types among bundled flagella of normal strain SL23 with time in methylcellulose solution*

Time in methylcellulose solution (min.)	Cells observed	Bundle flagellated cells (%)		Dispersely flagellated cells (%)
		Single	Multiple	
0	105	0	0	100
10	100	15	85	32
180	106	33	67	34

Transformation of the shape of bundled flagella in small-amplitude strain SJ712

The wavelength, amplitude and helical unit length, which is defined as a contour length of one wave of flagellar bundle, were comparable in strains SL 23, SJ 30 and SJ 706 to those of dispersed flagella and did not change during the period of observation. On the other hand, if strain SJ 712 was suspended in 0·5% methylcellulose solution, in the bundled flagella a transformation of curvature from small-amplitude to curly occurred. Dark-field photomicrographs of this transformation of flagellar bundles are shown in Pl. 3, where it can be seen that the transition is initiated at the distal end of the flagellar bundle and proceeds proximally to its base. On this organism, one helical unit of the small-amplitude transformed to curly in approximately 45 min. Continuous observation showed that the transformed part had twirled around the axis of the original flagellar bundle. An overnight culture of SJ 712 was examined in the dark-field microscope 80 min. after suspension of the cells in 0·5% methylcellulose solution. The fractions of the cells with small-amplitude, curly and dimorphously bundled flagella of the former two flagellar shapes were 59, 36 and 5% respectively. But when the cell had been previously fixed with 0·5% formalin the transformation was never observed.

The morphological change of the flagella of this strain had not previously been observed electron-microscopically on preparations stained with 2% potassium phos-

photungstate (Iino & Mitani, 1966). The reason was made clear when the solution of 0·5 % methylcellulose in which the small-amplitude strain was suspended was dropped on a grid, and the preparation was observed in the electron microscope. Two morphologically different types of flagellar bundles, one shorter and the other longer, were identified (Pl. 4). In methylcellulose, some of the cells of this strain also formed flagellar bundles and the rest dispersed, as has been shown for the other strains. The longer the exposure to methylcellulose was, the larger the number of cells with bundled flagella of shorter helical unit length. Three hundred minutes after the cells had been suspended in methylcellulose the flagellar bundles were examined by electron-microscope and photographed; and their helical unit length was measured. As a control, the flagellar bundle of the cells kept for 10 min. in methylcellulose was observed. The distribution diagrams of the helical unit length of the flagellar bundle of these two samples indicated that the fraction of shorter helical unit length was increased 12 times

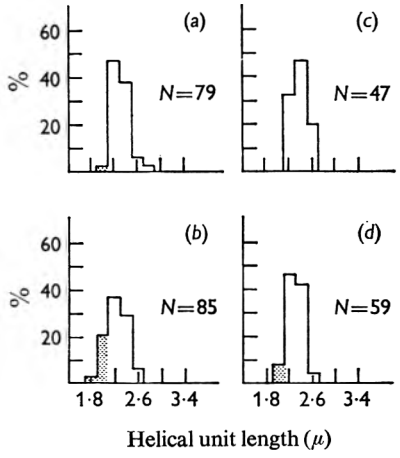

Fig. 1

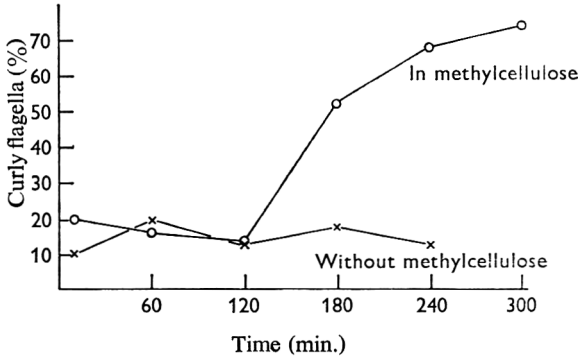

Fig. 2

Fig. 1. Distribution diagram of helical unit length of flagella in Salmonella SJ 712: bundled flagella (*a*) 10 min., (*b*) 300 min., and dispersed flagella (*c*) 10 min., (*d*) 300 min. after the bacteria had been suspended in methylcellulose solution. *N* denotes the number of flagellar waves observed. Dotted parts show the fraction of the bacteria with shorter helical unit length.

Fig. 2. Transformation of bundled flagella from small-amplitude to curly in Salmonella SJ 712 as observed by electron microscopy: ○, with methylcellulose; ×, without methylcellulose. Number of bacteria observed at each time interval varied from 92 to 116.

in the preparation which remained 300 min. in methylcellulose (Fig. 1*a*, *b*). Further timed experiments indicated that the cell fraction with the short wavelength flagella started to increase 120 min. after the cells had been suspended in methylcellulose solution (Fig. 2).

From these results, it was inferred that the longer helical unit type corresponded to the small-amplitude type, and the shorter to the curly type observed by dark-field microscopy. The increase in the proportion of organisms of shorter helical unit length was taken as a manifestation of the transformation of the flagellar bundle as demonstrated by electron microscopy. However, this experiment alone did not allow a decision as to whether the effect of methylcellulose on the transformation was on the

individual flagella or on the flagella when bundled. Therefore the helical unit lengths of the dispersed flagella on the preparations kept both 10 and 300 min. in methylcellulose solution were measured. The change of the helical unit length was very slight even though the cells were suspended for 300 min. in that solution (Fig. 1 *c*, *d*). The observations are interpreted to mean that the transformation of the flagella of this strain is promoted in methylcellulose solution on flagellar bundles but not on individual flagella. Although dimorphous flagellar bundles have been seen by dark-field light microscopy they have not hitherto been examined in the electron microscope. Furthermore, the distribution pattern of Fig. 1 is much sharper than that without methylcellulose (cf. Fig. 3 of Iino & Mitani, 1966).

DISCUSSION

In an earlier paper (Mitani & Iino, 1965), bundles of peritrichous flagella were demonstrated electron-microscopically on a Salmonella strain with curly flagella. Bundles were also observed on a normal strain, but much less frequently than on the curly strain, and the observed bundles were rather loose and irregular. The present investigation shows that when the organisms are suspended in methylcellulose solution, flagellar bundles are clearly seen in the electron microscope in flagellated strains of various morphological types, including normal, curly and small-amplitude. In a bundle, five or more of the component flagella arrange themselves parallel to each other, and the bundle gyrates helically with the characteristic wave. Within the bundled flagella of cells suspended in methylcellulose solution the component flagella are more tightly and regularly aggregated than in samples prepared without methylcellulose. Methylcellulose may be effective in bringing about the tight combination of component flagella in a bundle to each other and preserving the bundle during specimen preparation for electron microscopy.

The increase of single-bundled cells in methylcellulose solution with time may support the idea that in methylcellulose solution aggregation of flagella is enhanced and/or the dispersion of the aggregated flagella is inhibited (Stocker, 1956). This may be true even if the cells are non-motile; on the paralysed-curly strain, it was shown that the fraction of bundled flagella in methylcellulose was considerably larger than that without methylcellulose (cf. Table 1 of this paper and table 1 of Mitani & Iino, 1965).

For the observation of flagella the methylcellulose treatment has an additional advantage: it is effective in preserving the regular flagellar waves during the process of drying for electron-microscopical observation. It is noticeable when Fig. 1 of this report is compared with Fig. 2 of Iino & Mitani, (1966) that the scatter of helical unit length is narrower when measured on the samples suspended in methyl-cellulose solution. The difference in the dark-field and electron microscope on the cells of strain SJ712 suspended in methylcellulose suggests that certain types of flagella have a tendency to stretch and flatten even in the presence of methylcellulose.

As for the transformation of flagellar shape from small-amplitude to curly in a mutant Salmonella strain, SJ712, this occurred on bundled flagella when they were suspended in methylcellulose solution but not on dispersed ones. When methylcellulose is absent bundled flagella are presumed not to be transformed. The stress brought about in the component flagella by their tight association in a bundle may cause the transformation.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Dispersed normal flagella of *Salmonella abortusequi* SL 23. The cells had been suspended in distilled water (pH 6·8) and were negatively stained with potassium phosphotungstate (pH 7·0) for 10 min. × 14,400.

Fig. 2. Bundled flagella of SL 23, 300 min. after the cells had been suspended in a mixture of potassium phosphotungstate and methylcellulose. × 14,400.

Fig. 3. Dispersed flagella of SL 23 seen 300 min. after the cells had been suspended in a mixture of potassium phosphotungstate and methylcellulose. × 14,400.

PLATE 2

Fig. 4. Bundled flagella of curly strain SJ 30, 10 min. after the cells had been suspended in a mixture of potassium phosphotungstate and methylcellulose. × 14,400.

Fig. 5. Bundled flagella of paralysed-curly strain SJ 706, 90 min. after the cells had been suspended in a mixture of potassium phosphotungstate and methylcellulose. × 14,400.

PLATE 3

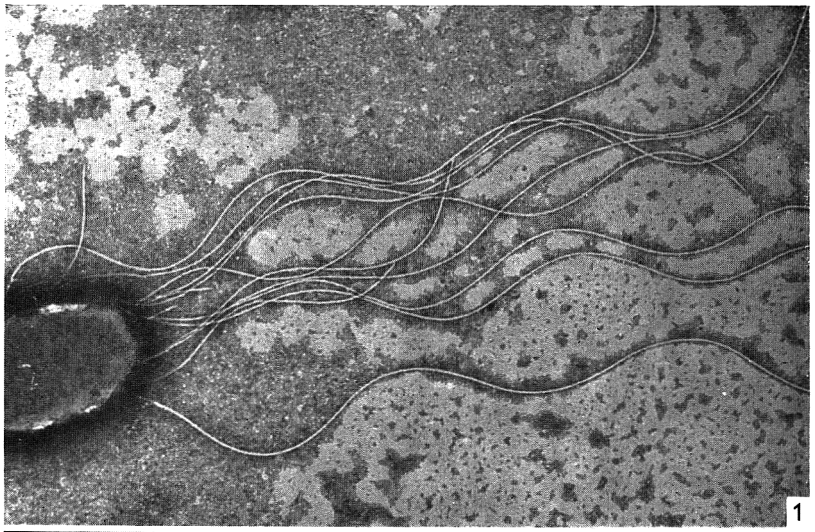
Progressive transformation of bundled flagella from small-amplitude to curly in SJ 712 as seen by dark-field microscopy, 90 min. (fig. 6), 120 min. (fig. 7), 135 min. (fig. 8) and 16·5 hr (fig. 9) after the cells had been suspended in methylcellulose solution. × 3200.

PLATE 4

Fig. 10. Dispersed flagella of small-amplitude strain SJ 712. The cells had been suspended in distilled water (pH 6·8) and were negatively stained by potassium phosphotungstate (pH 7·0) for 10 min. × 14,400.

Fig. 11. Long helical unit length of bundled flagella of SJ 712, 10 min. after the cells had been suspended in a mixture of potassium phosphotungstate and methylcellulose. × 14,400.

Fig. 12. Shorter helical unit length of bundled flagella of SJ 712, 300 min. after the cells had been suspended in a mixture of potassium phosphotungstate and methylcellulose. × 14,400.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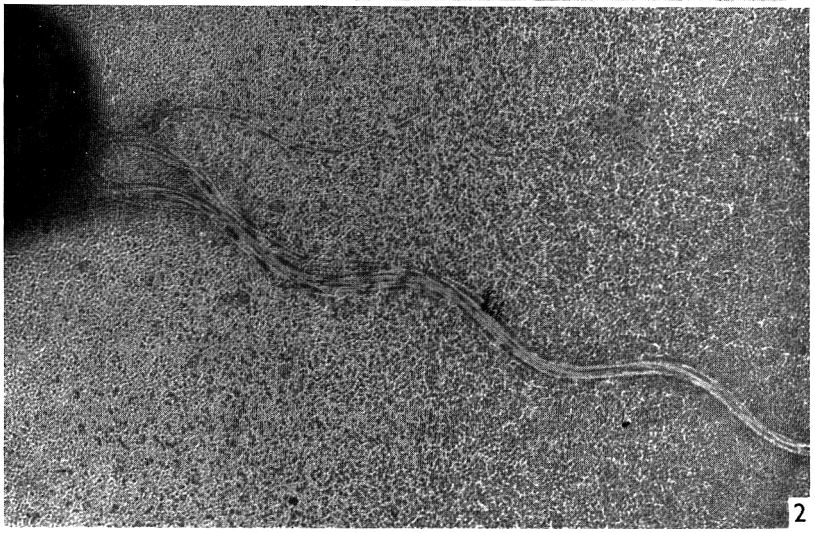
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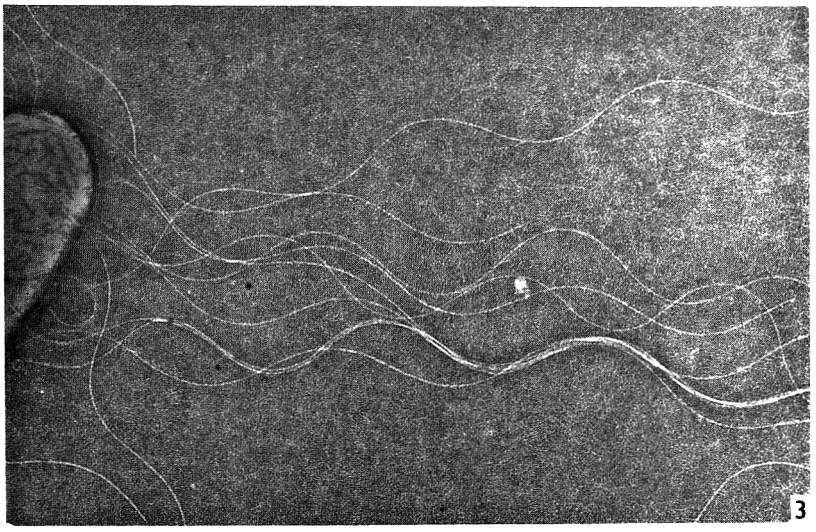
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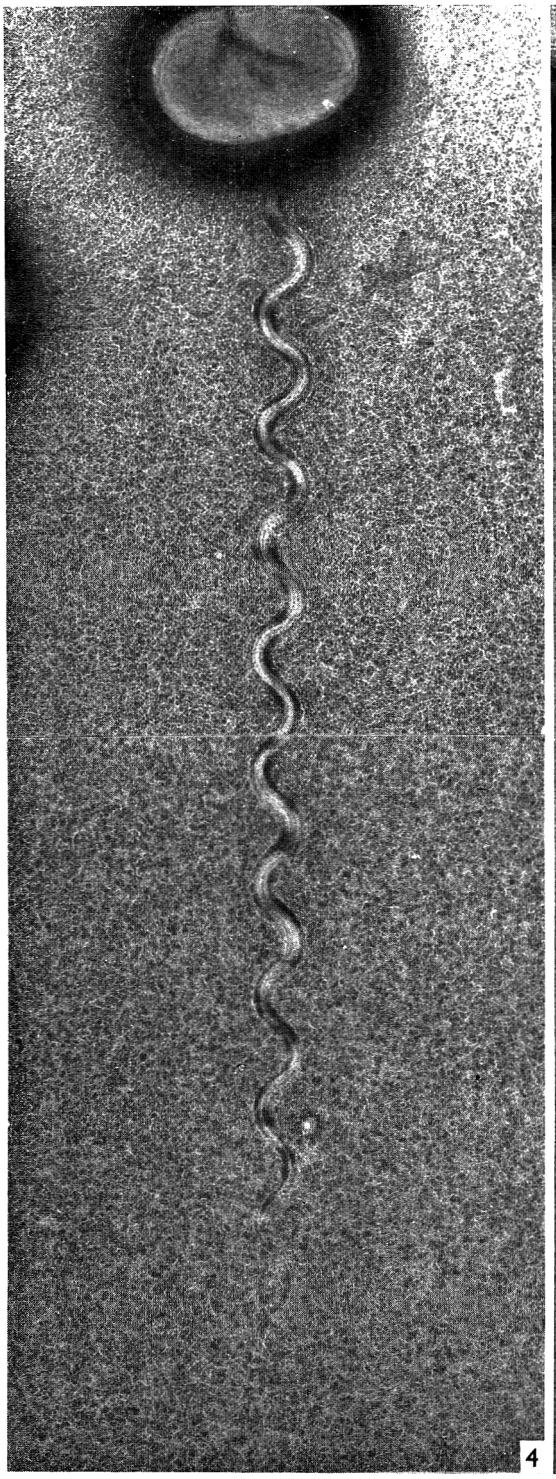
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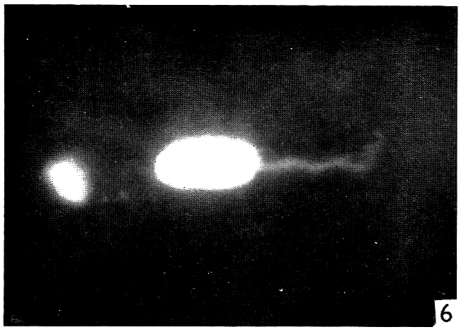
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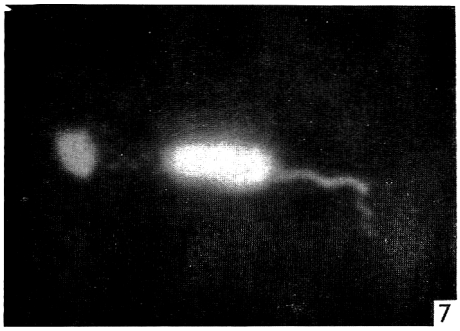
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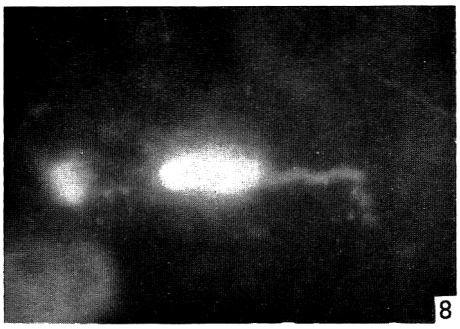
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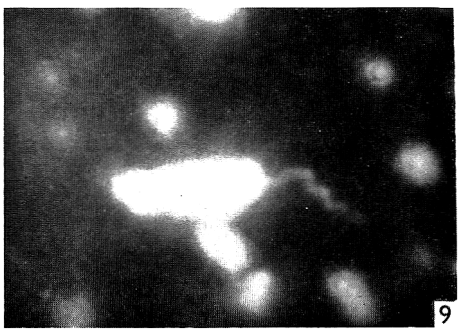
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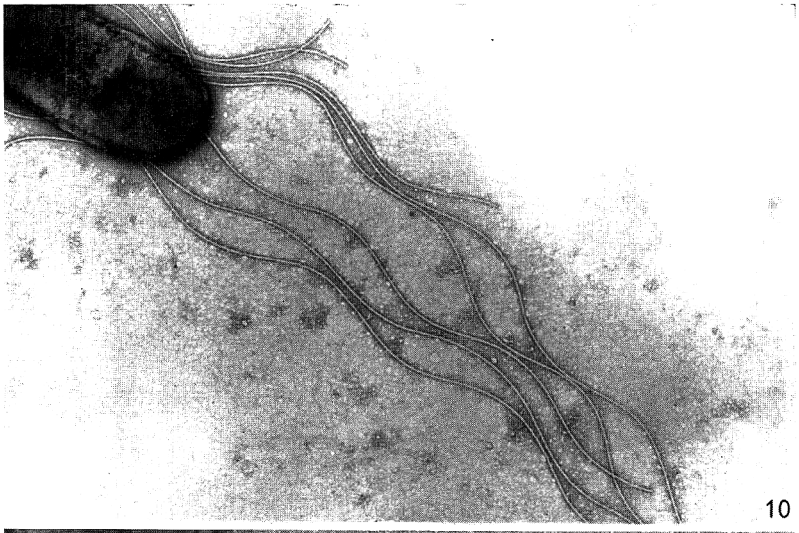

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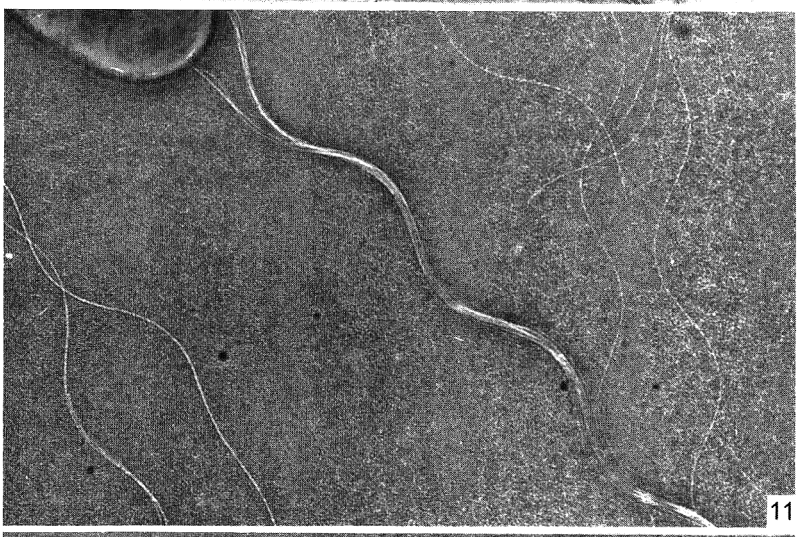

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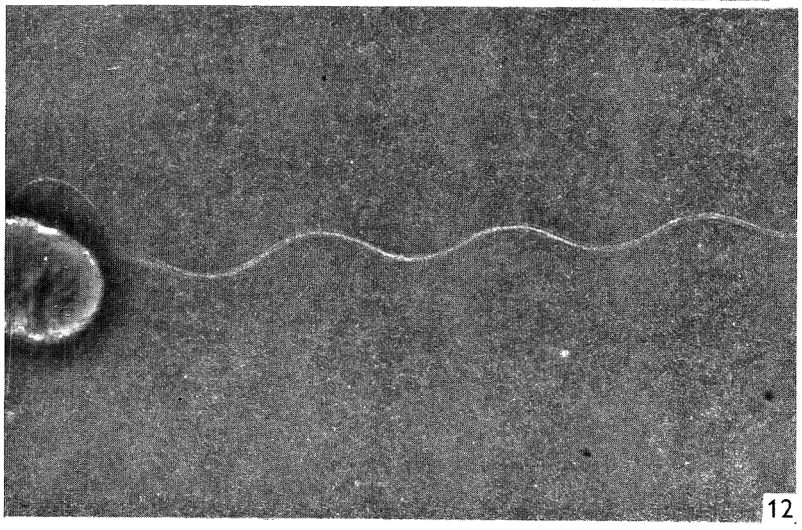

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Haemadsorption and Haemagglutination by Mycoplasmas

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SUMMARY

Factors concerned in demonstrating haemadsorption and haemagglutination and their occurrence among different mycoplasmas were investigated. Haemadsorption occurred best to colonies which had recently developed on agar at pH 6·5. Mycoplasmas isolated from various bird and animal sources, e.g. *Mycoplasma gallisepticum*, *M. agalactiae*, *M. bovigenitalium* and *M. pulmonis*, haemadsorbed with erythrocytes from a wide range of species. However, not all strains within a serotype haemadsorbed. Thus, the 'Negroni' strain of *M. pulmonis* did not. Haemadsorption could be inhibited by crowding of colonies on agar and by the addition of specific antiserum to the colonies. Generally, antiserum titres obtained by haemadsorption inhibition were low in comparison with those obtained by metabolic inhibition, and haemadsorption inhibition was not useful as a routine serological technique.

