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THE JOURNAL OF GENERAL MICROBIOLOGY

The *Journal* will publish accounts of original research in general microbiology, i.e. the study of bacteria, microfungi, microscopic algae, protozoa, and viruses in their biological activities and, more particularly, the fundamental aspects of the study of these forms, including structure, development, physiology, genetics, cytology, systematics and ecology. Writers of papers on a specialized aspect of their subject should describe their work so that its relevance to their own science and to microbiology in general will be apparent to readers who may be unfamiliar with the particular aspect.

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"Easy writing's curst hard reading."—*Richard Brinsley Sheridan*

"Easy reading's curst hard writing."—*The Editors, J. gen. Microbiol.*

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in English and should in general be divided into the following parts in the order indicated: (*a*) Summary: brief and self-contained; (*b*) Introduction; (*c*) Methods; (*d*) Results (illustrative protocols only should be included); (*e*) Discussion (if any), and general conclusions; (*f*) acknowledgements; (*g*) References.

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Nomenclature and Descriptions of Micro-organisms. Binomial Latin names of micro-organisms, the generic name only with a capital, must be used in accordance with International Rules of Nomenclature; in full at the first mention in each paragraph and in the Summary but in subsequent mention with the generic name abbreviated. Single initial letter abbreviations are used where they are not ambiguous. Binomials should be underlined in the typescript. Scientific epithets or trivial names are not underlined and should be without capitals.

Descriptions of new species of cultivable microbes should not be submitted unless an authentic specimen of a living culture has been deposited in a recognized culture collection.

The word 'generation' should not be used synonymously with 'subculture'. For an agreed use of terms like strain, type, variant, phase, etc., see the International Bacteriological Code of Nomenclature, Section 1, Rules 7 and 8.

Except for good reasons, micro-organisms should be designated by the names used in the works listed below. When other authorities are followed, they should be cited whenever obscurity might result from their use.

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

PLANT PATHOGENIC FUNGI AND PLANT DISEASES. *List of Common British Plant Diseases*, 1944. (Cambridge University Press.)

PLANT VIRUSES AND VIRUS DISEASES (1957). *Rev. appl. Mycol.* **35**, Suppl, 1–78.

BACTERIA. Author's references in naming are at present accepted provided that the designation is unambiguous and conforms with the International Bacteriological Code of Nomenclature (1949; *J. gen. Microbiol.* **3**, 444) and the Opinions issued by the International Committee on Bacteriological Nomenclature. If desired a synonym may be added in brackets when a name is first mentioned.

J. gen. Microbiol. (1961), **26**, 1–9
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Spermidine in the Bacterial Cell

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(*Received* 13 *September* 1960)

SUMMARY

The concentration of spermidine in extracts of various micro-organisms was determined; *Neurospora crassa* contained the highest concentrations of spermidine, while in *Pseudomonas aeruginosa*, *Escherichia coli* and *Saccharomyces cerevisiae* the concentration of spermidine was lower. Growing organisms and cell-free extracts of the micro-organisms tested were able to synthesize [^{14}C]spermidine from DL-[2-^{14}C]methionine. Cell-free extracts of *Pseudomonas aeruginosa* were most active in this respect. [^{14}C]Spermidine formed by growing *Bacillus subtilis* was localized in both the protoplasts and the bacterial cell walls. The kinetics of the adsorption of [^{14}C]spermidine on to cell walls and protoplasts of *B. subtilis* were also studied. The interpretation of these observations and their relation to the antibacterial effect and growth-promoting activity of spermidine are discussed.