The development of the haemagglutinin of *Mycoplasma gallisepticum* in liquid medium was studied in detail; a change in the pH value of the medium could be used as an index of its development and it was intimately associated with the organism. The centrifuged deposits of other mycoplasmas, from birds, cattle, goats, man, rodents and pigs, and which were grown in liquid medium also haemagglutinated, but generally to low titre. Haemagglutination occurred best in U-shaped cups at 37° and at pH 6·5–7·0 and could be inhibited by specific antiserum. There was lack of correlation between haemadsorption and haemagglutination; both these phenomena were exhibited by some mycoplasmas, others haemadsorbed only, and still others haemagglutinated only.

INTRODUCTION

Del Giudice & Pavia (1964) reported that erythrocytes from several animals adsorbed to *Mycoplasma pneumoniae* colonies on solid medium. The phenomenon did not occur with other prototype human oral mycoplasmas, or with miscellaneous mycoplasma strains. Haemadsorption was inhibited by sera from patients convalescing from primary atypical pneumonia and from whom *M. pneumoniae* had been isolated, and it was suggested that the method could be useful for sero-diagnosis and identification of *M. pneumoniae*. Several years earlier van Herick & Eaton (1945) had shown that a mycoplasma isolated from eggs haemagglutinated, and other workers found haemagglutination to occur widely among mycoplasmas from birds (Roberts, 1964; Dierks, Newman & Pomeroy, 1967). It may be inhibited by antibody (Jungherr, Luginbuhl & Jacobs, 1953) and the haemagglutination-inhibition technique has been used extensively, in particular with *M. gallisepticum*.

We suspected that the phenomena of haemagglutination and haemadsorption might be related. Since *Mycoplasma gallisepticum* readily agglutinated erythrocytes it seemed possible that its colonies, and perhaps those of other mycoplasmas, might

haemadsorb. Furthermore, it seemed unreasonable that haemagglutination should occur solely with mycoplasmas from birds. Therefore, mycoplasmas from various sources have been examined for colony haemadsorption and for their haemagglutinating ability, and the conditions under which the phenomena are best demonstrated have been investigated. The occurrence of haemadsorption and haemagglutination by mycoplasmas isolated from several animal and avian hosts is discussed in relation to their pathogenicity.

METHODS

Medium. The medium used for culture of the organisms was basically that described by Chanock, Hayflick & Barile (1962) and by Hayflick (1965). Liquid medium consisted of Difco PPLO broth (70 ml.), 25% (w/v) aqueous extract of DCL dried yeast (10 ml.) and unheated Burroughs Wellcome horse serum no. 6 (20 ml.). Thallium acetate (1/2000) and penicillin G (1000 units/ml.) were used as bacterial inhibitors. In addition, in order to detect growth of glucose-fermenting mycoplasmas, 0·1% glucose and 0·002% phenol red were added and the medium adjusted to pH 7·8; for mycoplasmas splitting arginine, 0·1% of this substrate and 0·002% phenol red were added and the medium adjusted to pH 7·0. Solid medium was prepared by adding 1 g. Oxoid Ionagar no. 2 to 100 ml. of liquid medium, since we found that various batches of Difco PPLO agar were inhibitory to mycoplasma growth.

Mycoplasmas. The mycoplasmas tested, the animal or bird species from which they were isolated, and those persons from whom strains were obtained are shown in Table 1.

Propagation. Mycoplasma colonies for haemadsorption tests were obtained in the following manner. A stock culture of the organism (stored at $-70°$) was diluted in liquid medium in serial tenfold steps, and 0·1 ml. amounts were spread on 5 ml. of solid medium in 5 cm. diameter plastic Petri dishes; excess moisture from the agar surface was removed by incubation for 1 hr at 37° before inoculation. Subsequently, the dishes were incubated at 37° in a humid atmosphere, which usually consisted of a mixture of 95% (v/v) N_2 and 5% (v/v) CO_2, for periods of from 2 to 4 days depending on the growth rate of the organism.

For haemagglutination tests mycoplasmas were grown in 50 ml. amounts of liquid medium contained in a 4 oz. flat medicine bottle. The cultures were incubated at 37° until the pH value of the medium had changed by at least 1 pH unit. The organisms were then centrifuged for 15 min. at 3750 *g*. The supernatant fluid was discarded, and the pellet washed 3 times in 10 ml. of pH 7·2 phosphate-buffered saline (PBS), resuspended in 1 ml. of PBS, and stored at $-20°$. PBS consisted of: 8·5 g. NaCl, 0·98 g. Na_2HPO_4, 0·74 g. $NaH_2PO_4.2H_2O$ in 1 l. water.

Erythrocytes. Erythrocytes were obtained from cattle, guinea pigs, fowl, man, monkeys, mice, rabbits, rats and turkeys. The blood was mixed with a citric acid–sodium citrate–glucose anticoagulant (ACD) immediately after collection. It was then centrifuged at 550 *g* for 10 min., and the pellet resuspended in 10 times its volume of glucose + gelatin + veronal solution (DGV; Clarke & Casals, 1958) and recentrifuged. This process was repeated twice more, the pellet being suspended finally to produce a 10% suspension of erythrocytes in DGV, which was stored at 4° until required.

Haemadsorption (h.ad.) and haemadsorption-inhibition (h.ad.i.). The stock 10%

Table 1. *Mycoplasmas investigated*

Mycoplasma	Source	Received from
M. anatis	Duck	D. H. Roberts
M. gallinarum (strain PG 16)	Fowl	D. H. Roberts
M. gallinarum (11 other strains)	Various birds	D. H. Roberts
M. gallisepticum (strain A 514)	Fowl	D. M. Berry
M. gallisepticum (strains S6, A 5969)	Fowl	D. H. Roberts
M. gallisepticum (5 other strains)	Fowl	D. H. Roberts
M. iners	Fowl	R. H. Leach
M. meleagridis	Turkey	D. H. Roberts
M. synoviae IOWA 695	Turkey	D. H. Roberts
WR 1, WR/W 120, WR/W 128	Turkey	D. H. Roberts
186	Fowl	D. H. Roberts
658	Fowl	D. H. Roberts
M. bovigenitalium (strains PG 11, PG 12, PG 48)	Cattle	R. H. Leach
M. bovigenitalium (strain 1836)	Cattle	P. Stuart
M. bovigenitalium (strain DAVIDSON 37)	Cattle	R. M. Lemcke
M. mycoides (strain GLADYSDALE)	Cattle	R. N. Gourlay
M. mycoides (strain T 3) DONETTA, D 12, N 29, 5M 331	Cattle	R. H. Leach
M. canis (strain PG 14)	Dog	R. H. Leach
M. maculosum (strain PG 15)	Dog	R. H. Leach
M. spumans (strain PG 13)	Dog	R. H. Leach
M. agalactiae (strain PG 2)	Goat	R. M. Lemcke
M. agalactiae	Goat	R. H. Leach
M. mycoides var. *capri*	Goat	R. H. Leach
M. hominis (2 strains)	Man	S. Stewart
M. hominis (strains PG 21, DC 63, V 2785)	Man	R. M. Chanock
M. fermentans (strain PG 18)	Man	R. M. Chanock
M. orale I (strain CH 19299)	Man	R. M. Chanock
M. orale I (3 other strains)	Man	S. Stewart
M. orale II (strain CH 20247)	Man	R. M. Chanock
M. pneumoniae (strain FH)	Man	R. M. Chanock
M. pneumoniae (6 other strains)	Man	B. E. Andrews
M. salivarium (strain PG 20)	Man	R. M. Chanock
M. salivarium (3 other strains)	Man	S. Stewart
M. arthritidis (strain PG 6)	Rat	J. G. Tully
M. hominis II (strain PG 27)	Man*	R. M. Chanock
M. histotropicus (strain SABIN C)	Mouse	J. G. Tully
M. neurolyticum (strain SABIN A)	Mouse	J. G. Tully
M. neurolyticum (strain PG 28)	Mouse	R. H. Leach
M. pulmonis (strain PG 22)	Mouse	R. H. Leach
M. pulmonis (strain PG 34)	Rat	R. M. Lemcke
M. pulmonis (strain KON)	Rat	R. M. Lemcke
M. pulmonis (strain M 1)	Mouse	R. M. Lemcke
M. pulmonis (strain 880)	Tissue culture†	R. H. Leach
M. pulmonis (Negroni agent strain)	Tissue culture†	R. M. Chanock
M. pulmonis (6 other Negroni strains)	Tissue culture†	B. E. Andrews
PG 5	Rat	J. G. Tully
M. granularum	Pig	Z. Dinter
M. hyorhinis (strains F, S7, SEPG, SEPSK, SEP 200)	Pig	Z. Dinter
M. hyorhinis (strain GDL)	Tissue culture‡	R. M. Chanock
B1, B2, B3, B4, B5, B6	Pig	Z. Dinter
M. laidlawii (strains A, B)	Sewage	R. H. Leach

* Isolated from man but now considered to be a strain of *M. arthritidis* (Edward & Freundt, 1965).

† Isolated from tissue cultures inoculated with human leukaemic material. Subsequently identified as strain of *M. pulmonis* (Fallon *et al.* 1965; Leach & Butler, 1966).

‡ Isolated from uninoculated HEp-2 cells (Butler & Leach, 1964) and identified as *M. hyorhinis* (Purcell *et al.* 1966*a*).

suspension of erythrocytes was diluted, either in PBS or in Difco PPLO broth, to produce a 0·5% suspension of which about 2 ml. was poured on to the agar surface. The cultures were then incubated, usually at 37°, for 15–30 min. Then, by means of a 'plate' microscope, giving a magnification of × 50 to × 150, colonies were observed with the erythrocyte suspension *in situ*, and again after the suspension had been removed and the agar surface washed with 5–10 ml. of PBS or PPLO broth.

For h.ad.i., a microtechnique was developed in order to conserve antisera. Mycoplasmas were inoculated on to the surface of 0·1 ml. amounts of agar medium contained in the cups of Linbro plastic disposo-trays (S-MRC-96; Linbro Chemical Co. Inc., New Haven, Connecticut, U.S.A.). The stock mycoplasma suspensions were diluted to produce about five to twenty colonies per cup after incubation at 37° in a humid atmosphere of 95% (v/v) N_2 and 5% (v/v) CO_2. Twofold antiserum dilutions (0·025 ml.) in PBS were made with Takátsy spiral loops and the 0·025 ml. amounts transferred to the agar. After incubation of the disposo-tray at room temperature for 30 min., 0·025 ml. of a 0·5% erythrocyte suspension was added to each cup and the tray incubated at 37° for 15–30 min. Excess erythrocyte suspension was then removed by dipping and agitating the tray in PBS. The colonies were then observed microscopically for haemadsorption.

In some instances, colonies which had developed on agar medium contained in Petri dishes were used. Blocks of agar about 1 cm. square and bearing the colonies were cut out and transferred to empty Petri dishes. The subsequent procedures were as described previously.

Haemagglutination (h.a.) and haemagglutination-inhibition (h.i.). Serial twofold dilutions of the mycoplasma suspension were made in PBS or PPLO broth diluent by using Takátsy loops and Linbro disposo-trays. Then 0·025 ml. of a 0·5% erythrocyte suspension and the same volume of diluent were added to give a total volume of 0·075 ml. in each cup. The trays were covered with 'Scotch' brand clear adhesive tape, shaken, and incubated, usually at 37°, for about 1 hr. Haemagglutination was graded as follows: ++++ = 100% agglutination, +++ = 75%, ++ = 50%, + = 25%. One h.a. unit was contained in the highest dilution of mycoplasma suspension at which approximately 75% or more of the erythrocytes were agglutinated.

For the h.i. test, serial twofold dilutions of antiserum in 0·025 ml. volumes were made in a disposo-tray, and 4 h.a. units of antigen in 0·025 ml. were added to each cup. The mixtures were incubated at room temperature for 30 min., and then 0·025 ml. of 0·5% erythrocyte suspension was added to each cup, and the tray incubated at 37° for about 1 hr. The antiserum titre was the highest dilution at which at least 75% of the erythrocytes were not agglutinated.

Metabolic inhibition (m.i.). These tests were performed as described in detail previously (Taylor-Robinson, Purcell, Wong & Chanock, 1966; Purcell, Taylor-Robinson, Wong & Chanock, 1966*b*).

RESULTS

Haemadsorption

The basic technique used to detect haemadsorption is described in the Methods section. However, various factors which might influence the demonstration of haemadsorption were investigated.

Agar medium. The constituents were not varied, but media at pH 8·0, 7·0 and 6·5 were used. Agar medium did not gel adequately at pH 6·0, and therefore was not used. The number of colonies which developed on agar medium at each pH value was unaffected, except in one instance, namely, mycoplasma B6 colonies which did not develop surface peripheral growth at pH 8·0. Since erythrocytes adhered to the periphery and not to the central 'nipple' portion of the colonies of this mycoplasma, it was necessary to use agar at a low pH value in order to demonstrate haemadsorption. Also, growth of other mycoplasmas on agar at pH 6·5 produced colonies, the surface growth of which did not detach easily from the agar when an erythrocyte suspension was added. For these reasons agar at pH 6·5 was used in subsequent experiments.

Culture atmosphere. The effect of the atmosphere in which the culture was grown on subsequent haemadsorption was investigated with *Mycoplasma pneumoniae* and *M. agalactiae*. Colonies of these mycoplasmas developed just as well in an atmosphere containing a mixture (v/v) of 95% air + 5% CO_2 as in a mixture of 95% N_2 + 5% CO_2. The rate and extent of haemadsorption to colonies which had developed under either condition was the same. Since colonies of most mycoplasmas develop best, or in some instances, only in an atmosphere (v/v) of 95% N_2 + 5% CO_2, this was used for subsequent cultures.

Age of colonies. Agar cultures of several mycoplasmas were incubated for 4 days at 37° and duplicate cultures were incubated for 10 days, and at each of these times the colonies were tested for haemadsorption. Colonies of mycoplasmas which haemadsorbed strongly after 4 days, haemadsorbed weakly after 10 days, i.e. fewer erythrocytes attached. Colonies of *Mycoplasma agalactiae* which haemadsorbed strongly at 4 days did not do so at all after 10 days, due to the development of a crystalline film which covered the colonies and agar at this time. Colonies of mycoplasma B6 which haemadsorbed weakly at 4 days did not do so at all at 10 days. Subsequently, for routine use, haemadsorption tests were performed with colonies which had recently developed or had been incubated only until there was no obvious increase in colony size. This usually took 3 or 4 days.

Crowding of colonies. The number of colonies on agar medium was an extremely important factor in the demonstration of haemadsorption. In preliminary experiments with several mycoplasma strains, in particular *Mycoplasma agalactiae*, *M. gallisepticum* and *M. pneumoniae*, it was noticed that discrete colonies at the edge of a crowded area of colonies haemadsorbed, but those in the crowded area did not. Therefore, serial tenfold dilutions of the organisms were inoculated on agar medium so that, after incubation, the number of colonies in each agar dish ranged from confluency to a few only. After the addition of erythrocytes, isolated colonies haemadsorbed but colonies which were crowded did not. This is illustrated in Fig. 1 and in Pl. 1, figs. 1, 2. Because of this phenomenon, care was taken in other experiments to use agar cultures with not more than about 100 colonies.

Species of erythrocytes. Del Giudice & Pavia (1964) reported that erythrocytes from several animal species adsorbed to *Mycoplasma pneumoniae* colonies. This was confirmed in the present experiments since erythrocytes of all the species tested adsorbed to colonies of this mycoplasma (Table 2). However, because of the difficulty of testing such a wide variety of erythrocytes against all the mycoplasmas, only a few species of erythrocytes were tested. The following were used: the 'homologous' species of

erythrocytes, i.e. erythrocytes from the bird or animal species from which the mycoplasma was originally isolated, and one or more heterologous species, in particular, guinea-pig erythrocytes. In most instances, a colony which adsorbed the homologous erythrocytes adsorbed the heterologous erythrocytes also. Exceptions to this were observed with *M. bovigenitalium* strain 1836, which adsorbed guinea-pig, but not bovine erythrocytes, and the porcine mycoplasma B1 which adsorbed pig, but not guinea-pig erythrocytes.

Suspending fluid and age of erythrocytes. Washed guinea-pig erythrocytes stored at 4° in DGV were resuspended in PBS (pH 7·2) or in Difco PPLO broth at pH 6·5, 7·0 and 8·0. The adsorption of erythrocytes, suspended in these various fluids, to colonies of *Mycoplasma pneumoniae*, *M. gallisepticum*, mycoplasma B6 and *M. laidlawii* A was tested at 37°. Erythrocytes did not adsorb to colonies of *M. laidlawii* A under any conditions and no difference in the rate or extent of haemadsorption to colonies of the other mycoplasmas was noted with erythrocytes in the various suspensions. Therefore, further experiments were made with pH 7·2 PBS as the erythrocyte suspending fluid. In addition, erythrocytes stored at 4° in DGV for various periods up to 1 month adsorbed as readily as fresh erythrocytes.

Table 2. *Effect of temperature on haemadsorption by three mycoplasmas*

Incubation		Mycoplasma	*M. laidlawii*
Time	Temperature	*M. gallisepticum*	DONETTA	A
15 min.	37°	++++*	++	–
	22°	++	+	–
	4°	++	–	–
180 min.	37°	++++	++	–
	22°	++++	++	–
	4°	++++	–	–

* ++++, ++, + = 100%, 50%, 25% respectively of colony surface covered by adherent guinea-pig erythrocytes.

Table 3. *Mycoplasmas which haemadsorb with homologous and guinea-pig erythrocytes*

M. gallisepticum (all strains)
IOWA 695
WR/W120, WR/W128
658
M. bovigenitalium (strain 1836* only)
DONETTA
M. canis
M. spumans
M. agalactiae (both strains)
M. pneumoniae (all strains)
M. histotropicus
M. pulmonis (strains PG22, PG34 and M1 only)
B1†
B6

Other mycoplasmas shown in Table 1 did not haemadsorb.
* Adsorbed guinea-pig but not bovine erythrocytes.
† Adsorbed pig but not guinea-pig erythrocytes.

Temperature of reaction. The effect of temperature on haemadsorption to colonies of three distinct mycoplasmas was investigated. The results are shown in Table 2. Adsorption to colonies of *Mycoplasma gallisepticum* was most rapid at 37° but occurred at 4° also. Similarly, adsorption to colonies of mycoplasma DONETTA was most rapid at 37°, but was not observed at 4°. Adsorption to colonies of *M. laidlawii* A

was not observed at any temperature after prolonged incubation. Incubation at 37° was used for subsequent tests.

Haemadsorption among different mycoplasmas. A number of mycoplasma strains belonging to 39 serotypes (Table 1) were then examined for colony haemadsorption using the conditions outlined above. Fourteen of the serotypes were observed to haemadsorb, as shown in Table 3. Colonies of all the strains of *Mycoplasma gallisepticum* and *M. pneumoniae* that were tested haemadsorbed. It is noteworthy, however, that not all strains of other serotypes did likewise. Thus only one of five strains of *M. bovigenitalium* did so.

Haemadsorption inhibition

The inhibition of erythrocyte adsorption to colonies of *Mycoplasma pneumoniae* by antiserum was reported by Del Giudice & Pavia (1964). In the present investigation, inhibition was investigated with rabbit antisera to three of the haemadsorbing mycoplasmas, including *M. pneumoniae*. In a preliminary test with *M. pneumoniae* antiserum, inhibition was not demonstrated in the absence of unheated guinea-pig serum. The subsequent h.ad.i. tests were performed, therefore, with guinea-pig serum. The results, together with those of metabolic inhibition tests performed on the same antisera, are shown in Table 4. The homologous h.ad.i. antiserum titre for each mycoplasma was low in comparison with the metabolic inhibition titre. Because of these low titres, cross h.ad.i. tests to determine specificity were not performed.

Table 4. *Haemadsorption inhibition with hyper-immune rabbit sera*

	Homologous antiserum titre (reciprocal) measured by	
Mycoplasma	Haemadsorption inhibition	Metabolic inhibition
M. gallisepticum (S6)	40	640
M. agalactiae	20	10,240 or >
M. pneumoniae (FH)	40	320

Haemagglutination

Several factors which could affect haemagglutination were investigated.