INTRODUCTION

The polyamines spermidine

$$(NH_2(CH_2)_3NH(CH_2)_4NH_2)$$

and spermine

$$(NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2)$$

are widely distributed in nature. They have been found in animal tissues (Dudley & Rosenheim, 1925; Dudley, Rosenheim & Starling, 1927; Harrison, 1931; Rosenthal & Tabor, 1956), in micro-organisms, especially Gram-negative bacteria (Herbst, Weaver & Keister, 1958; Weaver & Herbst, 1958), and in some viruses (Ames, Dubin & Rosenthal, 1958; Bawden & Pirie, 1959; Ames & Dubin, 1960). Conjugated polyamines such as acetylated spermidine were found in *Escherichia coli* (Dubin & Rosenthal, 1960*a*); a spermidine-glutathione conjugate from *E. coli* has also been described (Dubin & Rosenthal, 1960*b*). Other conjugates of polyamines have been found in a phospholipid from human malignant tumours (Kosaki, Ikoda, Kotani, Nakagawa & Saka, 1958) and in spider poisons (Fischer & Bohn, 1957*a*).

Very little is known about the biosynthesis of polyamines. The condensation of putrescine with methionine in the presence of adenosine triphosphate and magnesium sulphate has been proposed as a pathway for the biosynthesis of spermidine in *Escherichia coli* (Tabor, Rosenthal & Tabor, 1957), and in *Neurospora crassa* (Greene, 1957). The enzymes involved in this condensation reaction have been purified from *E. coli* (Tabor, Rosenthal & Tabor, 1958; Tabor & Tabor, 1960). Weaver & Herbst (1958) have found that *Haemophilus parainfluenzae* produced spermidine from

propane-1:3-diamine. Quantitative determinations of spermidine in animal tissues were made by Rosenthal & Tabor (1956) and Fischer & Bohn (1957*b*). Very little, however, is known about the quantities of polyamines found in micro-organisms.

The purpose of the present investigation was to determine quantitatively the amount of spermidine present in extracts of various micro-organisms, to isolate spermidine from different constituents of the bacterial cell, and to study the mechanism of spermidine absorption to the bacterial cell.

METHODS

Organisms. Strains of the following micro-organisms were used: *Bacillus subtilis* (ATCC 6633); *Pseudomonas aeruginosa* (Bachrach, 1957); *Escherichia coli* 59; *Neurospora crassa* 34455; *Saccharomyces cerevisiae* 81. All these strains were from the collection of the Department of Clinical Microbiology, Jerusalem.

Media. Neurospora crassa was grown in the defined medium of Fries described by Nason & Evans (1953). All the other micro-organisms were grown in the medium of Davis (1949) after adjustment to pH 7·0.

Conditions of growth. The micro-organisms were grown in quantities of 1 l. liquid medium in a 3 l. flask agitated on a rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). After 20 hr. of incubation at 37° the organisms were harvested by centrifugation (Sharples supercentrifuge, or MSE high-speed centrifuge) and washed three times with physiological saline.

Preparation of cell-free extracts. The micro-organisms were subjected to the action of a 10 kcyc. Raytheon sonic oscillator for 25 min. Debris and intact organisms were removed by high-speed centrifugation (13,000 *g* for 10 min.).

Preparation of protoplasts. These were prepared according to Fitz-James (1958). Organisms were washed three times with saline and suspended in 0·1 M-phosphate buffer (pH 6·1) containing 0·5 M-sucrose and 0·016 M-magnesium sulphate. Lysozyme (1 mg./ml.) was then added, and the suspension was incubated at 37° for 30–60 min. The formation of protoplasts was observed by phase-contrast microscopy. Whole organisms were removed by centrifugation (3000 *g*) and the protoplasts separated from the supernatant fluid by a second centrifugation (10,000 *g*).

Preparation of cell walls. These were prepared according to Salton & Horne (1951). Organisms were subjected to the action of a Mickle tissue disintegrator for 60 min. Unbroken organisms were removed by centrifugation (3000 *g*) and the cell walls separated by a second centrifugation (10,000 *g*). The purity of the preparation was checked by electron microscopy.

Chemicals. Spermidine phosphate was obtained from Hoffman-La-Roche (Basle, Switzerland). Putrescine dihydrochloride, DL-methionine and adenosine triphosphate (ATP), were the products of Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). 1-Fluoro-2:4-dinitrobenzene was purchased from L. Light and Co. Ltd (Colnbrook, Bucks, England). DL-[2-^{14}C]methionine was obtained from California Corporation (Los Angeles, U.S.A.).

Spermidine estimations. Paper chromatography was the most convenient method for the qualitative and quantitative estimation of the amines. Butanol + acetic acid + water (50 + 25 + 25 by vol.; Baker, Harborne & Ollis, 1952) was the solvent system used. The papers were sprayed with 0·2% (w/v) ninhydrin in butanol.

Quantitative determinations were carried out by eluting the coloured complex (Giri, Radhakrishnan & Vaidyanathan, 1952).

Ion exchange chromatography. A solution of 10% (w/v) trichloroacetic acid solution was added to an equal volume of the sample to be examined. The precipitate formed was removed by centrifugation and the residual trichloroacetic acid extracted with ether. Samples of the supernatant fluid were then passed through a column of Dowex 50 H^+ (6 mm. diameter; 60 mm. high) and the spermidine eluted from this column by a gradient of HCl (prepared by the dropwise addition of 2·5 N-HCl to 300 ml. water in a mixing vessel) at a flow rate of 1 ml./min. Fractions of 3·5 ml. were collected in an automatic fraction collector (LKB-Produkter, Stockholm, Sweden) and the eluate containing spermidine (fraction between 210–270 ml.) was concentrated in vacuum, neutralized and estimated colorimetrically as the 2:4-dinitrophenyl derivative (Rosenthal & Tabor, 1956).

Steam distillation. Samples containing spermidine were steam distilled in a Markham still after the addition of potassium hydroxide (Dudley, Rosenheim & Rosenheim, 1924).

Protein estimations. Proteins were estimated by the spectrophotometric method of Layne (1957) with a Unicam Model SP 500 Spectrophotometer (Unicam Instruments Ltd, Cambridge).

Radioactivity estimations. Spermidine was identified by paper chromatography and the radioactive areas on the paper were counted either directly with a mica end-window Geiger–Müller tube, or eluted with water and subsequently assayed for radioactivity by means of an end-window lead-shielded Geiger–Müller tube (Tracerlab). Spermidine was also separated by the ion exchange method, converted into the 2:4-dinitrophenyl derivative, extracted with ethyl acetate and assayed for radioactivity by means of a Geiger–Müller counter.

RESULTS

Quantitative determinations of polyamines in various micro-organisms

Extracts of micro-organisms obtained after disruption by sonic oscillation were examined for polyamines by paper chromatography. The polyamines detected were separated by the ion-exchange method and assayed colorimetrically. As shown in Fig. 1, 21 μg. spermidine were found per mg. protein in an extract of *Neurospora crassa*. The concentration of spermidine in extracts of *Pseudomonas aeruginosa* was appreciably lower (12·5 μg./mg. protein). The lowest concentration was obtained with *Saccharomyces cerevisiae* (only 7 μg./mg. protein).

Synthesis of [^{14}C] *spermidine by growing organisms and by cell-free extracts*

The micro-organisms were grown for 20 hr. at 37°, harvested, washed with saline and resuspended in 4·0 ml. of medium to which phosphate buffer and [2-^{14}C]methionine had been added. After further incubation, the organisms were harvested and disrupted. The spermidine in the extracts was separated by ion-exchange chromatography, and its radioactivity determined. *Neurospora crassa* contained the highest concentration of [^{14}C]spermidine, while the lowest concentration was found in *Pseudomonas aeruginosa* (Fig. 2).

After ascertaining that growing organisms were capable of producing spermidine

from putrescine, it was attempted to synthesize this polyamine by extracts obtained from the various micro-organisms. Putrescine, [2-^{14}C]methionine, ATP and magnesium sulphate were incubated with the various extracts. The spermidine formed was separated by ion-exchange chromatography and its radioactivity determined. The highest concentrations of [^{14}C]spermidine were obtained with extracts of *Pseudomonas aeruginosa* (Fig. 3).