Growth of mycoplasma. Since it was impractical to study in detail the growth and development of haemagglutinin for each mycoplasma, a detailed examination of a known haemagglutinating mycoplasma, namely *Mycoplasma gallisepticum*, was performed. This organism was grown in liquid medium and samples were removed at 24 hr intervals and examined for pH value, number of viable organisms (colony forming units), and haemagglutinin with chicken erythrocytes. In addition, each sample was centrifuged at 3750 *g* for 15 min. and the haemagglutinin titre of the supernatant fluid and the deposit was determined. The results are shown in Fig. 2. The maximum number of viable organisms occurred after 3 days of incubation, when the pH value of the medium had fallen from 7·6 to 7·0. However, the maximum haemagglutinin titre of the deposit was not attained until after 5 days of incubation, when the pH value had fallen to 6·2. Haemagglutinin was not detected in the supernatant fluid after centrifugation of any of the samples, indicating its association with the whole organism.

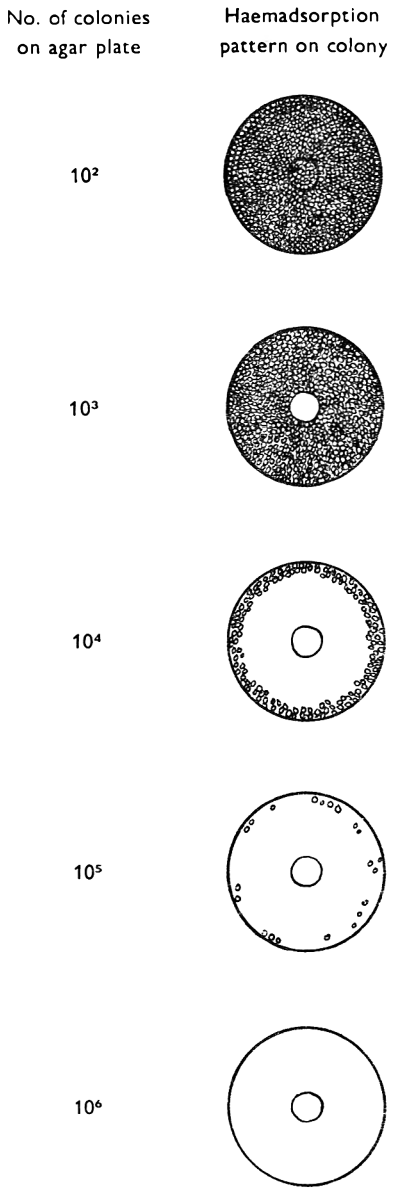

Fig. 1

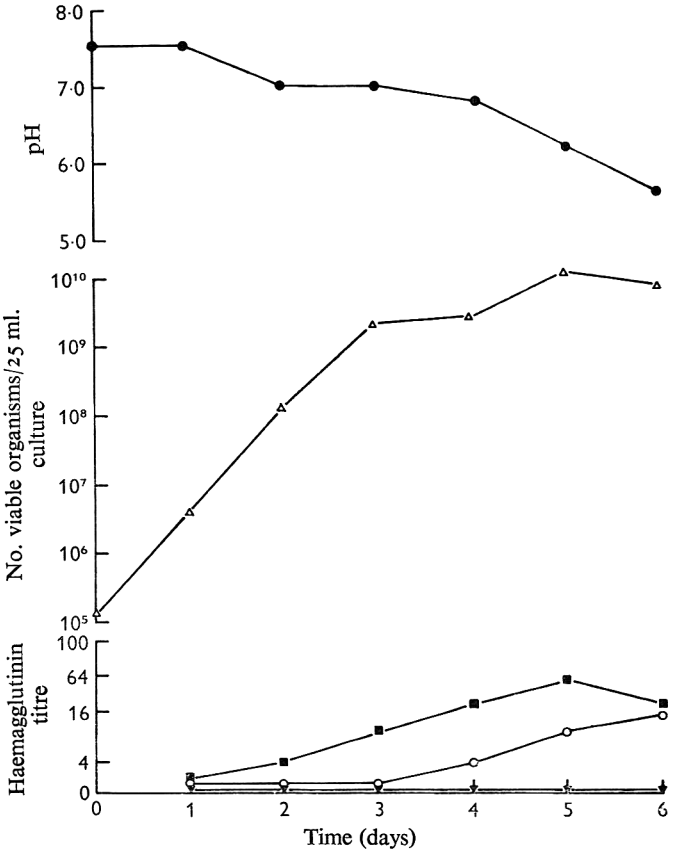

Fig. 2

Fig. 1. Inhibitory effect of increasing numbers of colonies on the adsorption of guinea-pig erythrocytes to *Mycoplasma pneumoniae* colonies. × 150.

Fig. 2. Development of haemagglutinin and viable organisms in a liquid culture of *Mycoplasma gallisepticum* (strain A5969). ●, pH; △, number viable organisms/25 ml. culture; ■, haemagglutinin titre of centrifuged deposit from 25 ml. culture; ○, haemagglutinin titre of culture before centrifugation; ↓, haemagglutinin titre of supernatant fluid after centrifugation.

As a result of the above experiment, cultures of other mycoplasmas were incubated until the pH value had changed by at least 1 unit or until there was no further change in pH value as judged by a change in colour of the indicator. The cultures were then centrifuged as before and the deposits tested for haemagglutinin.

Diluent and pH value. Preliminary haemagglutination tests with several mycoplasmas, colonies of which had been observed to haemadsorb, were made with Difco PPLO broth adjusted with HCl to pH 5·5, 6·0, 6·5, 7·0, 7·5, and PBS (pH 7·2). Haemagglutination titres were higher at pH 6·5–7·0 than above; at pH 5·5 lysis of erythrocytes occurred. Haemagglutination titres and patterns were the same when broth at pH 7·0 and PBS (pH 7·2) were used. Most subsequent tests were performed with PBS as diluent.

Temperature of reaction and type of cup. Several tests were made at 37°, 22° and 4° in U-shaped and V-shaped cups. Haemagglutination titres were optimal at 37° and the patterns were most distinct and easily readable in U-shaped cups.

With the conditions as outlined above, namely PBS diluent at pH 7·2, U-shaped cups and incubation at 37°, deposits of all the available mycoplasmas were examined in haemagglutination tests with erythrocytes from several species. The results are presented in Table 5. In general, h.a. titres were of a low order and a mycoplasma which agglutinated erythrocytes did not necessarily do so with erythrocytes of all the species tested.

Table 5. *Mycoplasmas which haemagglutinate*

Mycoplasma	Reciprocal haemagglutination titre* with erythrocytes from			
	Fowl	Man	Mouse	Guinea-pig
M. anatis	0	2	2	2
M. gallisepticum (S6)	4	8	8	32
M. gallisepticum (A514)	nt†	nt	nt	32
IOWA 695	0	4	nt	2
WR 1	64 or >	64 or >	16	64 or >
M. bovigenitalium (PG 11)	0	0	0	2
DONETTA	0	2	0	0
M. agalactiae	0	2	8	4
M. pneumoniae (FH)	0	2	0	0
M. neurolyticum (PG 22)	0	0	2	0
PG 5	0	0	0	2
B 1	2	16	16	16
B 2	0	0	0	4
B 3	0	0	2	0
B 5	0	4	2	4

* Highest dilution of antigen which caused agglutination of approximately 75% or more of the erythrocytes.

† nt = not tested.

Haemagglutination inhibition

Because of the low h.a. titres, exhaustive inhibition tests with specific rabbit antisera were not made. However, those mycoplasmas for which it was possible to use 4 h.a. units were tested and, in addition, mycoplasma PG 5. The results, together with those of metabolic inhibition tests performed on the same antisera, are shown in Table 6. With the exception of mycoplasma WR 1, the titres obtained by h.i. were much lower

than those obtained by m.i. Heterologous h.i. titres were not determined in each case, but antiserum to mycoplasma WR I did not inhibit haemagglutination by *Mycoplasma gallisepticum* and vice versa.

Table 6. *Haemagglutination inhibition with hyperimmune rabbit sera*

Mycoplasma	Homologous antiserum titre (reciprocal) measured by: Haemagglutination inhibition	Homologous antiserum titre (reciprocal) measured by: Metabolic inhibition
M. gallisepticum (A 514)	80	640
WR I	160	20
PG 5	320	nt
B I	80	1280

DISCUSSION

Several factors were shown to be important for the demonstration of haemadsorption. However, the most interesting discovery was that haemadsorption with some mycoplasma strains was inhibited when there were large numbers of colonies crowded on agar medium. The occurrence of this phenomenon may explain the failure of others to detect haemadsorption for mycoplasmas other than *Mycoplasma pneumoniae*. Our observation is similar to that of Somerson, Purcell, Taylor-Robinson & Chanock (1965), who found that β-haemolysis of guinea-pig erythrocytes in an agar overlay was completely inhibited by crowding of colonies of *M. pneumoniae*. There is an apparent anomaly in the occurrence of inhibition of haemadsorption by crowding of colonies on agar medium and the lack of inhibition of haemadsorption to confluent sheets of the same mycoplasma adherent to glass under liquid medium as shown by Taylor-Robinson & Manchee (1967*a*). This may be due to the fact that nutrients and waste products are freely diffusible in a liquid medium but not in a solid medium, and the exhaustion of a particular nutrient or the accumulation of a waste product may be responsible for the inhibition of haemadsorption when there are a large number of colonies on agar.

Although colonies of all strains of *Mycoplasma gallisepticum* and all strains of *M. pneumoniae* haemadsorbed, this was not true for other mycoplasmas tested. The fact that one strain within a serotype haemadsorbed did not mean that other strains within the same serotype would do so, and such differences were not due to variation in the number of colonies on agar. Notable examples of this were seen with strains of *M. bovigenitalium* and *M. pulmonis*. Thus, the PG 34 strain of the latter haemadsorbed, but the 'Negroni' strain of the same serotype did not. It is interesting that some strains of *M. pulmonis* haemadsorb, since Berg & Frothingham (1961) reported that tissue culture cells infected with this organism haemadsorbed. Whether colonies of a non-haemadsorbing mycoplasma strain could acquire by continuous passage the ability to haemadsorb or, conversely, whether those of a haemadsorbing mycoplasma could lose this ability is unknown. In this respect, colonies of recently isolated strains of *M. pneumoniae* haemadsorb and so do those of the FH strain after over 300 passages in liquid and on solid medium; whether the FH strain haemadsorbed originally is unknown.

The titres obtained in haemadsorption-inhibition tests were much lower than in

comparable metabolic-inhibition tests. Because of the low haemadsorption-inhibition titres, cross-tests to determine specificity were not performed. In addition, because of its insensitivity we have not been able to use the technique as a routine one for measuring antibody. Haemadsorption by itself, however, has a useful place in a mycoplasma diagnostic scheme. After isolating a mycoplasma we determine, among other characteristics, its ability to haemadsorb. Since only a few mycoplasmas haemadsorb, this limits the range of specific antisera required to identify a haemadsorbing mycoplasma; as a first choice, we select antisera prepared against those mycoplasmas known to haemadsorb and use them in disc growth-inhibition (Clyde, 1964) or metabolic inhibition (Taylor-Robinson *et al.* 1966; Purcell *et al.* 1966*b*). Furthermore, we have found the phenomenon of value in examining a mixed mycoplasma population. About 10% of the colonies produced by a culture of *Mycoplasma hyorhinis* haemadsorbed. These haemadsorbing colonies, which apart from this characteristic were indistinguishable from the others, were cloned and identified as *M. agalactiae*. This eliminated the possibility that the haemadsorbing colonies were mutants of *M. hyorhinis*. In fact, of the many mycoplasma strains examined, we have not observed both haemadsorbing and non-haemadsorbing colonies belonging to strains of a particular serotype although, as noted previously, different strains may show one or other characteristic. This may be due to the picking of single colonies for the production of stock cultures.

Table 7. *Correlation between haemadsorption and haemagglutination*

	Presence (+) or absence (−) of	
Mycoplasma	Haemadsorption	Haemagglutination*
M. gallisepticum (A 514)	+	+ (32)
M. agalactiae	+	+ (2)
M. pneumoniae	+	+ (8)
B I	+	+ (16)
M. pulmonis (PG 22)	+	−
M. pulmonis (PG 34)	+	−
WR I	−	+ (64 or >)
M. neurolyticum	−	+ (2)
M. fermentans	−	−
M. laidlawii A	−	−

* Haemagglutination titres are shown in parentheses.

Mycoplasmas from various sources were found also to haemagglutinate although usually at a low titre. Those that did so best were those isolated from birds, such as *Mycoplasma gallisepticum* and mycoplasma WR I. *M. pneumoniae* haemagglutinated at a much lower titre than that reported by Feldman & Suhs (1966). Their test is performed in the presence of horse serum which contains agglutinins for the particular erythrocytes used in the test. It appears that the haemagglutination is due to an additive effect of these agglutinins and *M. pneumoniae* agglutinin, each of which alone is present in a subagglutinating concentration (John, Stahl & Fulginiti, 1966). It is possible that if this principle were applied to mycoplasmas that we have found to haemagglutinate to low titre, higher titres might be attained. In several tests the haemagglutination that we have observed could be inhibited with specific antiserum, and the inhibition was specific where tests to determine this were performed.

Of considerable interest is the lack of correlation between haemadsorption and

haemagglutination. This is shown in Table 7. Some mycoplasmas haemadsorb and haemagglutinate; others haemadsorb but do not haemagglutinate. In such instances, inability to haemagglutinate might be due to too low a concentration of antigen, although this seems unlikely, since the antigens of *Mycoplasma pulmonis* strains were derived from cultures containing 10^8 viable organisms/ml. More striking are those mycoplasmas which do not haemadsorb under any circumstances and yet agglutinate in the case of mycoplasma WR 1 a variety of erythrocytes to high titre. It was suspected initially that haemadsorption might be a more sensitive expression of haemagglutination. Clearly this is not so in all cases, and underlines the fact that the mechanisms involved are not understood. The occurrence of tiny projections on the surface of *M. gallisepticum* particles has been observed in electron micrographs by Chu & Home (1967). The appearance is similar to that of myxoviruses, and the projections are possibly haemagglutinin in nature. It is also possible that an erythrocyte receptor mechanism similar to that known to exist for myxoviruses may play a part in mycoplasma haemagglutination and haemadsorption. Thus, it has been indicated that in the haemagglutination reaction, a sialic acid at the surface of turkey erythrocytes provides binding sites or receptors for *M. gallisepticum* (Gesner & Thomas, 1965), although in other experiments (Roberts, 1967) the removal of receptors on chicken erythrocytes for this mycoplasma was not achieved. In view of these contradictions, further investigations are warranted.

Finally, it is worth considering the possible relationship of haemadsorption and haemagglutination to mycoplasma pathogenicity. *Mycoplasma pneumoniae* infections are often associated with the production of a cold-agglutinin response. Somerson, Walls & Chanock (1965) have postulated that the organism alters the erythrocyte membrane in some way, rendering it antigenic, and that adsorption of the organism to the erythrocyte is the first stage in this process. Furthermore, mycoplasmas adhere to cells other than erythrocytes. Thus, adherence to neutrophils, eosinophils, monocytes, lymphocytes, tissue culture cells and spermatozoa has been demonstrated (Zucker-Franklin, Davidson & Thomas, 1966*a*, *b*; Taylor-Robinson & Manchee, 1967*b*). In addition, not only *M. pneumoniae* but also *M. gallisepticum*, *M. pulmonis* and *M. agalactiae* are pathogenic for their particular bird or animal host. All these mycoplasmas haemadsorb and it is possible that this or their ability to adhere to other tissue cells is the first stage in pathogenesis. Clearly, however, haemadsorption cannot be considered a factor in the pathogenesis of all mycoplasma infections. Thus, both strains of *M. mycoides* did not haemadsorb and this mycoplasma is highly pathogenic, being a major cause of cattle disease in several parts of the world. Gourlay & Shifrine (1966) have suggested that the pathogenicity of this organism is due to the production of a hypersensitivity reaction in the animal host.

We thank those persons from whom we obtained mycoplasmas and Mrs Susan Beveridge for technical assistance.

Note added in proof. Subsequent serological studies have shown that mycoplasma strains B1, B2 and B5 are similar to *Mycoplasma gallinarum*, strain B4 is similar to *M. laidlawii* and strain B6 is similar to *M. iners*.

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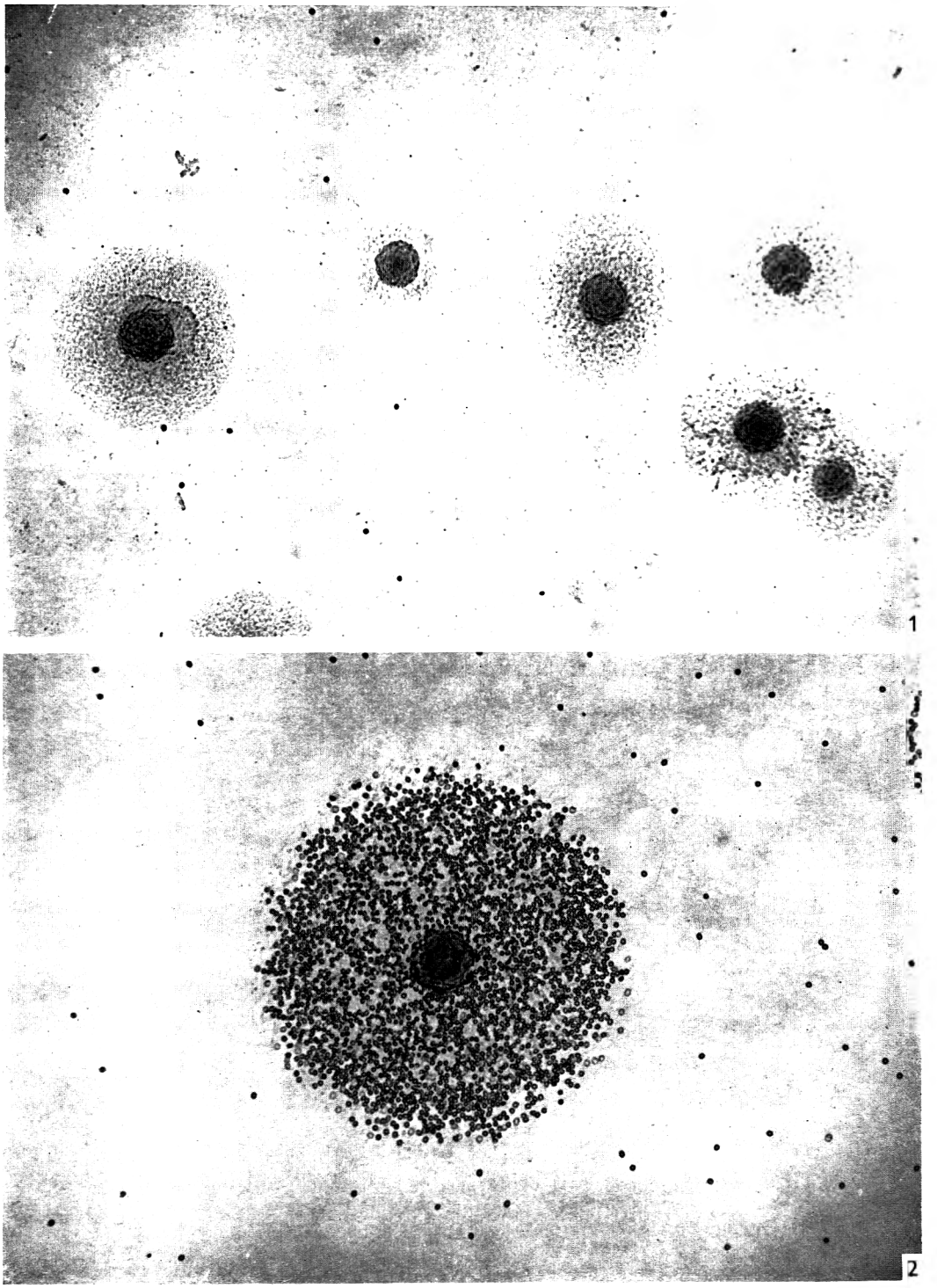
EXPLANATION OF PLATE

Fig. 1. Representative colonies from an agar plate which contained 8000 colonies of *Mycoplasma agalactiae*; incubated at 37° for 15 min. with guinea-pig erythrocytes. No adsorption observed after excess erythrocytes removed. × 250.

Fig. 2. A representative colony from an agar plate which contained 80 colonies of *Mycoplasma agalactiae*; incubated with guinea-pig erythrocytes. Adsorption after excess erythrocytes removed. × 250.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The Effect of the Treatment of Crude Tetanus Toxin with Ganglioside Cerebroside Complex on Sphincter Paralysis in the Rabbit's Eye

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SUMMARY

Crude tetanus toxin paralyses the cholinergic innervation of the sphincter pupillae of the rabbit eye. To test whether this activity was due to tetanospasmin or to a non-spasmogenic fraction with a peripheral effect, the action of crude tetanus toxin and of the non-spasmogenic fraction prepared from it were compared in rabbits. The paralytic action of the crude toxin was not due to its content of the non-spasmogenic fraction and was probably due to tetanospasmin itself. A convenient method for the preparation of a mixture of the four most abundant gangliosides is given. The preparation of a ganglioside cerebroside complex and its use for the removal of tetanospasmin from a solution of crude tetanus toxin are described.

INTRODUCTION

Ambache, Morgan & Payling Wright (1948*a*) showed that 1–2 days after injection of a crude preparation of tetanus toxin (13,330 mouse LD 50/mg.; 330 LD 50 per injection) into the anterior chamber of the eye of the rabbit the reflex constriction of the pupil in response to light was abolished; the effect was still produced after sympathetic denervation of the iris. Since stimulation of the oculomotor nerve did not cause constriction of the pupil in the toxin-injected eye and yet the sphincter pupillae would still contract readily (within 10–15 seconds) when acetylcholine was injected into the anterior chamber of the eye, it was concluded that the toxin was paralysing the cholinergic innervation of the sphincter pupillae. In further experiments (Ambache, Morgan & Payling Wright, 1948*b*) it was shown that tetanus toxin decreased the acetylcholine content of the aqueous humour, and to lesser extent of the iris, while leaving the cholinesterase content of the iris unaffected. At the same time, the sphincter pupillae of the intoxicated eye became hypersensitive to carbaminoylcholine injected subcutaneously. It was deduced that the toxin was interfering with the liberation of acetylcholine at the nerve endings in the sphincter pupillae.