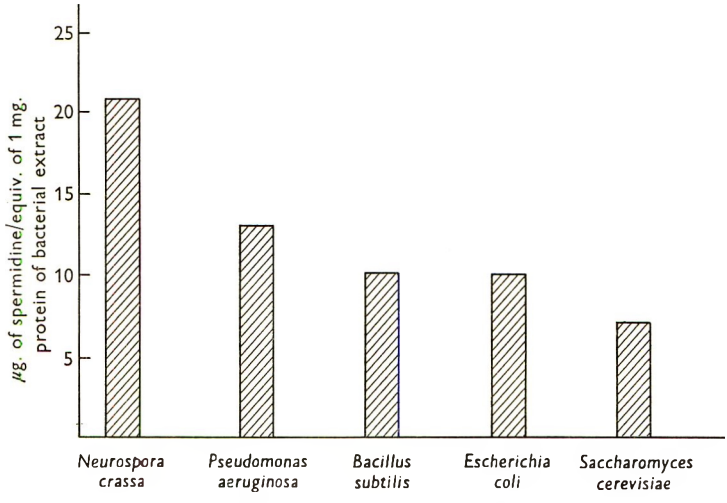

Fig. 1. Spermidine in extracts of various micro-organisms.

Localization of spermidine in Bacillus subtilis

Bacillus subtilis was chosen for study because of the detailed techniques elaborated for the separation of its cellular constituents. The localization of spermidine in the cell walls and protoplasts was examined by incubating intact bacilli in a medium fortified with either [2-^{14}C]methionine or [^{14}C]spermidine prepared from [2-^{14}C]methionine in the presence of *Pseudomonas aeruginosa* as described above. *B. subtilis* was grown in 2000 ml. medium at 37° for 20 hr. The bacteria were harvested, washed and

Table 1. [^{14}C]*Spermidine in cell walls and protoplasts of* Bacillus subtilis *grown in the presence of* DL-[2-^{14}C]*methionine or* [^{14}C]*spermidine*

Bacteria were grown for 20 hr. at 37°, harvested and resuspended in 10·0 ml. of medium supplemented with 2·5 μmole DL-[2-^{14}C]methionine ($1·5 \times 10^5$ c.p.m.) or 1·0 ml. of [^{14}C]spermidine (5×10^3 c.p.m.) and incubated for another 14 hr. The [^{14}C]spermidine in the cell walls and protoplasts of these bacteria was determined.

Preparation	mg. of protein/ml. of preparation	mg. of dry wt./ml. of preparation	c.p.m./ml. of preparation	c.p.m./mg. Protein	c.p.m./mg. Dry wt.
Cell walls incubated with:					
[^{14}C]Methionine	0·9	10·5	2·160	2·400	206
[^{14}C]Spermidine	1·7	19·8	2·082	1·225	105
Protoplasts incubated with:					
[^{14}C]Methionine	2·9	5·8	243	84	42
[^{14}C]Spermidine	3·1	6·2	220	71	36

resuspended in 10 ml. of medium to which [2-^{14}C]methionine had been added. After another 14 hr. of incubation the bacteria were again harvested and protoplasts and cell walls prepared. The [^{14}C]spermidine in the samples was separated by steam distillation. The distillate was concentrated and examined by paper chromatography.

The amount of [^{14}C]spermidine bound to an equivalent of 1 mg. dry wt. of the cell wall was higher than that bound to the same equivalent of protoplast. The amount of [^{14}C]spermidine localized in the cell walls of *Bacillus subtilis* was also higher when [^{14}C]methionine was added to the growing organisms (Table 1).

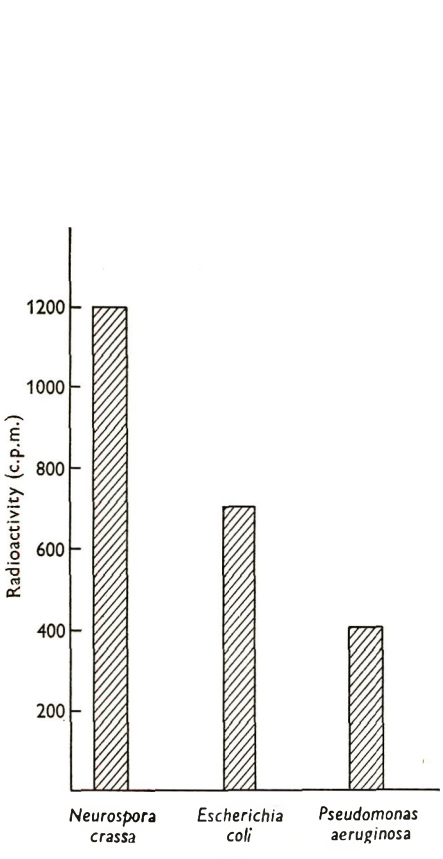

Fig. 2

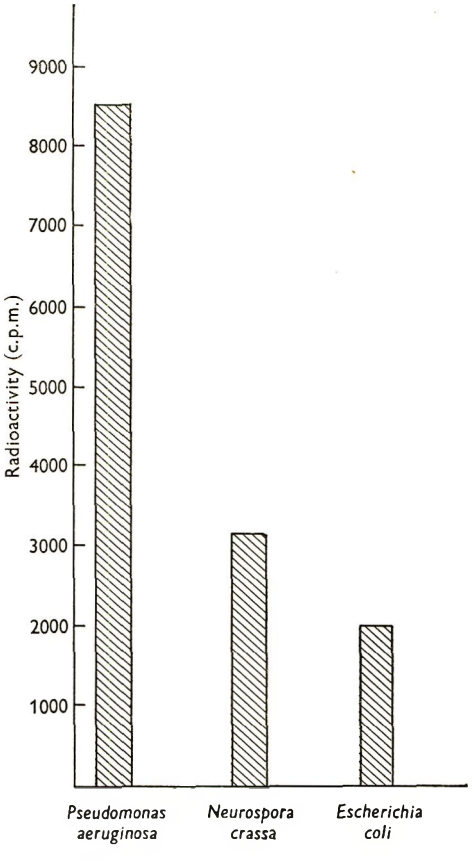

Fig. 3

Fig. 2. Synthesis of [^{14}C]spermidine by growing cells of various micro-organisms. The bacteria were grown for 20 hr. at 37°, harvested and resuspended in 4·0 ml. of medium, supplemented with 3·0 ml. of 0·067 M-phosphate buffer pH 7·9; 2·5 μmole of DL-[2-^{14}C]-methionine ($1{\cdot}5 \times 10^5$ c.p.m.) and incubated for another 14 hr. The cells were then harvested, disrupted and the concentration of [^{14}C] spermidine in the extracts determined.

Fig. 3. Synthesis of [^{14}C]spermidine by cell-free extracts of various micro-organisms. The reaction mixtures contained: 1·0 ml. of 0·067 M-phosphate buffer pH 8·0; 1·0 μmole of DL-[2-^{14}C]methionine ($0{\cdot}6 \times 10^5$ c.p.m.); 1·0 μmole of putrescine; 30 μmole of $MgSO_4$; 2·5 μmole of ATP and 3·0 ml. of cell-free extract (equiv. of 50 mg. of protein). Incubation was carried out at 37° for 60 min.

Binding of [^{14}C]*spermidine by the cell walls and protoplasts of* Bacillus subtilis

Having established that [^{14}C]spermidine was bound to the cell wall of *Bacillus subtilis* and to a smaller degree to its protoplast, the kinetics of this reaction were studied. Figure 4 shows that cell walls of *B. subtilis* rapidly bound [^{14}C]spermidine (prepared biologically by *Pseudomonas aeruginosa*) as indicated by the decrease in radioactivity of the supernatant fluid, and the increase of radioactivity in the precipitate (i.e. cell walls). The binding of [^{14}C]spermidine by protoplasts is shown in Fig. 5.