Feigen *et al.* (1963) found that partially purified preparations of tetanus toxin contained a non-spasmogenic fraction (called NSP) which increased the frequency (whilst leaving the amplitude unaffected) of miniature end-plate potentials recorded from intercostal muscles in the isolated hemithorax of the mouse. This is a presynaptic action and so was the paralytic effect of crude tetanus toxin in the eye. It was there-

fore an obvious possibility that the non-spasmogenic fraction (NSP) might be responsible for the paralytic action of crude tetanus toxin in the eye. The work described in this paper was done to test this possibility; a preliminary account has been published (Ambache & Mellanby, 1966).

The non-spasmogenic fraction from crude tetanus toxin used by Feigen *et al.* (1963) was prepared by mixing a solution of the crude toxin with a suspension of protagon (a water-insoluble complex of various water-insoluble sphingolipids and water-soluble ganglioside) and then centrifuging off the protagon with the tetanospasmin adsorbed on it. The ability of protagon to fix tetanospasmin is due to its content of ganglioside (van Heyningen, 1959), but pure ganglioside cannot conveniently be used to remove tetanospasmin from a preparation of tetanus toxin since ganglioside, like the toxin, is water-soluble. When, however, ganglioside is complexed with cerebroside it is rendered water-insoluble and can then be used to adsorb tetanospasmin from solution. The capacity of the complex to combine with tetanospasmin is highest when the proportion of cerebroside to ganglioside in the complex is 3:1 (van Heyningen & Mellanby, 1968). In the present work, the action of a crude preparation of tetanus toxin on the rabbit iris was compared with the action of an equal weight of non-spasmogenic fraction prepared by treating the same toxin preparation with ganglioside cerebroside complex. In addition, the action of highly purified tetanus toxin (60,000,000 mouse LD 50/mg.) on the rabbit eye was tested.

METHODS

Toxin preparations. Two preparations of tetanus toxin were obtained from Dr R. O. Thomson of the Wellcome Research Laboratories, Beckenham, Kent. Preparation A was a purified tetanospasmin containing 60,000,000 mouse LD 50/mg.; preparation B was a fraction, obtained from a saline extract of tetanus bacilli by ammonium sulphate and acid precipitation, containing a relatively high concentration of non-spasmogenic fraction (NSP) and a low concentration of tetanospasmin. It contained 150 mouse LD 50/mg. The toxins for injection were dissolved in 0·1 M-phosphate (pH 7) containing 0·2% (w/v) gelatin (as protective colloid), and diluted appropriately in this buffer.

Assay of toxicity. Serial twofold dilutions of the solutions prepared for the injections were made in 0·1 M-phosphate (pH 7) containing 0·2% (w/v) gelatin; 0·5 ml. doses were injected into pairs of Swiss albino mice.

Preparation of non-spasmogenic fraction from crude tetanus toxin

(*a*) *Ganglioside.* Fresh whole beef brain was homogenized in a Waring Blendor with acetone (3 l./kg.) for 3 min. and the mixture filtered. The residue was extracted again with acetone (1 l./kg. original brain) and dried in a current of air. The dry brain was extracted with chloroform + methanol (1 + 2, v/v; 2 l./kg. dry brain) at 60° with continuous vigorous agitation for 30 min. The extract was filtered off and the residue re-extracted with the same volume of solvent. The pooled extracts were left to stand at 4° overnight. The material which settled out was filtered off and dissolved in chloroform + methanol (4 + 1, v/v). This solution contained about 90% of the ganglioside but only about 40% of the total dry weight of the original extract.

Before proceeding to the next stage, the dry weight of the solution was determined and adjusted to 5 g./100 ml. If this solution was not clear it was filtered. Silicic acid

was added (1 g./g. dry weight) and the mixture stirred briskly with a glass rod for about 2 min. The suspension was then centrifuged at about 1000 rev./min. for 15 min. and the supernatant fluid discarded. The silicic acid was washed 3 times with chloroform + methanol (3 + 1, v/v; 8 ml./g. silicic acid). The ganglioside was then eluted twice with 8 ml. methanol/g. silicic acid. The solution of ganglioside in methanol was then evaporated to approximately one sixth of its volume on a rotary evaporator with bath temperature just below 50°.

The ganglioside was now transferred to an aqueous phase by partition dialysis: the methanol solution was mixed with 2 volumes of chloroform and dialysed (against 2 changes of 20 volumes of distilled water) for 48 hr. The upper phase was concentrated on a rotary evaporator to 20 ml. for each kg. original fresh brain, and then freeze-dried. The material thus obtained was usually contaminated with a small amount of silicic acid. This was removed by redissolving the material in a small volume of water, centrifuging at 30,000 rev./min. for 30 min., and freeze-drying the supernatant fluid.

The yield of ganglioside was about 0·5 g./kg. fresh whole brain. The sialic acid content varied from 23 to 28 %. Ascending thin-layer chromatograms on silicic acid, developed in chloroform + methanol + 2·5 N-aqueous ammonia (60 + 35 + 8 by vol.) and stained with bromthymol blue or Ehrlich reagent, showed 4 ganglioside bands. This was therefore a convenient method for preparing a mixture of the 4 most abundant gangliosides.

(*b*) *Cerebroside.* Cerebroside was prepared from beef brain by the method of Carter & Fujino (1956).

(*c*) *Ganglioside cerebroside complex.* Cerebroside and ganglioside were mixed in the proportion of 3:1 and dissolved in a mixture of chloroform + methanol (3 + 1, v/v; 3 ml./100 mg. ganglioside) with gentle warming. The solvent was then evaporated off under reduced pressure at 60° (water-bath temperature). The complex was resuspended in water (2·7 ml./100 mg. ganglioside) by stirring with a glass rod, to give a thick white suspension.

(*d*) *Treatment of toxin with the ganglioside cerebroside complex.* To a solution containing 12 mg. toxin preparation/ml. in water, 0·25 vol. of the above ganglioside cerebroside suspension was added. The mixture was allowed to stand at room temperature for 5 min. and then centrifuged at 35,000 *g* (20,000 rev./min. in 40·2 head of Spinco, Model L) for 20 min. The supernatant fluid was decanted and treated in the same way 3 times with the same amount of ganglioside/cerebroside suspension as before. The supernatant fluid was then freeze-dried and the white powder obtained constituted the non-spasmogenic fraction (NSP) used in this work. This fraction contained 0·6 % of the toxicity (as measured in mice) of the original toxin preparation.

Assay of non-spasmogenic activity. This was done by Dr G. A. Feigen in his laboratory as described in Feigen *et al.* (1963).

Injection of rabbits. The preparations of toxin or NSP, dissolved in 0·1 M-phosphate (pH 7·0) containing 0·2 % (w/v) gelatin, were injected into the anterior chamber of the eye of albino rabbits as described by Ambache (1951). In each case, 0·05 ml. of solution was injected. Toxin A was diluted so that this volume contained 0·01 μg. (1000 LD 50); toxin B to contain 25 μg. (3·75 LD 50); NSP, prepared from B, to contain 25 μg. in 0·05 ml. (0·05 LD 50).

Measurement of pupil diameter. The transverse diameter of the pupil was measured

by using calipers held just in front of the eye. First, the diameter was measured in diffuse daylight and then the reaction to light was obtained by measuring the pupil diameter again after 30 sec. of direct illumination from a 100 W lamp held 10 cm. from the eye. Measurements were not made during the first day following the injection because during this period the irides sometimes showed signs of vascular engorgement.

RESULTS

Tetanus toxin was injected into one eye of each of 4 rabbits. In two of these animals the other eye was injected with gelatin buffer and in the other two animals the second eye was injected with the non-spasmogenic fraction. A fifth rabbit was given non-spasmogenic fraction into one eye and gelatin buffer into the other. The diameter of the pupil of each eye was measured at intervals for 410 hr after injection, firstly in diffuse daylight and then during direct illumination.

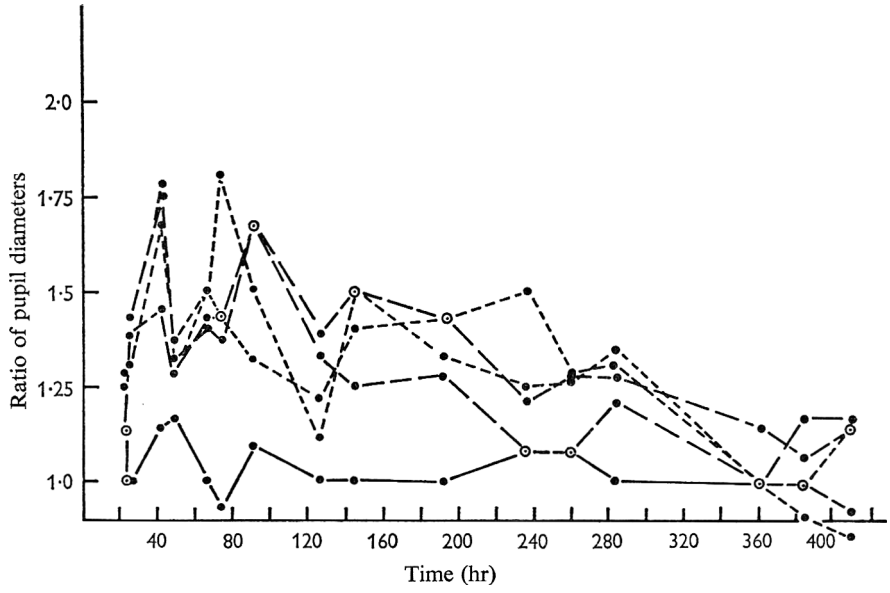

Fig. 1. Ratio, in diffuse light, of the diameter of the pupil of the eye injected with either buffer (first and second rabbits, broken lines) or NSP (third and fourth rabbits, dotted lines) to the diameter of the pupil of the other eye of the same animal injected with tetanus toxin, plotted against the time after the injection. The solid line represents the ratio of the diameters of the pupils of the eyes of the fifth rabbit injected on the one side with buffer and on the other with NSP. (For dosages see legend of Table 1.)

In Fig. 1 the results obtained in diffuse daylight are illustrated. The ratio of the diameter of the pupil of each eye injected with tetanus toxin to the diameter of the pupil of the other eye of the same animal injected either with buffer (broken lines) or with NSP (dotted lines) is plotted against the time after the injections. The fifth line (5) represents the ratio of the diameter of the pupils of the eyes of the fifth rabbit which were injected with NSP and buffer respectively. It can be seen in Fig. 1 that the ratio of the pupil diameters in the animal where NSP was compared with buffer remained around a value of one. On the other hand, the ratios in the rabbits where the effect of tetanus toxin was investigated were always above a value of one. The

pupil of an eye injected with tetanus toxin and observed in diffuse daylight was around 50 % larger than the opposite pupil regardless of whether that had been injected with buffer only (broken lines) or with NSP (dotted lines). Hence the NSP no longer possessed the paralytic activity, present in the original crude toxin preparation, which causes an increased pupil diameter observable in diffuse daylight. Figure 2 issimilar to the previous

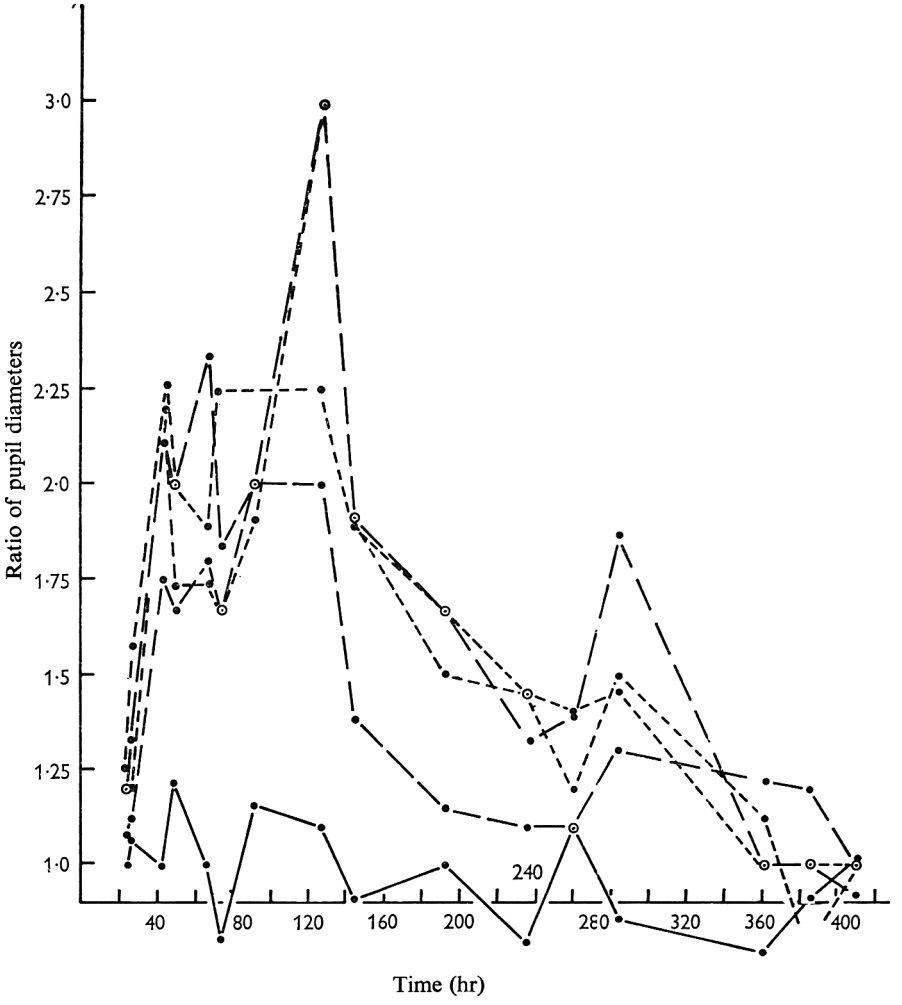

Fig. 2. Details as in Fig. 1, but experiment in direct light from 100 watt lamp held 10 cm. in front of eye.

graph except that here the ratios are derived from measurements of the pupil diameters in direct light. Again, where the ratio is obtained from the animal with NSP in one eye and buffer in the other, the ratio is near to one. In the animals where the ratio is obtained from comparing tetanus toxin injection with either NSP or buffer, the ratios are well above one, and are considerably higher than those obtained in diffuse daylight. Again, there was no difference between the ratios whether tetanus toxin was compared with NSP or with buffer.

The ratios are higher in direct than in diffuse light because the tetanus toxin, but not

Table 1. *A comparison of the effect of crude tetanus toxin on the light reflex in the rabbit eye with that of either non-spasmogenic fraction (NSP) or buffer*

Rabbits 1 and 2 were injected with 25 μg. untreated crude tetanus toxin (B; containing 3·75 mouse LD 50 in 0·05 ml. of 0·1 M-phosphate (pH 7) containing 0·2% gelatin) in one eye and gelatin buffer (0·05 ml.) in the other eye. Rabbits 3 and 4 were also injected with crude tetanus toxin in one eye (as above) but the other eye was injected with 25 μg. non-spasmogenic fraction (NSP prepared from toxin *B*; containing 0·05 mouse LD 50 in 0·05 ml. gelatin buffer). Rabbit 5 was injected with 25 μg. NSP in one eye (as above) and gelatin buffer (0·05 ml.) in the other eye. (For technique of injection, see Ambache 1951.) The pupil diameters were measured at times after the original injection as indicated in the table: (i) in diffuse light; (ii) in response to a 100 W lamp shone directly on the eye from a distance of 10 cm. The values are the percentage decrease in pupil diameter produced by this light.

Rabbit no.	Eye injected with	Time after injection (hr)																
		23	25	42	48	66	73	90	126	144	192	42 to 192*	236	260	284	362	385	410
		% decrease in pupil diameter on exposure to light																
1	Toxin	33	50	13	17	10	0	0	17	10	17	11±7	15	21	24	21	8	23
2	Toxin	25	40	10	5	0	0	0	0	0	0	2±4	6	0	17	38	19	31
3	Toxin	40	25	0	0	0	0	0	0	0	10	1±4	11	6	6	25	19	25
4	Toxin	29	21	0	0	5	0	0	10	5	6	3±4	13	14	47	25	30	17
3	NSP	38	23	25	21	14	14	31	55	27	14	25±14	8	14	15	25	19	14
4	NSP	38	30	11	36	25	20	33	56	25	25	29±13	25	10	25	50	18	29
5	NSP	33	36	25	21	22	33	36	44	31	18	29±9	33	15	33	42	36	28
1	Buffer	31	39	27	36	29	14	17	44	19	7	24±12	17	23	29	36	23	29
2	Buffer	38	36	25	38	40	25	17	56	21	14	29±14	14	7	43	29	13	14
5	Buffer	35	46	14	25	22	23	40	50	23	18	24±12	23	17	22	25	17	25

* Mean (42–192 hr inclusive) ± standard deviation.

the buffer or NSP, diminished the reflex constricted of the pupil in response to light. This effect is illustrated in Table 1, where it is shown that over a period lasting from a day or so after injection up to about 10 days, tetanus toxin greatly depressed the % decrease in pupil diameter which occurred in response to direct illumination; neither NSP nor buffer had such an effect. In Table 2 the means (and their standard deviations) are shown for the light response in each of the injected eyes when the values obtained during the period of maximal effect (42–192 hr) are considered. Application of the *t* test showed that even the response of the eye of rabbit 1 injected with toxin ($11 \pm 7\%$) was significantly lower than the response with buffer ($P = 0{\cdot}02$) and the other three values with toxin injection (rabbits 2–4) were of course more significant ($P < 0{\cdot}005$). There was no difference between the values with NSP and those with buffer.

The action of highly purified tetanus toxin on the rabbit eye was similar to the action of crude toxin. Thus, 42 hr after injection of 0·01 μg. of purified toxin (1000 mouse LD 50) the reflex response to light in the injected eye was abolished. The response remained absent for about 5 weeks, after which time it gradually returned.

DISCUSSION

The present work shows that the treatment of crude tetanus toxin with ganglioside/cerebroside complex, which leaves its non-spasmogenic activity unaffected (or increased) not only removes 99% of the tetanospasmin but also removes the ability to paralyse the cholinergic innervation of the sphincter pupillae. It can be concluded that the non-spasmogenic factor, able to increase the frequency of intercostal miniature end-plate potentials in a hemithorax preparation from the mouse, is not responsible for the paralysis observed in the eye after injection of tetanus toxin into the anterior chamber. It therefore still remains open for an *in vivo* action of this non-spasmogenic fraction to be demonstrated.

Since highly purified tetanus toxin had a similar effect to crude toxin, it is likely that this effect in the eye is due to tetanospasmin itself. In the present work with crude toxin the effect on pupil diameter was as great as that obtained by Ambache *et al.* (1948*a*) and was produced with approximately a hundredth of the toxicity.

We wish to thank Dr W. E. van Heyningen for advice and encouragement; Dr R. O. Thomson of the Wellcome Research Laboratories for supplying the preparations of tetanus toxin; Dr G. A. Feigen of Stanford University for assaying samples of NSP; Miss Jane Gray for technical assistance. This work was done during the tenure of a contract (Task No. 103-474) between Dr W. E. van Heyningen and the Office of Naval Research of the United States Department of the Navy.

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Nitrogen-fixers—Pseudomonads and other Aerobic Bacteria—from Rhodesian Soils

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SUMMARY

Very numerous small transparent colonies appeared on plates of nitrogen-poor agar inoculated with soils from various places in Rhodesia. Eighty-three cultures of bacteria were isolated from these colonies, all but two of them small Gram-negative rods. Two cultures contained Gram-positive cocci. Thirty-nine of the isolates fixed nitrogen, ten of them fixing as much as or more than a Beijerinckia culture grown under the same conditions. The Gram-negative isolates are not all alike; their identity is uncertain, but some might be Pseudomonas species, some Achromobacter (or Acinetobacter), and one good fixer might be a Flavobacterium. There are also a few isolates which attack glucose fermentatively. Nitrogen-fixers of these types—aerobic bacteria with very small colonies—appear to be numerous and widely distributed in Rhodesian soils.

INTRODUCTION

In the course of ecological studies on bacteria of various physiological groups in soils in the neighbourhood of Salisbury, Rhodesia (Meiklejohn, 1965, 1967), plates were prepared for counting Azotobacter and Beijerinckia species by Jensen's method (1940). These plates, of nitrogen-poor sucrose agar, were examined after two or three days of incubation, to see whether Azotobacter colonies had developed. On many of the plates, prepared from soils of varying properties and from many different sites, numerous very small transparent colonies appeared during the first 2 or 3 days. As these small colonies grew abundantly on nitrogen-poor agar, it was thought possible that some of them might be nitrogen-fixers.

In some preliminary experiments, an isolate from such a small transparent colony (culture B), derived from soil sample 10 (see Table 3), was compared for nitrogen-fixing ability with a Beijerinckia culture isolated from the same soil sample. The results of two such experiments, testing different concentrations of molybdenum and of calcium, are given in Table 1. From this table it is apparent that culture B fixed about as much nitrogen as the Beijerinckia culture, and that, like Beijerinckia, culture B seemed to require molybdenum, but not calcium, when grown in a nitrogen-poor medium.

Culture B was found not to be a pure culture, so it was purified, and cultures 1, 7 and 10 were derived from it. These were all found to be nitrogen-fixers (see Table 3), and were used as the first cultures of a series systematically isolated from soil samples and tested for their ability to fix nitrogen (or rather, for ability to fix enough nitrogen to be detectable by a Kjeldahl determination).