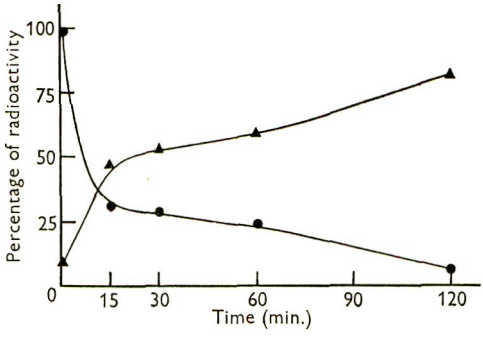

Fig. 4

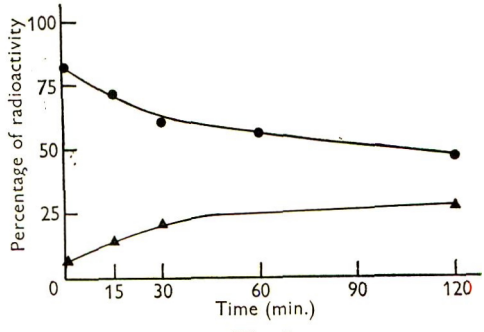

Fig. 5

Fig. 4. Binding of [^{14}C]spermidine by cell walls of *Bacillus subtilis*. The reaction mixture contained 1·0 ml. of [^{14}C]spermidine ($5{\cdot}0 \times 10^3$ c.p.m.) and 4·0 ml. of cell wall suspension (equiv. of 5·2 mg. of protein or 61 mg. dry wt.). Samples were taken at the time intervals indicated, centrifuged and the radioactivity of the supernatants, ●, and the precipitates, ▲, determined.

Fig. 5. Binding of [^{14}C]spermidine by protoplasts of *Bacillus subtilis*. Experimental conditions were as given in Fig. 4, but 4·0 ml. of protoplast suspension (equiv. of 10·0 mg. of protein or 20 mg. dry wt.) was employed. ●, Supernatant; ▲, precipitate.

DISCUSSION

The amine content of *Escherichia coli* depends on growth conditions such as the composition and pH value of the growth medium. In the absence of spermidine in the growth medium, 1 g. wet wt. organism was found to contain about 200μg. spermidine (Dubin & Rosenthal, 1960*a*). Brewers yeasts contain 130μg. spermidine/g. wet wt. (Fischer & Bohn, 1957*b*). These amounts are similar to those found in rat or rabbit livers (Rosenthal & Tabor, 1956). The use of wet weights of organisms for the comparison of the amounts of spermidine in different organisms, however, might lead to considerable errors. We therefore used the protein content of the examined bacterial extracts as a criterion in the evaluation of our results. The amount of spermidine in the extract is not equal to the total spermidine content of an organism, for insoluble constituents of the cells may retain a considerable amount of this compound which cannot be completely extracted even with 0·1 N-hydrochloric acid (Razin & Rozansky, 1959). The results represented in Fig. 1 indicate that spermidine is present in similar concentrations in the extracts of the Gram-positive and Gram-negative organisms tested. Extracts of *Pseudomonas aeruginosa* produced more [^{14}C]spermidine from [2-^{14}C]methionine than the equivalent extracts of *Neurospora crassa* and *E. coli* (Fig. 2). The quantities of [^{14}C]spermidine which accumulated

within the organisms grown on [2-^{14}C]methionine were smaller than those produced by extracts (Fig. 3). This is not surprising and might be explained by the limited capacity of the whole organism to store the polyamine. Decomposition of spermidine by *P. aeruginosa* (Razin, Gery & Bachrach, 1959) may also cause a decrease in its intracellular concentration.

The attachment of polyamines to bacteria was described by Razin & Rozansky (1959), and Mager (1959) studied the binding of spermidine to protoplasts. The receptors on the cell wall might be located in the plastic film of lipoprotein described by Mitchell (1959) or in some bacteria in the lipoprotein particles like those responsible for the adsorption of bacteriophages (Anderson, 1960). The active sites in the protoplasts might be associated with its ribonucleic acid content (Cohen & Lichtenstein, 1960) or with the cytoplasmic lipoprotein layer (Luria, 1960).

Experiments described in this paper indicate that *Bacillus subtilis* grown in the presence of methionine or spermidine contained spermidine in cell walls and protoplasts. Cohen & Lichtenstein (1960) found that polyamines were attached to ribosome particles of *Escherichia coli* prior to cell disruption, and were not bound during or after the formation of the extract. If this concept is general, one could exclude the possibility of redistribution of the polyamines during the disintegration, and assume that the location of the polyamines in disintegrated and intact cells is the same.

Spermine and spermidine are known to inhibit the growth of various bacteria (Gurevitch, Rozansky, Weber, Brzezinsky & Eckerling, 1951; Rozansky, Bachrach & Grossowicz, 1954) and fungi (Razin, Cohen, Rozansky, 1958). As both sensitive and resistant bacteria absorb these polyamines (Razin & Rozansky, 1959), the selective toxicity is not explained by absorption alone. Detoxification of spermidine by acetylation (Dubin & Rosenthal, 1960*a*) does not seem to solve the problem, for acetylated polyamines were also found in *Staphylococcus aureus* (Rosenthal & Dubin, 1960), which is sensitive to spermidine. Recent studies on the mode of action of streptomycin indicate that its initial uptake by the bacterial cell occurs outside the cytoplasmic membrane. The secondary uptake is caused by the cytoplasmic membrane, resulting in changes of the cell permeability (Anand & Davis, 1960). Furthermore, streptomycin-resistant bacteria do not bind streptomycin on their cytoplasmic membrane (Anand, Davis & Armitage, 1960). Streptomycin resembles spermidine in its basic property; both are bound to acidic components like nucleic acids (Cohen, 1946; Fraser & Mahler, 1958; Felsenfeld & Huang, 1960) and their biological activity is counteracted by constituents of serum. One may speculate that the polyamines inhibit the growth of sensitive micro-organisms by interfering with the normal function, synthesis, or permeability barrier, of the cytoplasmic membrane.

The growth-promoting effect of polyamines for certain bacteria (Herbst & Glinos, 1955; Martin, Pelczar & Hansen, 1952; Sneath, 1955; Traub, Mager & Grossowicz, 1955; Kihara & Snell, 1957) remains still to be explained. The degradation of spermidine to β-alanine and other compounds by *Pseudomonas aeruginosa* (Razin, Gery & Bachrach, 1958, 1959; Bachrach, 1960) and by *Mycobacterium smegmatis* (Bachrach, Persky & Razin, 1960) appears to apply to these two micro-organisms and does not explain the growth-promoting activity for other bacteria. It seems possible that polyamines may enhance bacterial growth by affecting the permeability barrier of the cells (e.g. by preventing leakage of nutrients from the cell). Similar effects have been obtained with mitochondria (Tabor, 1960; Herbst & Whitherspoon, 1960) and bacterial protoplasts (Mager, 1959).

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A New Genus of the Actinomycetales: *Micropolyspora* gen. nov.

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SUMMARY

Three filamentous micro-organisms are described. They are typical Actinomycetales forming branching hyphae, 1·5 μ or less in diameter, which are differentiated into a substrate (primary) mycelium and an aerial (secondary) mycelium. They have an unusual mode of sporulation since they form chains of conidia both on the substrate and on the aerial mycelium. A new genus, *Micropolyspora*, is proposed. The type species is *M. brevicatena*. The new genus is part of the family Actinomycetaceae. The abolition of the family Streptomycetaceae is proposed.