METHODS

Isolation of cultures

Forty-nine samples of surface soil (down to 4 in.) were collected from selected sites in the following seven places: Atlantica Ecological Research Station, 16 miles W. of Salisbury; College Farm, in the Mazoe valley, 24 miles N.; Glen Rosa farm, near Raffingora, 90 miles N.; Grasslands Research Station, Marandellas, 50 miles S.E.; Henderson Research Station, Mazoe valley, 20 miles N.; Mount Pleasant vlei, and the University College site (U.C.R.N.), both in the northern outskirts of Salisbury.

Table 1. *Effect of molybdenum, and of calcium, on nitrogen fixation by culture B and a Beijerinckia culture*

	Total nitrogen (mg./25 ml.)					Nitrogen fixed	
		Beijerinckia		Culture B		(mg./25 ml.) (mean)	
Medium	Control (not inoculated)	(1)	(2)	(1)	(2)	Beijerinckia	Culture B
+2 p.p.m. Mo	1·75	11·06	—	12·25	11·73	9·31	10·24
+0·01 p.p.m. Mo	1·68	8·16	10·50	7·88	7·00	7·65	5·76
No Mo	1·68	4·38	3·94	5·61	3·05	2·48	2·65
Sucrose medium without yeast extract. Cultures incubated 25 days at 30°.							
+100 p.p.m. Ca	1·75	6·13	7·28	4·94	4·46	4·95	2·95
+20 p.p.m. Ca	1·60	6·70	6·55	4·81	4·38	5·03	2·99
No Ca	1·81	4·08	6·34	3·94	5·83	3·40	3·07
Sucrose medium without yeast extract. Cultures incubated 28 days at 30°.							

Suspensions of 1/10, 1/40 and 1/160 in sterile water were made from each soil sample, and 1 ml. portions spread over the surface of pre-dried agar plates (Jensen, 1940). The plates were incubated at 30° for 2 days, and then colonies were picked to agar slopes, and afterwards purified by making poured plates and picking colonies again. The medium used for the isolation and purification of the cultures was the 'sucrose agar' of Brown, Burlingham & Jackson (1962) (g./l.): sucrose, 5; K_2HPO_4, 0·8; $MgSO_4.7H_2O$, 0·2; $FeSO_4.7H_2O$, 0·04; $CaCl_2.2H_2O$, 0·5; $Na_2MoO_4.2H_2O$ 0·005; agar 15; deionized water, 1000 ml.

Stock cultures were maintained on the same medium, but with the addition of 10 ml 0·1% yeast extract (Difco) per litre.

Testing for nitrogen fixation

Culture conditions: The isolates were tested in batches, by growing them on a nitrogen-poor liquid sucrose medium, of the same composition as the medium used for plates, without agar but with yeast extract. Forty ml. of this medium in a 250 ml. conical flask was inoculated with 1 ml. of a suspension made by emulsifying the growth on a 7-day-old agar slope culture with 10 ml. sterile liquid medium. The flasks were stoppered with cotton wool, which was covered with metal foil, and were incubated at 30° for 5 weeks. With each batch of cultures, a Beijerinckia culture prepared in the same way and a flask of uninoculated sterile medium were also incubated.

Estimates of total nitrogen were made by Kjeldahl digestion, followed by determination of the ammonia formed by the micro-diffusion method of Conway (1950).

Ten ml. samples of the cultures, and of the uninoculated control, were digested with sulphuric acid, with selenium dioxide as a catalyst. After cooling, the contents of each Kjeldahl flask were diluted to 50 ml., and 1 ml. samples of this taken for ammonia determinations. Three Conway units were prepared for each culture, and for the control, and 2 blank units, with reagents but no test solution, were also prepared. All the units were incubated overnight at 25° and the ammonia estimated by titration with 0·01 M-hydrochloric acid.

Because the uninoculated controls always contained a small amount of nitrogen, the estimates from the inoculated flasks were corrected for this amount, to give an estimate of nitrogen fixed (in 5 weeks at 30°). In the tables this is given as mg. nitrogen/25 ml. culture.

Observations and tests on nutrient agar slope cultures

The isolates were subcultured to nutrient agar (Oxoid) slopes for observations and biochemical tests. The following observations were made: *colour* of growth on slope; *motility*, on hanging drop from a 24 hr culture; *Gram stain*, *cell length* and *shape*, on Gram-stained films.

Biochemical tests made by methods described by Cowan & Steel (1965) were:

Oxidation–fermentation (O/F) test—Hugh & Leifson's medium with 1% glucose, incubated at 28° for up to 14 days, *Escherichia coli* as fermentative control.

Catalase activity—a few drops of 3% hydrogen peroxide run down a 24–72 hr slope, *Staphylococcus albus* as positive control.

Oxidase activity by Kovacs method; *Pseudomonas aeruginosa* as positive control, *E. coli* as negative control.

Gelatin liquefaction by Frazier method: *P. aeruginosa* as positive control, *E. coli* and an uninoculated plate as negative controls.

Nitrate reduction was tested on tubes of Meiklejohn medium (1940) containing Durham tubes, which were incubated at 30° for 7 days, and then examined for gas formation and tested for nitrate with Griess-Ilosvay reagent. The medium contained (g./l.): KNO_3, 1; glycerol, 3·6; KH_2PO_4, 0·5; K_2HPO_4, 0·5; $CaCl_2.2H_2O$, 0·1; NaCl, 0·1; $MgSO_4.7H_2O$, 0·3; $FeCl_3$, 0·01; deionized water, 1000 ml.

RESULTS

The nitrogen-poor agar plates made from all the 49 soil samples were covered with very numerous tiny colonies after two days' incubation at 30°. The colonies were raised and transparent, looking like little drops of clear syrup on the agar surface. Plates inoculated with 1/10 and 1/40 suspensions were too crowded to count, but an approximate count could usually be done on plates made from 1/160 suspensions. Table 2 gives approximate counts from 14 soil samples, which give rough estimates of 32,000 to 128,000 organisms per gram soil. All the samples but one also contained Beijerinckia sp., which were very much fewer than the small-colony organisms.

Nitrogen-fixing ability of isolates

Eighty-three cultures were isolated from the small transparent colonies, and all of them were bacteria. Most of them grew very slowly in the liquid medium used to test for nitrogen fixation, and it was therefore assumed that they were slow fixers, and the

incubation period was prolonged to 5 weeks. Nitrogen determinations at the end of 5 weeks showed that 39 of the 83 isolates could fix nitrogen; there were 10 good fixers (as good as Beijerinckia or better), 19 moderately good, and 10 poor fixers.

Soils yielding good nitrogen-fixers. There were 7 soil samples which yielded good nitrogen-fixers (Table 3). They had three things in common: none of them was very

Table 2. *Numbers of small transparent colonies and of Beijerinckia*

Soil sample	Place	Colonies per plate 1/160 dilution (approx.)	Estimated numbers per gram soil	Beijerinckia per gram soil
416	Henderson	500	80,000	29
420	Henderson	400	64,000	0
433	College Farm	800	128,000	1,905
434	College Farm	400	64,000	13,000
556	College Farm	200	32,000	288
557	College Farm	300	48,000	465
558	College Farm	200	32,000	645
559	College Farm	300	48,000	542
560	College Farm	300	48,000	606
562	College Farm	200	32,000	5,143
573	Glen Rosa	200	32,000	384
574	Glen Rosa	200	32,000	5,362
575	Glen Rosa	500	80,000	2,055
577	Glen Rosa	300	48,000	438

Table 3. *Soil samples yielding good nitrogen-fixers*

Sample	pH	Place: type of soil	Cultures isolated	N fixed (mg./25 ml.)
456	7·6	Atlantica, termite md. nr. stream, brown clay loam	456B	5·60
425	6·8	Henderson, Rhodes grass pasture, yellow-brown clay loam	425	3·62
430	6·4	Henderson, Rhodes grass pasture, red clay loam	430	3·56
10	6·4	Atlantica, grass near stream, dark brown loam	10	3·50
			1	2·45
			52A	2·03
			7	1·35
420	6·0	Henderson, Panicum pasture, brown clay loam	420	3·40
537	6·2	Atlantica, under *Brachystegia spiciformis*, brown sandy loam	537B	2·75
			537A	2·70
536	7·2	Atlantica, under *Combretum molle*, brown loam	536B	2·22
			536A	1·75
423	6·2	Henderson, Panicum pasture, yellow-brown clay loam	Beijerinckia	2·80*
428	6·4	Henderson, *Desmodium discolor*, light brown clay loam	Beijerinckia	2·24†

* Mean of 7 determinations; † Mean of 8 determinations.

acid, all but one (537) were heavy soils, and all were covered with permanent vegetation. Four of the 7 were under grass, 2 were from among the roots of trees, and one (456) was from a termite mound which had bushes and trees, including a large *Acacia galpinii*, growing on it.

The 10 good nitrogen-fixers fixed from 2·03 to 5·60 mg. N/25 ml. culture in 5 weeks at 30°; average nitrogen fixation by the two Beijerinckia cultures grown under the same conditions was 2·80 mg. N/25 ml., and 2·24 mg. N/25 ml.

Table 4. *Soil samples yielding moderate nitrogen-fixers*

Sample	pH	Place: type of soil	Cultures isolated	N fixed (mg./25 ml.)
569	6·2	UCRN, sprouting grass, brown organic	569B	1·98
451	6·0	Grasslands, nr. river, dark brown clay	451	1·94
559	6·1	College farm, Rhodes grass, brownish yellow loam	559A	1·93
			559B	1·40
574	6·6	Glen Rosa, burnt bush, brown organic	574B	1·93
454	7·6	Atlantica, Termite md. in wood, light brown clay	454	1·89
560	6·4	College farm, Rhodes grass, yellow-brown loam	560A	1·80
562	6·1	College farm, Rhodes grass, red-brown clay loam	562A	1·80
571	6·2	UCRN, mown grass, brown loam	571B	1·75
557	6·2	College farm, Rhodes grass, brownish yellow loam	557B	1·68
556	6·1	College farm, Rhodes grass, brownish yellow loam	556B	1·63
577	6·4	Glen Rosa, grass fallow, light brown sandy loam	577A	1·58
539	6·4	Atlantica, under *Terminalia sericea*, brown organic	539A	1·52
433	6·0	College farm, burnt grass, brown sandy loam	433	1·23
570	6·5	UCRN: Termite md., brown clay loam	570B	1·17
			570A	0·96
573	6·2	Glen Rosa, grass after maize, light brown loam	573B	1·05
			573A	0·88
575	7·2	Glen Rosa, bush, dark brown organic	575A	1·00

Table 5. *Soil samples yielding poor nitrogen-fixers, or none*

Sample	pH	Place: type of soil	Cultures isolated	N fixed (mg./25 ml.)
434	6·0	College farm, wood, brown sandy loam	434B	0·81
416	5·8	Henderson, pasture, dark brown organic	416	0·63
566	6·0	UCRN, top end, mown grass, brown organic	566A	0·58
314	c. 5·6	Grasslands, maize, brown sandy	34A	0·53
558	6·2	College farm, Rhodes grass, brownish yellow loam	558A	0·53
			558B	0·53
450	6·1	Grasslands, Hyparrhenia, brown sandy	450B	0·40
457	6·6	Atlantica, Hyparrhenia, brown loam	457B	0·35

Soil samples yielding only isolates that did not fix nitrogen: 2 from Atlantica, 4 from College Farm, 2 from Glen Rosa, 2 from Grasslands, 5 from Henderson, 2 from Mount Pleasant vlei, 2 from University College site (UCRN). Total 19.

Table 6. *Characteristics of the nitrogen-fixing isolates*

Group	Culture no.	Gram reaction	Shape	Cell length (μ, approx.)	Motility	Colour (nutrient agar)	Reactions in tests*					N-fixing ability†
							O/F test	Catalase	Oxidase	Nitrate to nitrite	Gelatin liquefaction	
A 1	‡425	−	Oval rod	1·0	+	Yellow	F	+	−	+	−	Good
	539A	−	Short rod	1·2	+	Orange	F	+	−	+	−	Moderate
	573A	−	Short rod	1·3	−	Orange	F	+	−	−	−	Poor
	573B	−	Short rod	1·3	−	Orange	F	+	−	+	−	Moderate
A 2	571B	−	Short rod	1·3	−	Pink	(F)	+	+	+	+	Moderate
B 1	10	−	Short rod	1·0	+	Buff	(O)	+	−	+	−	Good
	430	−	Short rod	1·0	+	Buff	(O)	+	+	+	−	Good
	433	−	Short rod	1·0	+	Buff	O	+	(+)	−	−	Moderate
B 2	559A	−	Short rod	1·1	+	Green	O	+	+	−	+	Moderate
	559B	−	Short rod	1·1	+	Green	O	+	+	−	+	Moderate
	560A	−	Short rod	1·0	+	Green	O	+	+	−	+	Moderate
B 3	34A	−	Short rod	1·0	+	Yellow	O	?	?	+	+	Poor
	434B	−	Short rod	1·0	+	Yellow	O	+	+	−	+	Poor
	457B	−	Oval rod	1·0	+	Yellow	O	+	−	−	+	Poor
B 4	52A	−	Short rod	1·0	+	White	O	+	+	−	−	Good
	416	−	Short rod	1·0	+	Grey	(O)	−	+	−	−	Poor
	556B	−	Short rod	1·4	+	Grey	O	+	+	−	−	Moderate
	558B	−	Short rod	1·1	+	Grey	(O)	+	+	−	−	Poor
	562A	−	Oval rod	0·8	+	Grey	(O)	+	+	−	−	Moderate
	566A	−	Short rod	1·1	+	Grey	O	(+)	−	−	+	Poor
B 5	450B	−	Short rod	1·0	+	Grey	O	?	?	+	+	Poor
	451	−	Short rod	1·0	+	Grey	O	?	?	+	+	Moderate
	454	−	Rod	1·0	+	Grey	N	(+)	−	+	+	Moderate
	537A	−	Oval rod	0·5	+	Grey	(O)	+	+	+	+	Good
	557B	−	Short rod	1·1	+	Grey	(O)	+	+	+	−	Moderate
C 1	456B	−	Short rod	1·0	−	Yellow	(O)	+	+	−	−	Good
C 2	1	−	Short rod	1·0	−	Buff	(O)	+	(+)	−	−	Good
	7	−	Short rod	1·0	−	Buff	(O)	+	(+)	−	+	Moderate
	536B	−	Short rod	1·6	−	Buff	N	(+)	+	−	−	Good
	558A	−	Short rod	0·7	−	Grey	N	+	+	−	−	Poor
	569B	−	Short rod	1·1	−	Grey	(O)	+	(+)	−	−	Moderate
	575A	−	Short rod	1·0	−	Grey	N	+	+	+	−	Moderate
	577A	−	Short rod	1·7	−	Grey	O	+	+	−	+	Moderate
C 3	420	−	Short rod	1·0	−	Yellow	N	(+)	−	−	−	Good
	536A	−	Short rod	0·8	−	Green	N	+	−	−	+	Moderate
	537B	−	Rod	1·5	−	White	N	+	−	+	−	Good
	574B	−	Oval rod	0·7	−	Grey	N	−	−	−	−	Moderate
D	§570A	+	Coccus	2·8	−	Buff	(F)	+	−	−	−	Poor
	§570B	+	Coccus	2·8	−	Buff	(F)	+	−	+	−	Moderate

* Reactions in tests: F, fermentative attack; (F) slow fermentative attack; O, oxidative attack; (O), slow oxidative attack; N, no attack; +, positive; (+), weakly positive; −, negative.

† Nitrogen-fixing ability: for details see Tables 3–5.

‡ Culture 425 gives acid on glucose and sucrose, not on lactose (1 % in peptone water).

§ Cultures 570A and 570B contained Gram-negative cells as well as Gram-positive.

Soils yielding moderate nitrogen-fixers. Two moderate nitrogen-fixers (7 and 536A) were obtained from soils that also yielded good fixers. The other 17 isolates in this group came from 16 soil samples of differing properties, with nothing in common except that none was very acid (Table 4). The 19 isolates classed as moderate nitrogen-fixers fixed from 1 to 1·98 mg. N/25 ml.; that is, their performance ranged from strains fixing nearly as much nitrogen as Beijerinckia to strains fixing only half as much.

Soils yielding poor nitrogen-fixers, or none. Seven soil samples, of differing properties, yielded isolates of poor nitrogen-fixing ability (less than 1 mg. N/25 ml.). There were also 19 soil samples from which no nitrogen-fixers of the small-colony type were isolated, though they all contained Beijerinckia species.

Characteristics of the isolates

On the nitrogen-poor sucrose agar all the isolates looked much alike, with tiny glassy colonies on plates, and colourless gummy streaks on slopes. But on nutrient agar, on which most of them grew quite well, differences in colour appeared.

The isolates also differed in other respects, and were not a uniform group, though nearly all of them were small Gram-negative rod-shaped bacteria. Two cultures only, from soil sample 570, contained large Gram-positive cocci (group D). The Gram-negative isolates can be divided into three groups, A, B and C, which can be further subdivided (see Table 6):

Group A: attacking glucose fermentatively in the O/F test.
- A 1 Fast fermenters
- A 2 Slow fermenters

Group B: attacking glucose oxidatively or not at all: motile.
- B 1 Buff on nutrient agar
- B 2 Green on nutrient agar
- B 3 Yellow on nutrient agar
- B 4 White or grey: nitrate not reduced
- B 5 Grey: nitrate reduced to nitrite

Group C: attacking glucose oxidatively or not at all: non-motile.
- C 1 Yellow on nutrient agar: oxidase-positive
- C 2 Buff or grey: oxidase-positive
- C 3 Oxidase-negative

Nitrogen-fixing ability does not seem to be connected with any other of the properties studied; 13 isolates, 5 of which fix nitrogen, are in group A; group B contains 20 fixers and 20 non-fixers, and group C 12 fixers and 15 non-fixers. Both of the Gram-positive cultures (D) fixed nitrogen, and there was one non-fixer which grew so badly on nutrient agar that it could not be grouped. The data for the 39 nitrogen-fixers are given in Table 6, and it can be seen that good, moderate and poor fixers are found in all of the groups A, B and C.

DISCUSSION

The bacteria which form small transparent colonies on nitrogen-poor sucrose agar were found in many soil samples collected from various places in eastern Rhodesia, including all of the 49 samples described in this paper. The numbers of small colonies

(Table 2) indicate a large population, of tens of thousands per gram of soil. Since nearly half of the eighty-three colonies picked at random contained nitrogen-fixers, it is probable that aerobic nitrogen-fixers of this type are both widespread and abundant in eastern Rhodesian soils, and account for more nitrogen fixation than organisms from either of the genera *Azotobacter* or *Beijerinckia*.

Species of the genus *Azotobacter* are rare in eastern Rhodesian soils, probably because most soils are too acid. We have found *A. chroococcum* at Henderson, but consistently in one soil only, a red clay from the bottom of the Mazoe valley. Species of the genus *Beijerinckia* are widespread in Rhodesian soils, but usually in small numbers (Table 2), and seem to be more numerous in poor soils (Meiklejohn, 1965). They are slow fixers of nitrogen, and no more efficient than the best of the new isolates. The average nitrogen fixed by the two Beijerinckia cultures used as controls in this work was 2·80 mg. and 2·24 mg. N/25 ml. Ten of the small-colony isolates fixed more than 2 mg. N/25 ml., the best of all, culture 456B, fixing 5·6 mg. N/25 ml.

The identity of the new isolates is doubtful. They are not all alike (Table 6), but none of them can be identified as a species of the genera *Azotobacter* or *Beijerinckia*, or as *Derxia gummosa* (Jensen, Petersen, De & Bhattacharya, 1960), from all of which they differ in colony type, in cell size, and in other characters.

The new isolates are smaller in cell size than any species of *Azotobacter*, even *A. vinelandii* (Jensen, 1954), except for the two in group D, which are Gram-positive. The isolates in groups A, B and C are all small rods, the largest being only 1·7 μ long. *Azotobacter chroococcum* forms colonies on nitrogen-poor sucrose agar in 2 to 3 days (Brown *et al.* 1962), but the colonies are large and opaque, and turn brown after some time, unlike the small glassy colourless colonies of the new isolates.

Beijerinckia colonies, unlike those of the new isolates, are very slow to appear on sucrose agar plates. They are usually not visible until after 10–14 days' incubation at 30°. They are white, opaque, and very sticky, even rubbery (Jensen, 1954). Beijerinckia organisms have a very characteristic appearance, with an oil drop at each end, which was never shown by any of the new isolates.

Derxia gummosa (Jensen *et al.* 1960) is a slow nitrogen-fixer isolated from an Indian soil. It forms yellow colonies, which become large and dark brown later, on nitrogen-poor mannitol agar. The cells are rod-shaped and large, 3–6 by 1–1·2 μ in young cultures, bigger than any of the new isolates. Also *D. gummosa* is catalase-negative and fermentative, a combination of characters not found in any of the new isolates.

The new isolates, however, resemble the small-celled aerobic nitrogen-fixers described by Skinner (1928), Stapp (1940), Anderson (1955), Voets & Debacker (1956) and V. Jensen (1956). Stapp (1940) isolated a small Gram-negative nitrogen-fixing rod from vegetable material sent from Egypt, and called it *Azotomonas insolita*. It was motile, colourless on nutrient agar, and fermented sugars with acid and gas. Nitrogen-fixing strains of the fermenting species *Aerobacter aerogenes* were isolated by Skinner (1928) in U.S.A., and V. Jensen (1956) in Denmark. (Mahl, Wilson, Fife & Ewing (1965), however, found that several small-celled nitrogen-fixers originally classified as strains of *A. aerogenes* could be identified serologically as types of *Klebsiella pneumoniae*.)

Non-fermenting small-celled aerobic nitrogen-fixers have also been described. In 1955, Anderson found a nitrogen-fixing Pseudomonas in soils from Idaho, in which

it was much more abundant than Azotobacter. The growth on Martin's nitrogen-poor agar was colourless and transparent at first, becoming yellow or orange after 2 weeks. Anderson called it *Pseudomonas azotocolligans*, n.sp. (Anderson, 1955). In the following year, Voets & Debacker isolated, from a greenhouse soil in Belgium, a non-pigmented Pseudomonas which fixed about 1 mg. N/100 ml. medium in 7 days; they called it *P. azotogensis* n.sp. (Voets & Debacker, 1956).

Voets' & Debacker's culture, and 5 other Pseudomonas cultures, were examined by Proctor & Wilson (1958), who found that all of them, and also all of eight Achromobacter cultures (one of which had been isolated in Denmark by V. Jensen), fixed nitrogen. The aerobic fixation by the Pseudomonas cultures was from 3 to 50 μg./ml. culture in 4 days at 30°. The Achromobacter cultures fixed from 3 to 17 μg. N/ml. under the same conditions.

But, though the new isolates resemble those previously described, they cannot be definitely identified as any of them. The cultures in group A 1, which ferment glucose rapidly in the O/F test, could belong to some genus in the Enterobacteriaceae, as they are fermenting, catalase-positive and oxidase-negative; but not to the genus *Aerobacter* or *Klebsiella*. Nor can they be *Azotomonas insolita*, because they are pigmented on nutrient agar and give no gas on sugars.

The motile, oxidative cultures in group B are nearly all catalase- and oxidase-positive, and could be Pseudomonas species, the non-pigmented forms in groups B 4 and B 5 resembling the *P. azotogensis* of Voets & Debacker (1956).

Of the non-motile oxidative forms in group C, culture 456B, which is put by itself in group C 1, forms yellow pigment on nutrient agar and might be a Flavobacterium (Table 6). This culture was the best nitrogen-fixer of the new isolates, (5·6 mg. N/25 ml.). The cultures in group C 2, which are oxidase-positive, could belong to the genus *Achromobacter*, and those in group C 3 to the genus *Acinetobacter* (Cowan & Steel, 1965).

The identity of the Gram-positive cocci in the two cultures from sample 570 is a complete puzzle (group D). The cultures also contained Gram-negative cells, and it is possible that they, not the cocci, are the real nitrogen-fixers.

It may seem rather extraordinary that we should have isolated 39 cultures of nitrogen-fixers, and also 44 cultures which resembled one or other of the 39 in every respect save that they could not fix nitrogen. This, however, has been observed by Skinner (1928), who isolated 23 strains of *Aerobacter aerogenes*, only 3 of which fixed nitrogen, and by V. Jensen (1956). He had two *A. aerogenes* strains which were identical, except that one fixed nitrogen and the other did not. Mahl *et al.* (1965), in addition to their nitrogen-fixing strains of *Klebsiella pneumoniae*, tested 19 strains which were found not to fix nitrogen.

It might be asked why, since these small-celled aerobic nitrogen-fixers seem to be abundant and widespread in Rhodesian soils, nothing of the kind has been observed elsewhere, except by Anderson (1955) in Idaho. The reason may well be that this type of nitrogen-fixer either does not occur, or is very rare, in soils in Europe, and perhaps in northern North America too. Brown *et al.* (1962) found that nitrogen-poor agar plates inoculated on the surface with a variety of soils from Britain yielded, after 2 or 3 days of incubation, either Azotobacter colonies or nothing. There was never any abundant development of small colonies, such as we observed in Rhodesia. Colonies of other organisms than Azotobacter sometimes developed on plates inoculated with

soils from Britain, but these did not appear till the plates had been incubated for a week or longer.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its fiftieth General Meeting at Churchill College, Cambridge, on Thursday, Friday and Saturday 14, 15 *and* 16 *September* 1967. *The following communications were made:*

ORIGINAL PAPERS

The Nitrogen Sources of *Bacteroides amylophilus*. By P. N. HOBSON, E. I. MCDOUGALL and R. SUMMERS (*Rowett Research Institute, Bucksburn, Aberdeen*)

Some rumen bacteria have been shown to grow in media containing ammonia as main source of nitrogen and with others ammonia has been found to be essential even in the presence of amino acids. Chemical analysis suggested that a strain of *Bacteroides amylophilus*, growing in continuous culture in a medium containing tryptose and ammonia, produced almost all of its cell nitrogen from the ammonia (Hobson, P. N. & Summers, R., (1967), *J. gen. Microbiol.* **47**, 53). Also *B. amylophilus* will grow, in batch culture, in a medium containing only ammonia and cysteine, although the presence of tryptose markedly decreases the lag phase (Blackburn T. H., Ph.D. Thesis, Aberdeen, 1965). *B. amylophilus* was grown in batch cultures at 38° in two media. Both contained maltose and the same basal salts-bicarbonate solutions and were incubated under CO_2. Medium (1) contained tryptose (32·8 % of medium N), cysteine (21·3 % of medium N) and $(^{15}NH_4)_2SO_4$ (45·9 % of medium N). In medium 2 tryptose was omitted. Nitrogen from the cultures was analysed in an MS 10 mass spectrometer (AEI Ltd., Manchester). In medium (1) the $(NH_4)_2SO_4$ added contained 31·7 % ^{15}N. Ammonia distilled from the uninoculated medium contained 29·2 % ^{15}N (dilution with tryptose ammonia). After 15 hr incubation the cells contained 25·6 % ^{15}N and the supernatant ammonia 27·7 %; after 33 hr the values were 24·9 and 27·4 %. These correspond to 92·4 and 91·1 % of the cell nitrogen obtained from ammonia nitrogen. In medium (2) the ammonia contained 31·7 % ^{15}N. At 16 hr the cells contained 29·0 % ^{15}N and the supernatant 31·1 %; at 20 hr the values were also 29·0 and 31·1 %. These show 93·3 % of the cell nitrogen obtained from ammonia.

These results suggest that part of the nitrogen of the cells might come from utilization of the medium cysteine as a source of sulphur amino acids. However, in a medium as 2 but with cysteine replaced by Na_2S and ascorbic acid (medium 3) the medium NH_3 contained 31·5 % ^{15}N and the supernatants and cells at 16 and 20 hr 31·3, 29·0 and 31·4, 29·7 % ^{15}N respectively. Kjeldahl digestion of the uninoculated medium 3 gave ammonia of 30·9 % ^{15}N, while the free ammonia of the medium again contained 31·5 % ^{15}N. The bacteria thus seem to be utilizing an unidentified nitrogenous impurity in the medium, or traces of nitrogen gas contained in the CO_2 atmosphere of the culture.

We thank the Wellcome Trust for provision of the mass spectrometer.

The Internal Structure of Poly-β-Hydroxybutyrate Granules from *Bacillus megaterium*. By D. J. ELLAR, D. G. LUNDGREN, R. H. MARCHESSAULT and K. OKAMURA (*Department of Bacteriology and Botany, Biological Research Laboratories and College of Forestry at Syracuse University, Syracuse, New York*)

Poly-β-hydroxybutyrate (PHB) is a water insoluble polymer found as discrete granules in a wide variety of bacteria (Lundgren, D. G. *et al.* (1965). 89, 245) and in one species of blue-green algae (Carr, N. G. (1966), *Biochim. biophys. Acta* **120**, 308). Its role as a storage product in the endogenous metabolism of bacteria has been reviewed (Dawes, E. A. & Ribbons, D. W. (1964), *Bact. Rev.* **28**, 126). Studies have shown that rupture of the membrane-like

coating surrounding the granule and distortion of the underlying polymer morphology resulted in a decreased susceptibility of the granule to enzymic hydrolysis (Merrick *et al.* (1965), *J. Bact.* **89**, 234). This observation, together with the report that chemically purified polymer is not attacked by the intracellular hydrolytic enzyme system (Merrick, J. M. & Doudoroff, M. (1964), *J. Bact.* **88**, 60), suggested that a unique polymer morphology within the native PHB granule might determine its ability to be degraded enzymically.

This unique morphology was demonstrated when PHB granules isolated from *Bacillus megaterium* KM were treated with low concentrations of acetone and examined with the electron microscope. Although acetone is not a solvent for PHB, the granules were disrupted by 5 % aqueous acetone liberating fibrils and ribbon-like structures. These 150 Å diameter fibrils displayed a high degree of flexibility and were observed to aggregate into parallel bundles. In certain instances the close packing of the fibrils produced smooth, ribbon-like structures 50 Å in height. Treatment of the granules with 100 % acetone produced large, pointed lath-like crystals, identical with crystals of chemically purified PHB. X-ray diffraction analysis and calorimetric melting-point determinations revealed a lower degree of crystallinity in the native granule compared to purified polymer crystals and supported the concept of the 150 Å fibril as the unique PHB morphology *in vivo*. The role of this fibril in the intracellular synthesis of the PHB granule has been considered.

Cellular Lipid and the Anti-Staphylococcal Activity of Phenols. By W. B. Hugo and Irene Franklin (*Department of Pharmacy, The University, Nottingham*)

In previous communications (Hugo, W. B. & Stretton, R. J. (1964), *Nature, Lond.* **209**, 940; (1964), *J. gen. Microbiol.* **42**, 133) it was shown that when the lipid content of certain bacteria was increased by growth in the presence of glycerol the organisms became more resistant to penicillins (benzylpenicillin, quinacillin, methicillin and cloxacillin) as judged by MIC values.

In this communication the influence of cellular lipid on the resistance of *Staphylococcus aureus* (Oxford strain) to the homologous series of 4-*n*-alkyl phenols, from phenol to 4-*n*-hexylphenol, was investigated. Some of the findings reported here were reported briefly elsewhere (Hugo, W. B. & Franklin, I. (1966), *IX Int. Congr. Microbiol., Moscow* (Abst. Communic), p. 21). Cellular lipid builds up with successive subcultures in the presence of glycerol (3 %), but rapidly reverts to the control or normal level after one subculture in the absence of glycerol. Accordingly, MIC values were always determined in the presence of 3 % glycerol.

Lipid content was determined by solvent extraction and by observing changes in electrophoretic mobility in the presence of 10^{-4} M sodium dodecyl sulphate (SDS). Electron micrographs of thin sections of control and fattened cells also provided evidence that a thickening of extra-cytoplasmic layers had occurred.

The effect of a series of phenols on: (1) ability to grow (MIC values); (2) membrane integrity as measured by leakage of 260 mμ absorbing material and Pi; (3) electrophoretic mobility; and (4) the uptake of phenol from solution by cells, was determined. In summary it was found that differences in response as between normal and fattened cells began with the 4-*n*-alkyl (butyl) phenol and for fattened cells as compared with controls the MIC value was greater and both the amount of cytoplasmic material leaking and the uptake of phenol were less.

Electrophoretic mobility studies showed that fattened cells move more slowly to the anode than controls, but in the presence of phenols the mobilities of cells in general, were more greatly enhanced, especially after treatment with *n*-butyl phenol, furthermore, this enhancement was greater for fattened cells.

All these facts are explicable and reconcilable if we assume that the deposited lipid is at or near the cell surface and is not a part of or at least the functional part of a metabolically active organelle such as the cytoplasmic membrane; and that the more polar alkyl phenols are orientated at the cell water interface. Increased lipid might be expected to hold more drug molecules at the surface without inward penetration, hence the apparent smaller drug uptake; but the functional areas of the membrane are protected hence the decrease in leakage. The

protective effect of fat on growth inhibition manifest in a decreased MIC value also follows.

The decrease in mobility of fattened cells in the absence of drug could be explained by a possible masking of aniogenic groups on the cell surface by lipid but the greater enhancement of negative mobility in the presence of phenols could be explained (and reconciled with the smaller drug uptake) by assuming the phenols are locked at the cell surface thus causing a mobility increase.

The Adsorption of Tetrachlorosalicylanilide by Sensitive and Resistant Bacteria. By W. A. HAMILTON (*Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford*)

Bacteriostasis of *Staphylococcus aureus* results from the uptake of $0{\cdot}75 \times 10^5$ molecules of tetrachlorosalicylanilide (TCS) per cell (Hamilton (1967), *Biochem. J.* **103**, 73 P); Woodroffe & Wilkinson ((1966), *J. gen. Microbiol.* **44**, 353) have shown that this adsorption is reversible and takes place on the membrane of sensitive cells.

At the minimum inhibitory concentration (MIC), the amounts of the germicides tribromosalicylanilide (TBS), trichlorocarbanilide (TCC) and monochlorophenoxysalicylanilide (GL 31) adsorbed by *S. aureus* are $2{\cdot}0 \times 10^5$, $5{\cdot}1 \times 10^5$ and $7{\cdot}2 \times 10^5$ molecules/cell respectively. From their studies with other membrane active antibacterials such as cetyl trimethylammonium bromide (CTAB) and polymyxin, Salton ((1951), *J. gen. Microbiol.* **5**, 391) and Few & Schulman ((1953), *J. gen. Microbiol.* **9**, 454) have shown that the adsorption of large amounts of these germicides by the sensitive cell is an important feature of their mode of action. Polymyxin-resistant organisms were found not to adsorb the antibiotic (Newton (1954), *J. gen. Microbiol.* **10**, iii).

The MIC of TCS with *S. aureus* is $0{\cdot}15$ μg./ml. and with *Escherichia coli* is $30{\cdot}0$ μg./ml. We have examined the adsorption of $1{\cdot}0$ μg./ml. ^{14}C-TCS by 6×10^9 cells/ml. The maximum adsorption of $1{\cdot}37 \times 10^5$ molecules/cell with *S. aureus* was obtained immediately. Adsorption of the germicide by *E. coli* is variable and occurs slowly; the maximum values obtained were $1{\cdot}5 \times 10^5$ molecules/cell, but these were only reached after 5–10 hr incubation. However spheroplasts prepared from *E. coli* adsorbed $1{\cdot}46 \times 10^5$ molecules/cell, and this adsorption occurred immediately.

We postulate that all membrane-active antibacterial compounds, i.e. detergents, phenols, polypeptide antibiotics and the germicides TCS, TBS, TCC, GL 31, have a common mode of action in which the non-specific physicochemical adsorption onto the membrane is a critical step. Resistance to these compounds results from their non-adsorption, which is a property of the cell wall.

Growth Inhibition by Lactose in *Escherichia coli*. By W. H. HOLMS (*Department of Biochemistry, University of Glasgow, Glasgow*)

The strain of *Escherichia coli* (ATCC 15224, ML 308, $i^-z^+y^+$) used in this work is derepressed for β-galactosodase and β-galactoside permease. These enzymes are gratuitous in defined media lacking lactose but are subject to catabolic repression. Glucose as energy source gives cells with only 25 % of the enzyme levels obtained with glycerol (Holms, W. H. (1966), *J. gen. Microbiol.* **45**, ii).

When cells of low enzyme content (trained to glucose) were washed and used to inoculate various media they grew immediately on glucose, galactose, glucose + galactose or lactose. Cells of high enzyme content (trained to glycerol) grew immediately on glycerol, glucose, galactose and glucose + galactose but showed a long lag before growth on lactose. Lactose imposed a lag even when glycerol, glucose or galactose was also present. Addition of lactose to cells growing exponentially on glycerol inhibited growth for about 130 min. When cells of high enzyme content were inoculated into glucose medium the lag obtained on lactose addition declined exponentially as the culture grew. In general, the lag produced by lactose increased with the β-galactoside permease content of the cells.

Cells of high enzyme content were inoculated into a medium containing only enough galactose to support about three generations of growth. When lactose was added at the end

of logarithmic growth there was a lag of 650 min., but when it was added 1 hr later or during logarithmic growth the lag was only 80 min. Changes in permease level were insufficient to account for these variations in lag.

The results are consistent with the following hypothesis. Lactose inhibits growth in this mutant by preemption of the available energy for operation of the permease system. The lag is proportional to the level of the permease system and inversely proportional to the energy available for its operation.

Influence of Extracellular Products on the Growth of Mixed Microbial Populations in Mg^{2+}-Limited Chemostat Cultures. By J. L. MEERS and D. W. TEMPEST (*Microbiological Research Establishment, Porton, Wiltshire*)

Assuming the basic equations for the growth of micro-organisms in a chemostat to be correct (Herbert, D. (1956), *J. gen. Microbiol.* **14**, 601), then when two species are present in a chemostat, and there is no interaction between them other than competition for the growth-limiting nutrilite, the one which grows (at the imposed dilution rate) at the lower growth-limiting substrate concentration should completely displace the other species (Powell, E. O. (1958), *J. gen. Microbiol.* **18**, 259). However, mixed cultures of Mg^{2+}-limited *Bacillus subtilis* and *Torula utilis* behaved paradoxically; neither species grew when inoculated (in low concentration) into Mg^{2+}-limited cultures of the other species. And similar results were obtained when Mg^{2+}-limited cultures of *Bacillus megaterium* were mixed with either *T. utilis* or *B. subtilis*.

No growth inhibitors could be detected in any of the above culture fluids but extracellular products were present which stimulated growth and the uptake of magnesium from environments containing low concentrations ($< 0{\cdot}5$ μg./ml.) of this cation. Some specificities were apparent (e.g. growth of the two Bacillus species was most stimulated by their own extracellular fluids), and these specificities probably account for the paradoxical 'take-over' patterns described previously.

The concentration of growth-promoting substance(s) in the extracellular fluids of Mg^{2+}-limited *B. subtilis* cultures varied with population density. Thus, when the magnesium input concentration was lowered from 0·90 to 0·15 μg./ml. *B. subtilis* could no longer maintain itself against proportionately low concentrations of *T. utilis*. This fact, and the finding that addition of *B. subtilis* extracellular fluids to Mg^{2+}-limited *T. utilis* cultures enabled the bacillus now to outgrow the yeast, reinforces the conclusion that Mg^{2+} uptake by *B. subtilis* is more dependent on extracellular products than is Mg^{2+} uptake by *T. utilis*.

Continuous Culture of Mixed Populations. By P. J. COLLARD and JENNIFER GOSSLING (*Department of Bacteriology and Virology, University of Manchester*)

An attempt to develop an artificial ecosystem simulating conditions in the gut is described. Initially, a continuous flow culture system with only temperature, flow rate and medium controlled, was used. The temperature was 37° or 38·5° (± 1°). Various flow rates up to 1 change per hour were tried. Peptone (0·1 % or 1 %), sugar solutions, with yeast extract added for the more fastidious bacteria, were supplied as media. Counts were made twice daily for periods up to 6 weeks. The numbers of the different organisms in the system were estimated by plate counts, using selective media. Where possible control counts were made on different media.

Neither *Bacterioides* nor *Shigella sonnei* could be established in a culture of *Escherichia coli*. *E. coli* became established in a culture of *S. sonnei* and the *S. sonnei* disappeared. *Streptococcus faecalis* became established in a culture of *Lactobacillus acidophilus*, and *E. coli* in a culture of *S. faecalis*.

Starting with the culture of *L. acidophilus* and *S. faecalis*, the following were added at weekly intervals: *E. coli*, *Bacteroides*, *Clostridium perfringens*, *Candida albicans*. Only *Bacteroides* failed to establish itself. If the system was inoculated with all the organisms simultaneously, *L. acidophilus*, *S. faecalis*, *E. coli*, *Bacteroides*, *C. perfringens*, *C. albicans*, and *Staphylococcus albus* would grow together. *L. acidophilus* would not grow when the sugar concentration was low (0·05 %). At low sugar concentrations *Bacteroides* gave the highest

counts ($10^{8\cdot2}$ to $10^{8\cdot9}$ per ml.) followed by *E. coli*, *S. albus*, *C. perfringens*, *S. faecalis* and *C. albicans* in that order. Higher sugar concentrations ($\geqslant 0\cdot10\%$) enabled *L. acidophilus* to grow and caused an alteration in the relative numbers of the species, the counts for *E. coli* and *Bacteroides* falling and *C. perfringens* giving the highest counts.

The effects of adding antibacterial agents and other bacteria to the mixed culture are being studied.

Ancestral Relationships of Transmissible Bacterial Plasmids. By ELINOR MEYNELL (*M.R.C. Microbial Genetics Research Unit*), NAOMI DATTA (*Royal Postgraduate Medical School*), A. M. LAWN and G. G. MEYNELL (*Lister Institute, London, S.W.* 1)

Those plasmids of Enterobacteriaceae able to ensure their own transmission by conjugation consist of a sex factor often linked to one or more genes that determine a variety of characters like lactose fermentation, colicinogeny or drug resistance. We have previously suggested that all the sex factors so far examined are related either to F or to the sex factor of colicin factor I which may therefore be the archetypal plasmids from which the remainder are descended (Meynell, G. G., Lawn, A. M., Meynell, E. & Datta, N., (July, 1967), *Proc. Genet. Soc.*). Further evidence for this view will be presented.

The Radiation Target Size of Riley Virus Infectivity. By K. E. K. ROWSON, I. B. PARR (*Cancer Research Department, The London Hospital Medical College, London, E* 1) and TIKVAH ALPER (*M.R.C. Experimental Radiopathology Research Unit, Hammersmith Hospital, London, W.* 12)

Filtration of Riley virus through Gradocol membranes indicated a diameter of approximately 45 mμ for the infective particle (Rowson, K. E. K., Mahy, B. W. J. & Salaman, M. H. (1963), *Life Sciences* **7**, 479). However, it has been suggested that a small fraction of the virus particles in the plasma of infected mice may be much smaller and less dense (Adams, D. H. & Bowman, B. M. (1964), *Biochem. J.* **90**, 477; Crispens, C. G. (1964), *Virology*, **24**, 501; Riley, V., Campbell, H. A., Loveless, J. D. & Fitzmaurice, M.A. (1964), *Proc. Am. Ass. Cancer Res.* **5**, 53). Electron microscopic examination has confirmed the presence in Riley virus preparations of a particle of approximately 40 mμ diameter (De Thé, G. & Notkins, A. L. (1965), *Virology*, **26**, 512). However, it is not possible by microscopy to exclude the possibility of there being a smaller infective particle and so it was of interest to determine the size by an independent method.

Molecules possessing biologic activity will have this destroyed if they are exposed to ionizing radiations, and a 'target volume' may be calculated from the dose required to give a defined percentage inactivation. It has been found with a wide variety of macro-molecules and viruses that if they are irradiated dry this 'target volume' correlates very well with independent size determinations. Furthermore, inactivation curves for uniform populations are exponential with dose, whereas a heterogeneous population should yield a curve of a different form.

We have therefore carried out irradiation studies on freeze-dried Riley virus preparations. They consisted of mouse plasma obtained from mice either 24 hr or 72 days after infection and were exposed to the electron beam from the M.R.C. 8 MeV. linear accelerator. When the log of the surviving virus fraction was plotted against the dose of irradiation, straight lines were obtained and both virus preparations gave the same slope. Thus there was no evidence of either a small fraction of virus particles differing from the majority in their sensitivity to irradiation or of a difference between the virus in the early and late stages of the infection. Calculation of the target size of viral infectivity from the slope of the inactivation curve gave a value compatible with the complete viron having a diameter of 45 mμ.

The Absence of Inhibitor-destroying Activity in Neurotropic Influenza A Viruses. By D. HOBSON, E. A. GOULD, and H. I. FLOCKTON, (*Department of Bacteriology, University of Liverpool, Liverpool*)

Allantoic fluid pools of the neurotropic variant NWS (Stuart-Harris C. H. (1939), *Lancet* **1**, 497) of the ws strain of influenza AO virus differ from the parent strain in a reduced capacity

of the variant to elute from erythrocytes after adsorption, presumably because of lack of viral neuraminidase (Burnet, F. M. (1951), *J. gen. Microbiol.* **5**, 46.)

To determine if this finding might be correlated with the neurovirulence of NWS other strains of influenza A viruses, both neurotropic and non-neurotropic, have been compared for neuraminidase activity. The methods employed (Smith, W. & Cohen, A. (1956), *Br. J. exp. Path.* **37**, 612; Padgett, B. L. & Walker (1957), *J. exp. Med.* **106**, 53) measure the ability of virus suspensions to destroy the haemagglutination-inhibiting activity of certain mucopolysaccharides against a test virus (influenza B/Lee) heated 56°/30 min. to convert it, by loss of its own neuraminidase, to the inhibitor-sensitive indicator state.

In this system egg pools of AO/WS virus and representative strains of other influenza A families showed well marked inhibitor-destroying activity (IDA). In contrast, only NWS virus and the independently derived neurotropic variant WSN-F (Francis, T. Jun. & Moore, A. E. (1940), *J. exp. Med.* **72**, 717), or recombinant neurovirulent virus strains derived from them, were devoid of IDA.

The relationship of IDA against various substrates to mouse virulence and to other laboratory markers of influenza A viruses will be discussed.

Measuring the True Division Rate of *Salmonella typhimurium in vivo*. By JOAN WALSH and G. G. MEYNELL (*Guinness-Lister Research Unit, Lister Institute, London, S.W.* 1)

Viable counts on infected animals necessarily measure only the net result of microbial division and death. However, the true division rate can be estimated separately (Meynell, G. G. (1959), *J. gen. Microbiol.* **24**, 421; Meynell, G. G. & Subbaiah, T. V. (1963), *Br. J. exp. Path.* **44**, 197), which in turn gives the true microbial death rate *in vivo*. *Salmonella typhimurium* SR 120 was lysogenized by a non-excluding (*x*) mutant of phage P 22 (Walsh, J. & Meynell, G. G. (1967), *J. gen. Virol.* in the Press), the relevant markers being $c^+h^+xmg^s$. This was superinfected with phage P 22 carrying the distinguishing markers, $c_2hx^+mg^r$. The lysogenizing phage could therefore be counted on wild-type strain LT 2 on nutrient agar while the superinfecting phage could be counted on the host-range indicator, SL 1208, plated on nutrient agar containing 0·1 M Mg^{2+}. Thirty-one mice from two lines were given approx. one LD 50 by intravenous injection. After 1·5 hr the heart blood was virtually sterile and counts on homogenized spleen showed that 0·5–1·5 generations had elapsed since inoculation. The viable count subsequently doubled about every 24 hr; while the true doubling time was 8–10 hr, compared to the minimum of 0·5 hr observed *in vitro*. The rates of bacterial division and death varied from mouse to mouse, and the two rates may be positively correlated.

Classification of Oral Fusiforms. By A. W. HADI and C. RUSSELL. (*Department of Bacteriology and Virology and Turner Dental School, University of Manchester*)

The classification of fusiform organisms is confused (see Topley and Wilson's *Principles of Bacteriology and Immunity* (1964), 5th edn., vol. 1). Therefore, 152 representatives of fusiforms from saliva and gingival material were isolated on selective medium (Omata & Disraely, (1956), *J. Bact.* **72**, 677). Five groups were identified as follows.

Colonies of Groups I and II were 0·5–3 mm. diameter and greyish white. The characteristics of group I were similar to the description of *Fusobacterium nucleatum*, whereas group II corresponded to *F. polymorphum*. Both were slender (0·3–0·7 μ) Gram-negative rods with pointed ends, although group II cells tended to be filamentous and thicker than group I. Each group produced indole and H_2S and hydrolysed sodium hippurate. They fermented only few carbohydrates, producing acetic, butyric and lactic acids and acetylmethylcarbinol from glucose, with a terminal pH of 5·4. In the opinion of the authors, *F. nucleatum* and *F. polymorphum* should be regarded as one species.

Colonies of groups III and IV were 2·3 mm. diameter and light-blue. The characteristics of these groups corresponded to those of *Leptotrichia buccalis*. Cells, Gram-positive when young, gave Gram-positive filaments (0·8–1·5 μ) containing Gram-positive granules when older. Neither indole nor H_2S was formed, nor was sodium hippurate hydrolysed. Many carbohydrates were fermented and acetic and lactic acids were produced from glucose but neither

butyric acid nor acetylmethylcarbinol. The final pH was 4·6. Groups III and IV were considered variants of *L. buccalis* because of small but consistent differences in colonial morphology.

Colonies of group V were light-blue with a dark-blue centre and much smaller (pin-point to 1 mm) than those of the other groups. The group corresponded to *Bacteroides oralis*. Cells were Gram-negative with rounded ends, 2–4 $\mu \times 1\ \mu$. Neither indole nor H_2S was formed, many carbohydrates were fermented, terminal pH on glucose being 4·8.

Rugose Forms of an El Tor Vibrio. By M. J. CRUTCHLEY (*Wellcome Research Laboratories, Beckenham, Kent*)

Rugose forms of cholera strains were shown to be caused by mucilaginous secretions, containing bacteria, forming characteristic colonies (White, P. B. (1938), *J. Path. Bact.* **46**, 1). Rugose colonies occurred at all growth stages but gave the original colony form when subcultured. They predominated in platings of old peptone cultures and high peptone concentrations favoured their appearance in culture.

Present work showed that smooth *Vibrio cholerae* El Tor Ogawa (NCTC 10256, Vella, E. E. (1963), *Br. med. J.* p. 1203) gave rugose forms when grown 24 hr at 37° on agar containing 7 % horse or sheep blood. Rugose colonies subcultured onto nutrient agar gave smooth forms. Of blood constituents investigated 5 % or 10 % horse or sheep serum in agar did not give rugose colonies. Horse or sheep erythrocytes in agar, at the concentration of whole blood, gave rugose forms when smooth or rugose colonies were used as inocula. Ferric citrate in agar also gave rugose forms at concentrations ranging from 0·001 to 0·1 %. The latter gave rugose colonies in 24 hr but lower concentrations needed 72 hr. Ferric chloride at 0·01 % was effective, but higher concentrations inhibited growth. Sodium chloride (0·1 %) or citrate (0·1 %) had no effect. Rugosity due to blood or ferric salts was not prevented by adding EDTA (0·01 %), sodium citrate (0·1 %), or horse serum (5 %) to agar media. Peptone (5–10 %) in agar had no effect. The following salts of other metals were ineffective at concentrations indicated in agar (higher concentrations inhibited growth): lead nitrate (0·1 %, silver nitrate (0·01 %), cobalt chloride (0·01 %), nickel sulphate (0·01 %), and mercuric acetate (0·01 %). Cupric sulphate (0·01 %) gave rugose colonies from a rugose inoculum after 48 hr incubation.

Rugose forms, of this strain, are thus produced by free and combined iron in media.

The Attachment of Bacteria to Surfaces, and a Way of Measuring the Rate of Beat of Bacterial Flagella By P. S. MEADOWS. (*Department of Zoology, University of Glasgow, Scotland*)

The following observations were made on *Pseudomonas fluorescens* NCIB 9046 and on *Vibrio anguillarum* NCMB 829 as they attached to glass surfaces, and on *Aeromonas liquifaciens* NCIB 9233 as it attached to glass surfaces and to the surfaces of cheek epithelial cells and cultured mammalian cells. Continuous observation of individual bacteria (tape recorder) showed that they can attach, detach and re-attach a number of times. As they attach they can be classed into three states: (i) longitudinal axis at right angles to surface; nearest end 0–4 μ from surface (constant in a given individual); probably attached by polar flagella; (ii) longitudinal axis parallel to surface, about 1 μ from surface, just perceptible shivering; and (iii) longitudinal axis parallel to surface, on surface, no movement. Individuals may alternate between these states. In (i) bacteria often show a circular waggling movement. This may provide a way of measuring the rate of beat of bacterial flagella. Rates of between 2 and 13 beats per second have been recorded. Rates are constant for a given time (2–90 sec.) and then switch to another rate, or stop suddenly.

Experimental Induction of Delayed Ocular Reactions Resembling Post-Leptospirosis Ophthalmia. BY S. BEN-EFRAIM and M. TORTEN (*Israel Institute for Biological Research, Ness-Ziona, Israel*)

Ophthalmia, as a sequel to leptospirosis is a well-established clinical fact in some human and animal cases (Alston, J. M. & Broom, J. C. (1958), *Leptospirosis in Man and Animals*,

E. and S. Livingstone Ltd., Edinburgh and London). One of the assumptions put forward in order to explain the post-leptospirosis ophthalmia was the occurrence of a delayed type hypersensitivity reaction between cell-bound antibodies and leptospiral antigens presumably still present in the eye (Jubb, K. V. P. & Kennedy, P. C. (1963), *Pathology of Domestic Animals*, Academic Press). We tried to investigate whether an ocular hypersensitivity reaction can be produced experimentally in dogs following leptospiral infection and to what extent this reaction resembles histologically the ophthalmia already described. It was also our aim to check the specificity of the induced ocular reaction (genus or serotype specific) and compare it with the specificity of humoral antibodies, immediate and delayed intradermal reactions.

The experimental system consisted of dogs infected by two subsequent injections either with *Leptospira canicola* strain or *L. grippotyphosa* under conditions known to induce mild infections. The sera of infected animals were tested at various intervals by microscopic agglutination test (type specific antibodies) and by the fluorescent complement staining with the homologous strain and with the saprophytic strain of *L. patoc* for detection of genus specific antibodies. (Torten, M., Shenberg, E. & van der Hoeden, J. (1966). *J. infect. Dis.* **116**, 5). Ocular and skin tests were carried out at 120 days after the first injection, by injecting soluble extracts of homologous or heterologous strains.

Infection of dogs with *L. canicola* or *L. grippotyphosa* elicited formation of type-specific and genus-specific antibodies. Ocular injections of the homologous soluble antigen induced a delayed type reaction. Congestion of the conjunctiva, corneal oedema and clouding of the aqueous humour were observed. The histological picture is characteristic of delayed ocular reaction with typical change in the iris, ciliary body, choroid, retina and cornea. A marked increase of inflammatory cells in the ciliary body usually found in post leptospirosis iridocyclitis could also be observed.

Skin tests revealed also occurrence of delayed type reactions with the homologous strain. On the other hand, immediate reactions (active cutaneous anaphylaxis) were also obtained with heterologous strains.

These results indicate that a clinical condition resembling post-leptospirosis ophthalmia could be reproduced experimentally. The delayed ocular and skin reactions were strictly type specific. Genus specific antibodies were revealed by immunofluorescence tests and by occurrence of immediate cutaneous reactions.

Inhibition by Pyrimidine Analogues of the Synthesis of Folic Acid by Trachoma Agents. By P. REEVE and JANICE TAVERNE (*M.R.C. Trachoma Research Unit, Lister Institute of Preventive Medicine, London, S.W.* 1) and S. R. M. BUSHBY (*Wellcome Research Laboratories, Beckenham, Kent*)

Trimethoprim, a 2,4-diaminopyrimidine derivative, inhibits the growth of many species of bacteria. It competes with folic acid and inhibits dihydrofolate reductase; in combination with sulphonamides it shows a strong potentiating effect, as a consequence of the sequential blockade of the biochemical pathway that leads to the synthesis of coenzyme F. Since the trachoma agent is sensitive to inhibition by sulphonamides and presumably synthesizes folic acid, we tested its susceptibility to trimethoprim and the related anti-malarial compound pyrimethamine, alone and in conjunction with a sulphonamide. Both the pyrimidine analogues inhibited the growth of the trachoma agent in chick embryos and inhibitions was reversed by adding leucovorin calcium, providing evidence for the existence of a dihydrofolate reductase in the agent. Some potentiation of sulphonamide activity by trimethoprim was also observed.

Polymorphism in Methane-utilizing Bacteria. By HEATHER M. CHAPMAN and D. W. RIBBONS ('*Shell*' *Research Ltd., Milstead Laboratory of Chemical Enzymology, Broad Oak Road, Sittingbourne, Kent*)

Three main types of methane-utilizing bacteria have been described: the generally pigmented forms of *Pseudomonas methanica* (Dworkin & Foster (1956), *J. Bact.* **72**, 646); the microcolony-forming *Methanomonas methanooxidans* first described by Brown ((1958), Ph.D. Thesis, Louisiana State University), and the thermotolerant coccus *Methylococcus capsulatus* (Foster & Davis (1966). *J. Bact.* **91**, 1924).

The isolation and maintenance of methane-utilizing bacteria in pure culture is difficult. Two major causes of this are their slow growth rate which facilitates overgrowth by very low levels of contaminating micro-organisms, and their association with the faster-growing methanol utilizers. Some of the organisms to be described here are morphologically similar to *Methanomonas methanooxidans* or to *Methylococcus capsulatus*. Others share some of the characteristics of both, or appear to be new strains.

Strains of methane-utilizing bacteria have been isolated by enrichment and dilution to extinction; the procedures were similar to those described by Brown in 1958. The cellular and colonial morphology of five of these isolates will be illustrated and compared. The variations encountered in some of the isolates will be described in detail.

Synergistic Action of Polysorbate 80 and Polymyxin B Sulphate on *Pseudomonas aeruginosa*. By M. R. W. BROWN and B. E. WINSLEY (*Pharmaceutical Microbiology Group, School of Pharmacy, Bath University of Technology, Bristol*)

Previous work has shown a synergistic action between Polysorbate 80 and Polymyxin B sulphate on growth inhibition and lysis of *Pseudomonas aeruginosa* (Brown, M. R. W. & Richards, R. M. E. (1964), *J. Pharm. Pharmacol.* (Suppl.), **16**, 41 T). Polysorbate 80 has also been shown to increase leakage of 260 mμ-absorbing substances from suspensions of washed cells of this organism (Brown, M. R. W. & Winsley, B. E. (1966), *J. gen. Microbiol.* **45**, iv).

Using similar techniques, the effects upon leakage from suspensions of washed cells of *Pseudomonas aeruginosa* NCTC 6750 of mixtures of polysorbate and polymyxin were measured at 37°. Solutions of each surfactant were mixed in graded proportions; up to 25 units/ml. polymyxin and up to 0·13 % polysorbate were used. The leakage observed in the mixtures represented in every case about a threefold increase of the value predicted by addition of the leakages produced individually by each component of the mixture. The coefficient of variation for replicate determinations of leakage was approximately 3 %. The effects of these agents were determined concurrently on leakage and on viability of cell suspensions in a defined medium using replicate samples. Viability was estimated by rapidly diluting a sample in a lecithin medium to inactivate the residual polymyxin and spread-plate colony counts made. The extent of cell death appeared not to correlate with the amount of 260 mμ-absorbing substance lost by the cells. The results did not exclude however the possibility of a relationship between the initial maximum rate of loss of both viability and 260 mμ-absorbing substances.

Uptake of polysorbate by aqueous cell suspensions appeared to be complete in less than 5 min. at 20°; the residual polysorbate in solution was estimated by assay of a cell-free sample (method II, Stevenson, D. G. (1954), *Analyst, Lond.* **79**, 504). The amount of this surfactant taken up by a standard number of cells increased with solution concentration up to 0·3 %, the highest concentration used. Polysorbate added immediately before or several generation times before the addition of polymyxin to log-phase cultures produced the same synergistic effect on inhibition of growth rate and on lysis. Polysorbate alone had no observable effect on growth rate. These results suggest that polysorbate acts synergistically with polymyxin by means of an immediate effect on cell permeability rather than by a more gradual effect on metabolism.

The Characterization of a Temperature-induced Lesion in a Psychrophile. By N. L. MALCOLM (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Physical Properties of Ribosomes from a Protozoan. By G. A. M. CROSS (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

During studies of the mechanism of protein synthesis in a cell-free system from the parasitic flagellate *Crithidia oncopelti* (Cross, G. A. M. (1966), *J. gen. Microbiol.* **44**, iii, and to be published) it was discovered that purified ribosomes from this organism were unstable if suspended in buffered solutions containing magnesium ions at less than the optimum concentration for protein synthesis *in vitro*. This behaviour was surprising in view of the widely held notion that the 80s type of ribosome obtained from eucaryotic cells is resistant to dis-

sociation, in contrast to the strong dependence on magnesium ions for the structural integrity of bacterial (70S) ribosomes.

Ribosomes from *C. oncopelti* have a sedimentation constant of about 80S; in a cell-free system the protozoal ribosomes will incorporate amino acids in the presence of the complementary soluble enzyme fraction from mammalian cells, but not with the enzyme fraction from bacteria; furthermore, protein synthesis in the protozoal ribosomal system is insensitive to chloramphenicol and other specific inhibitors of bacterial protein synthesis, but is extremely sensitive to cycloheximide and related compounds.

The products of ribosome dissociation have been characterized by their sedimentation properties in the analytical ultracentrifuge and on sucrose density gradients. The role of nascent protein in the stability of the active ribosome-mRNA complex was investigated by labelling with radioactive amino acids *in vitro*, followed by sucrose gradient centrifugation. On lowering the magnesium ion concentration to 1·0 mM, all 80S ribosomes not carrying a nascent protein chain break down to 60S and 40S subunits. The stability of active ribosomes (mainly present as polyribosomal aggregates) under these conditions is due to the presence of a nascent protein chain and not to their association with mRNA. This was shown to be the case by removing nascent protein from the polyribosomes by treatment with puromycin. If the magnesium ion concentration is lowered to 0·1mM, all ribosomes and polyribosomes dissociate into two subunits, in a weight ratio of about 2·3:1, having sedimentation coefficients of 45S and 33S. Under these conditions roughly half of the radioactivity in the nascent protein chains is released. The remaining label is bound to both subunits. The large ribosomal subunit can also exist in a form sedimenting at 50S, and under certain conditions the small subunits are thought to dimerize.

The changes in sedimentation coefficient of the ribosomal subunits are visualized as conformational changes ('unfolding'). Dissociation is reversible to a certain extent; the 60S and 40S subunits will recombine to give a single component indistinguishable by its sedimentation coefficient from the undissociated ribosome. Under certain conditions the rate of ribosome dissociation is somewhat dependent on whether sodium deoxycholate is used during cell fractionation.

Peptide-bond Formation: the Effects of Antibiotics on the Puromycin Reaction. By E. CUNDLIFFE and K. MCQUILLEN (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Puromycin is an analogue of aminoacyl-adenosine, the terminus of charged transfer RNA. The drug causes the release of nascent peptides (as peptidyl-puromycin) from transfer RNA bound to ribosomes, apparently by taking part in the peptide bond-forming reaction. For this reason, the 'puromycin reaction' is a useful model for studying the formation of individual peptide bonds.

The present work was carried out *in vivo* using protoplasts of *Bacillus megaterium* containing nascent peptides which had been labelled for a short time with a mixture of [^{14}C]amino acids. Incorporation was stopped by the addition of one of several antibiotics at a concentration sufficient to inhibit protein synthesis promptly and completely. When puromycin was subsequently added, it was found that some antibiotics inhibited the release of nascent peptides whereas others did not.

Of the antibiotics used chloramphenicol, sparsomycin and erythromycin strongly inhibited the puromycin reaction *in vivo*, whereas chlortetracycline, pactamycin and bottromycin gave little or no inhibition.

Peptide-bond Formation: the Modes of Action of Chloramphenicol, Sparsomycin and Erythromycin. By E. CUNDLIFFE and K. MCQUILLEN (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

In the accompanying abstract the effects of various antibiotics on the puromycin reaction have been described. The experiments reported here were designed to investigate more precisely the modes of action of chloramphenicol, sparsomycin and erythromycin, which were previously shown to inhibit the puromycin reaction.

Accordingly, the effects of these three antibiotics on the puromycin reaction were tested in protoplasts of *Bacillus megaterium* pretreated with chlortetracycline (which does not itself inhibit the puromycin reaction). Under these conditions it was found that chloramphenicol and sparsomycin still inhibited the puromycin reaction whereas erythromycin no longer gave any appreciable inhibition.

These results will be discussed in connection with the 'translocation' model for peptide-bond formation. According to this type of model, peptide-bond formation involves the transfer of the growing peptide from t-RNA bound in one ribosomal site (the 'donor' site) to an amino acyl-t-RNA bound in another site (the 'acceptor' site) on the same ribosome. This reaction is catalised by an enzyme, peptidyl transferase. The resultant elongated peptide, attached to t-RNA, is then shifted back from the acceptor site into the donor site with an accompanying movement of the messenger RNA relative to the ribosome. This reaction involves translocation enzyme(s). The next amino acyl-t-RNA then binds to the vacant acceptor site and the process is repeated.

We assume that this model is substantially correct and make the further, plausible, assumptions: (i) that puromycin releases only those peptides attached to t-RNA bound in the donor site (Bretscher, M. S. & Marcker, K. A. (1966), *Nature, Lond.* **211**, 380); and (ii) that chlortetracycline inhibits the binding of amino acyl-t-RNA to the acceptor site (see Franklin, T. J. (1966), *Symp. Soc. gen. Microbiol.* XVI, 192).

Accordingly, we infer that chloramphenicol and sparsomycin inhibit the peptidyl transferase reaction whereas erythromycin may inhibit translocation.

SYMPOSIUM: POLYMORPHISM AND PLEOMORPHISM IN MICRO-ORGANISMS

Factors Influencing the Form of *Naegleria gruberi*. By E. N. WILLMER (*Department of Physiology, University of Cambridge*)

It has been known for many years that the soil amoeba, *Naegleria gruberi*, readily acquires flagella when cultures of it are treated with distilled water. The flagella are produced *de novo* and they are readily lost again when the ambient salt concentration is raised. While the mechanisms involved in these changes are far from being understood, it is clear that there are a great many factors that affect the form of the organism. The effect of ions appears to be of far more importance than purely osmotic effects and since ions, steroids, methonium compounds and other substances which would be expected to modify the properties of cell surfaces are particularly potent agents in determining the state of the organism, it is likely that the changes are initiated by changes in the surface properties of the organism. Although the electron microscope does not demonstrate any noticeable difference between the membranes of the amoeboid and flagellate phases it is quite clear from the behaviour of the organism in its two forms that the surface is in fact very different. In the amoeboid phase lobose pseudopodia may form anywhere on the surface, but, as the transformation occurs, they become restricted to the anterior end and finally disappear. Meanwhile filiform pseudopodia are produced posteriorly for a time until they too disappear and the fully transformed flagellate produces no pseudopodia at all.

In addition to these obviously surface effects it is clear that a polarity develops, or at least becomes accentuated, in the flagellate form. Moreover, the cell now produces filiform pseudopodia and flagella. These observations indicate that something is favouring a linear arrangement of proteins or other long molecules when the transformation is occurring. This linear orientation could be initiated by a different arrangement of lipids in the cell membranes, so that the proteins associate differently with the lipids; it could be the direct action of the transforming agents on the form of the protein molecules themselves; or it could be that, when the transformation occurs, new and more fibrillar proteins are produced in place of the initially more globular ones, whose presence is suggested by the behaviour of the cytoplasm

in the amoeboid phase. If the third suggestion is the correct one, then the effect could be the direct action of the transforming agent on the nuclear mechanism; or it could be the indirect result of a disturbance of equilibria within the cell, involving, for example, the redistribution of proteins on membranes and surfaces; and this disturbance of the protein equilibria could then secondarily affect the relationship between the proteins and the DNA–RNA mechanism that produces them.

The classes of agents favouring the transformation are probably more indicative of indirect action through alterations of the cell surfaces than of direction action on DNA or RNA, or on the masking or unmasking of parts of the coding-reading system.

Cyclical Transformation in Trypanosomes. By K. VICKERMAN and A. G. LUCKINS (*Department of Zoology, University College London*)

The sequence of morphological changes seen in trypanosomes as they develop in both invertebrate and vertebrate hosts reflect accompanying physiological changes in these flagellates. The most obvious morphological changes involve a shift in the position of the kinetoplast which is always associated with the basal body of the single flagellum. The kinetoplast represents the massed DNA of a single mitochondrion which extends from one end of the trypanosome to the other.

Sleeping sickness trypanosomes (*Trypanosoma brucei* group) multiplying in the blood of the experimental rodent host have the kinetoplast close to the posterior end of the body, and a simple tubular mitochondrion which lacks internal cristae as seen in electron micrographs. These flagellates are unable to utilize Krebs's cycle intermediates to maintain motility, though individual enzymes of the cycle can be detected in homogenates. Examined spectroscopically they lack cytochrome pigments and their respiration is insensitive to cyanide though large volumes of oxygen are consumed. Terminal respiration is carried out by a L-α-glycerophosphate dehydrogenase–L-α-glycerophosphate oxidase system. Pyruvate is excreted by the trypanosomes. It appears that the mitochondrion is inactive, oxidative phosphorylation does not occur, and all ATP synthesis is the result of aerobic glycolysis.

These trypanosomes when cultured *in vitro* adopt the form characteristic of the flagellates developing in the midgut of the tsetse fly vector. The kinetoplast lies in a more anterior position, but still behind the nucleus, and electron microscopy shows that the kinetoplast is part of a sub-pellicular network of mitochondrial tubes which have internal cristae. These forms can utilize Krebs's cycle intermediates, they possess cytochrome pigments and respiration is cyanide sensitive. From both morphological and physiological evidence the mitochondrion appears to be not only active but also more extensive in these culture (fly midgut) forms. Glucose and oxygen consumption in the midgut forms are one-tenth that of bloodstream forms. It might be expected that when the trypanosomes are moved from circulating to stagnant blood on entering the fly, a more economical form of respiration would be necessary. The activation and proliferation of the mitochondrion can be regarded as an adaptive response to the change of environment, and the change in position of the kinetoplast as the result of this mitochondrial reorganization.

The bloodstream population of trypanosomes in a chronic infection shows a marked polymorphism, for in addition to long slender multiplying trypanosomes, short stumpy forms are present and a whole spectrum of intermediate forms. Slender forms are abundant in the rising parasitaemia, stumpy forms at its remission. Both are found in clone strains, so the form variation is pleomorphism rather than genetic polymorphism, and it appears that slender forms change into stumpy forms. Stumpy forms are believed to be more likely to establish infection in the tsetse fly midgut. Using tetrazolium salts as acceptors, NADH oxidation has been demonstrated cytochemically in the mitochondrial tube of intermediate and stumpy, but not slender, forms. It appears that de-repression of the mitochondrion accompanies transformation to stumpy forms. This conclusion is supported by the presence of cristae in the mitochondrial tubes of stumpy forms and the ability of these trypanosomes to use α-oxo-glutarate as respiratory substrate for maintaining motility.

The stimulus inducing the slender-stumpy transformation is popularly believed to be the host's immune response as stumpy forms are associated with a remission. It is known that

antibodies depress the respiration of bloodstream trypanosomes and a change in surface permeability to glucose brought about by antibodies might be a key factor in bringing about the switch. Experimental infections in rats receiving either daily doses of cortisone sufficient to depress the immune response, or total body X-irradiation (400 r.), still show stumpy forms, however, and so this simple explanation is far from satisfactory.

Pleomorphism in Malaria Parasites. By ANN BISHOP (*formerly of the Molteno Institute, University of Cambridge*)

The malaria parasite has a complex life cycle, multiplying asexually in the vertebrate host and sexually in the mosquito vector. The fine structure of the infective stage, the sporozoite, which develops in the mosquito, includes an apical cup into which paired organelles appear to lead. It has been suggested that the function of these organelles is glandular and that their secretion may assist in the penetration of the host cell membrane (Garnham, Bird & Baker (1960), *Trans. R. Soc. trop. Med. Hyg.* **54**, 274). The sporozoites, when injected into the vertebrate host, invade the reticulo-endothelial cells in avian malaria, but in mammalian malaria their development is restricted to the parenchyma cells of the liver. Multinucleate parasites (exoerythrocytic schizonts) develop from the sporozoites, and the uninucleate merozoites produced by their division may enter similar cells or erythrocytes. In *Plasmodium falciparum* there is evidence that all the merozoites enter erythrocytes (haemotropic), whereas in *P. vivax* a few may re-invade the parenchyma cells so that two cycles run concurrently, one in erythrocytes and the other in the liver parenchyma. In *P. gallinaceum* erythrocytic merozoites may become histiotropic and reinvade the reticulo-endothelial cells. The factors which determine whether a merozoite will be histiotropic or haemotropic are not known. There are no morphological characters by which they can be distinguished but important physiological changes must occur when the parasite changes from one habitat to the other. Erythrocytic parasites break down and utilize haemoglobin and their cytoplasm contains the pigment haemozoin as a product of digestion, whereas exoerythrocytic parasites live in cells devoid of haemoglobin. Moreover, exoerythrocytic and erythrocytic parasites respond differently to antimalarial drugs which suggests either that their metabolism differs in some respect related to the mode of action of the drug, or that the drug is unable to penetrate into the exoerythrocytic parasite or the host cell containing it.

Differences in the mode of feeding in exoerythrocytic and erythrocytic parasites have been described. It was first shown by Rudzinska & Trager ((1957), *J. Protozool.* **4**, 190), in *P. lophurae*, that in erythrocytic parasites the ingestion of erythrocyte cytoplasm takes place by a form of pinocytosis. More recently, Aikawa, Hepler, Huff & Spring ((1966), *J. cell Biol.* **28**, 355) have described a 'cytostome' through which erythrocyte cytoplasm enters the parasite. In exoerythrocytic parasites the cytostome is smaller and does not appear to be functional, and pinocytosis has not been observed.

Gametocytes are formed in the erythrocytes but do not develop further unless the infected blood is ingested by a mosquito vector or is exposed to air. In the mosquito's gut the asexual parasites are digested, but the gametocytes develop into gametes. The gametocytes differ from the asexual parasites in that they are surrounded by a triple membrane whereas in asexual parasites the membrane is double.

The zygote is motile and has been observed to pass through the peritrophic membrane by annular waves of contraction of its body (Freyvogel (1966), *Acta trop.* **23**, 201). The oocysts develop on the gut wall. Their resistance to higher temperatures has been found to increase as development proceeds so that the sporozoites are already partly adapted to the body temperature of the future host before they leave the mosquito (Ball & Chao (1964), *J. Parasit.* **50**, 748).

Ciliated Protozoa. By G. H. BEALE (*Institute of Animal Genetics, University of Edinburgh*)

Many ciliates undergo changes from one morphological type to another, either during the normal course of development of the organism through a series of stages, or as a response to certain environmental changes which may occur at any time. Amongst the examples of such polymorphism in ciliates may be mentioned:

(1) The macrostome-microstome transformations in *Tetrahymena patula* and *T. vorax*.

(2) The changes in form of the parasitic marine apostomatous ciliates (e.g. *Gymnodinoides*) studied by Chatton and Lwoff, due to changes in the host animal (e.g. moulting), and to changes from one host species to another.

(3) Members of the group Suctoria in which there is a free-living ciliated 'embryonic' form and a parasitic non-ciliated 'adult' with tentacles, and;

(4) The various types of cysts which arise in many ciliates.

Although in some cases something is known about the environmental stimuli which initiate the change from one form to another, especially in regard to the *Tetrahymena* transformations, nothing is known about the genetic basis of these polymorphisms. However, in *Paramecium aurelia* there is an analogous phenomenon–antigenic transformation—induced by environmental changes of various kinds. Here considerable genetic and biochemical information bearing on the mechanism of transformation is available, and although of course there are no morphological consequences it may well be that the *Paramecium* system can serve as a useful basis for speculating about mechanisms underlying the morphological transformations which this symposium is concerned with.

Changes in Cell Properties in Cellular Slime Moulds. By B. M. SHAFFER (*Zoology Department, University of Cambridge*)

Collective amoebae, or cellular slime moulds (Shaffer, B. M. (1962, 1964), *Advan. Morphogenesis* **2**, 109; **3**, 301; Bonner, J. T. (1967), *The Cellular Slime Moulds*, Princeton University Press) exist in two main states: solitary or unicellular, and social or multicellular. In the former, the cells feed and divide; they repel rather than attract one another, and adhere poorly when they come in contact. In the social state, the cells attract one another chemotactically by secreting acrasin—unstable, still unidentified, and uncertainly related to the steroid stigmastenol, alkaloids, and a bacterial chemotactic factor—and they adhere strongly to one another. The resultant aggregates develop into fruiting bodies, the individual cells becoming carbohydrate-walled stalk or spores. Stalk cells die, and each spore can hatch out a single amoeba. The spores themselves produce a factor inhibiting this transition from resting to vegitative state. In most species, severe depletion of the external food supply leads, after some hours, to the transition from vegetative to social state. Cells cau be maintained in the vegetative state indefinitely by transferring them to fresh food. A factor diffuses from *Escherichia coli* inhibiting starving cells from aggregating (Shaffer, B. M. (1966). *J. Cell Sci.* 1, 391). A bacterial metabolite attracting the cells has been identified (Konijn, T. M., unpublished). Passage through the vegetative state is not prerequisite for becoming multicellular: in the absence of food, cells newly hatched from spores aggregate and fruit.

In a field of cloned *Polysphondylium violaceum* cells, all deprived of food simultaneously and initially averaging a few cell diameters apart, aggregation centres are seen to be started by single specialized founder cells, which round up, become adhesive, and attract their neighbours. These neighbours are induced themselves to become adhesive and secrete acrasin; consequently they do not crawl separately towards the centres but associate in continuous aggregation streams, and also recruit more distant cells, which extend the streams by joining on to their outer ends and in turn recruit still more distant cells. This mechanism is a chemotactic relay system. Which cells are induced in any given period to become adhesive and secrete acrasin thus depends on their spatial relationship to existing aggregations. This change is induced by a diffusible factor possibly identical with the chemotactic one. Founder cells are able to undergo a similar change apparently spontaneously. If a young centre is dispersed, the founder may re-establish a centre repeatedly. If the founder is removed, the

remaining cells cannot re-aggregate unless another founder develops before they become too old. Isolated founder cells do not develop further, but abruptly become ordinary motile solitary cells. The proportion of cells differentiating as founders greatly depends on conditions. It is inversely related to population density, as a factor diffusing from established aggregations inhibits unaggregated cells from becoming founders. Other species differ in the extent to which the founders are differentiated and the chemotactic relay operates. In all species examined, metabolites produced by the cells themselves act as centre suppressors. In some species the suppressor is volatile. Light increases the number of centres formed, possibly affecting both the suppressor mechanism and chemotaxis.

Transition to the final stage of fruiting-body differentiation is strongly promoted by desiccation and by unknown volatile slime-mould metabolites. The proportion of stalk to spore cells is little affected by approximately thousandfold differences in aggregate size, and mutilated aggregates may restore normal proportions. This cell differentiation has been studied immunologically and chemically. We have most information on cell-wall materials and their secretion (Wright, B. E., (1966), *Science*, **153**; Sussman, M. (1967), *Fed. Proc.* **26**, 77), but virtually none about the mechanism that switches cells into one of the two pathways.

Instead of building normal fruiting bodies, in some species cells may encyst individually; and one species, when wet, may construct spherical multicellular macrocysts.

Polymorphism in Fungi. By P. K. C. AUSTWICK (*Central Veterinary Laboratory, Weybridge*)

The success of the fungi as colonizers of dead and living organic material is a measure of their ready adaptability to an environment, a virtue perhaps acquired by the efficient genetic system and the extraordinary plasticity of the fungal cell. It is by the deployment of these two features that the fungi have achieved the bewildering variety of form which is the subject of this symposium. In Ainsworth and Bisbys' *Dictionary of the Fungi* 'pleomorphism' or 'polymorphism' is defined as 'having more than one independent form or spore stage in the life-history', which aptly summarizes the original concept of the brothers Tulasne when they first brought together the two morphologically different stages of the powdery mildews, 100 years ago. Now that we are seeking the controlling mechanisms of this polymorphism, it is necessary to understand both the various circumstances under which it occurs and the part it plays in the life of fungi.

A single species of a fungus may encompass a range of structure from uninucleate cell to complex fruiting body, and the limits to the polymorphism of its thallus and reproductive stages represent the genetic boundaries beyond which it cannot go without losing its entity or its life. The classical form of polymorphism, i.e. the alternation of morphologically different haploid and diploid generations, certainly plays a part in fungal life cycles and there are also alternations of generations of similar appearance. But the great range of fungal form beyond these cycles can only really be compared with that of such metazoans as the Platyhelminths, in which the complex life histories are bound up with multiple host parasitism. The height of reproductive polymorphism in the fungi is reached in plant pathogens such as the rust fungi, where four or five distinct spore types on different hosts may be involved in a single life cycle. The greatest range in vegetative morphology is perhaps found in certain of the fungi which attack animals and man, where it is called 'dimorphism' because the simple, and often yeast-like, phase *in vivo* is so unlike the mycelial state found *in vitro* or in the wild.

Although polymorphism may be manifest at macroscopic, microscopic or even submicronic levels the controlling factors still seem to be of two types—genetical and environmental. The genetic influence may be felt directly in the differentiation of haploid and diploid generations as in many ascomycetes and basidiomycetes, but occasionally in the yeasts and the phycomycetes the difference in chromosome numbers has no apparent effect on morphology. The wider genetic variations which mark the fungi as organisms apart, and particularly the presence of di- or heterokaryons, may also have little external effect, but they eventually determine the form of reproduction when suitable conditions arise. Environmental stimuli such as light, temperature, humidity and even flow rate of the supporting medium, have been investigated in attempts to assess their relative importance in the development of fungi and the reproducibility of induced morphological changes has attracted much attention as a tool

for the investigation of cell metabolism. It has been shown that the response of a hyphal tip to external stimuli as shown by acceleration, deceleration or cessation of growth or by changes in shape, may be more the result of exposure times measured in seconds and minutes than in hours or days. It seems that very delicate mechanisms are at work in fungal morphogenesis.

Knowledge of the metabolic changes accompanying polymorphism is accumulating rapidly following the use of more modern techniques, and, for instance, the shift from mycelial to yeast phase in *Blastomyces dermatitidis* has been found to involve a sixfold increase in oxygen consumption. Even the simple morphological change from prostrate hypha to erect conidiophore is filled with mystery for the hyphal metabolist, necessitating as it does complete rearrangement of the conduction of nutrients and metabolites, and provision for protection from dessication.

Langeron's view that fungal polymorphism was the principle obstacle to the aspiring mycologist perhaps still holds, but studied and interpreted by those who persevere, it can prove a most useful phenomenon wherever morphogenesis is of interest.

Morphogenesis in Procaryotic Organisms. By D. KERRIDGE (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

There are two aspects of bacterial development to consider; first the changes which occur within individual cells as an obligate part of the division cycle, and secondly the changes which occur in micro-organisms during growth of populations in batch culture. These developmental changes are modifications of the macromolecular composition of the cell and these in turn may result in morphological variations of 'polymorphism'.

Synchronously dividing bacteria have been used to determine the macromolecular composition of the cell at different stages of the division cycle, but in general these changes are only associated with a variation of cell size. Morphological changes during the division cycle occur in 'budding' and 'stalked' bacteria. In *Hyphomicrobium* spp. and *Rhodomicrobium* spp. cell division occurs by the production of a 'bud' at the end of a long hypha. Hyphal production is an obligate stage in division and the daughter cell may separate as a motile swarmer. As yet nothing is known of the factors controlling hyphal initiation and extension and bud formation (Murray, R. G. E. & Douglas, H. C. (1950), *J. Bact.* **59**, 157; Zavarzin, G. A. (1961), *Microbiology* (*USSR*), **30**, 774.; Hirsch, P. & Conti, S. F. (1964), *Arch. Mikrobiol.* **48**, 339, 358.; Starr, M. P. & Skerman, V. B. D. (1965), *Ann Rev. Microbiol.* **19**, 407; Conti, S. F. & Hirsch, P. (1965), *J. Bact.* **89**, 503). In *Caulobacter* spp., the stalk, like the hypha of budding bacteria, is an extension of the cell wall and membrane. Stalk formation is an obligate stage of the division cycle, but in these bacteria, division occurs by binary fission and results in the production of a stalked bacterium and a motile swarmer. The site of stalk synthesis is at its base and the length is affected by the phosphate ion concentration of the growth medium, but the mechanism by which the cell exerts a spacial and temporal control over stalk formation has yet to be elucidated (Stove-Poindexter, J. (1964), *Bact. Rev.* **28**, 131; Schmidt, J. M. & Stanier, R. Y. (1966), *J. Cell. Biol.* **28**, 423).

Morphological changes occurring during microbial growth, although under genetic control are often induced by alterations produced by the organisms in their environment. Examples, of this are microcyst formation by *Myxobacteria* and conidia formation by *Streptomycetes*. *Myxococcus xanthus* exhibits colonial morphogenesis, in that cells aggregate to form fruiting bodies and cellular morphogenesis, in that microcysts are produced from vegetative cells. Certain strains will grow in dispersed culture in liquid media and under these conditions do not form fruiting bodies, but can be induced to form microcysts by the addition of any one of a number of primary or secondary alcohols. The continued presence of inducer at high concentration is essential for morphogenesis and induction does not depend on incorporation of inducer into macromolecules, or indeed penetration into the cell. It is not a simple osmotic effect as a number of non-penetrating alcohols do not induce microcyst formation. There are no reports that compounds related to these artificial inducers occur naturally in themyxococcal fruiting bodies. It has been suggested that the inducers alter the DNA-membrane relationship and this results in the transcription of that part of the genome responsiblefor microcyst formation (Dworkin, M. & Sadler, W. (1966), *J. Bact.* **91**, 1516; Sadler, W. &

Dworkin, M. (1966), *J. Bact.* **91**, 1520). In a non-conidia producing strain of *Streptomyces griseus* transformation of the filamentous mycelium into conidia can be induced by a substance isolated from the growth medium of a normal strain. This substance (Factor C) binds to DNA *in vitro* and stimulates mRNA synthesis *in vivo* and it has been suggested that the morphogenetic effect of Factor C is due to an inhibition of repressor synthesis by the regulator genes associated with the production of conidia (Szabo, G., Bekesi, I. & Vitalis, S. (1966), *J. gen. Microbiol.* **44**, v; (1967), *Biochim. biophys. Acta* **145**, 159.

It is possible that in future, procaryotic organisms with their organizational simplicity and limited polymorphism, will provide valuable experimental material for studying the molecular basis of morphogenesis.

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