INTRODUCTION

Actinomycetes are filamentous bacteria which are often considered as intermediate between the true bacteria and the fungi. According to the latest edition of *Bergey's Manual* (1957) these organisms are grouped under the order Actinomycetales, which is subdivided into four families as follows:

I. Mycelium rudimentary or absent; no spores formed	Mycobacteriaceae
II. True mycelium produced	
A. Spores formed, but not in sporangia	
1. Spores formed by fragmentation of mycelium	Actinomycetaceae
2. Vegetative mycelium normally remains undivided	Streptomycetaceae
B. Spores formed in sporangia	Actinoplanaceae

Bergey's Manual recognizes three genera in the family Streptomycetaceae. They are characterized as follows:

I. Conidia produced in aerial hyphae in chains	*Streptomyces*
II. Conidia produced terminally and singly on short sporophores	
A. No growth between 50° and 65°	*Micromonospora*
B. Growth occurs between 50° and 65°	*Thermoactinomyces*

Since the publication of the 1957 edition of *Bergey's Manual*, other genera have been described, to include organisms which have novel modes of spore formation. These include: (1) *Waksmania* Lechevalier & Lechevalier (1957), in which pairs of spores are formed longitudinally on the aerial mycelium. The same genus, under the name *Microbispora*, was described at the same time in Japan by Nonomura &

Ohara (1957). (2) *Thermopolyspora*, in which short chains of spores are formed on the aerial mycelium of thermophilic organisms (Henssen, 1957).

The present paper reports the isolation and description of a type of actinomycete which produces spore-bearing substrate mycelia and aerial mycelia. The name proposed for the new genus is *Micropolyspora*. The type species is *M. brevicatena*. Two strains have been isolated and are deposited in the culture collection of the Institute of Microbiology, Rutgers, The State University, New Brunswick, N.J. (nos. 1084 and 1086). In addition another strain of the same genus was also isolated (no. 1085). This strain has cultural properties which differentiate it from the other two. Until more strains of this new genus are isolated and studied we shall refer to this culture as *Micropolyspora* sp. no. 1085.

METHODS

Isolation. Specimens of sputa taken in routine examination for *Mycobacterium tuberculosis* var. *hominis* were treated with 4% (w/v) NaOH and shaken on a mechanical shaker for 10 min. After neutralization with 2N-hydrochloric acid, the suspensions were centrifuged and the sediment used to inoculate two tubes of a Jensen–Holm modification of Lowenstein medium (*American Public Health Association*, 1950) and two tubes of penicillin blood agar (Tarshis, 1953). The tubes were incubated at 37°, horizontally for the first 72 hr. and then upright. They were examined weekly after the first 2 weeks.

Stains. Acid-fast staining was essentially as described on page 229 of Staining Procedures used by the Biological Stain Commission (Conn, Darrow & Emmel, 1960) except that one drop of Tergitol No. 7 (Carbide & Carbon Chemical Co., 30 East 42nd St., New York, N.Y.) was added to 30–40 ml. of carbol fuchsin just before staining. The Gram stains were done by covering smears for 1 min. with a 1% (w/v) aqueous solution of crystal violet and adding simultaneously five drops of 5% (w/v) aqueous sodium bicarbonate. After drainage, the slides were covered for 1 min. with an aqueous solution containing 2% (w/v) potassium iodide and 1% (w/v) mercury potassium iodide. After washing with water, the smears were decolorized with acetone, rinsed with water and stained for one minute with a 0·1% (w/v) aqueous solution of safranin Y.

Media. The composition of all media not listed here, and for which there are no references given in the text, will be found in the appendix of Waksman (1950) or in the *Difco Manual* (1953).

NZ-amine glycerol agar. NZ-amine A (casein hydrolysate; Sheffield Farms; 1267 6th Ave., New York), 5 g.; beef extract, 1 g.; glycerol, 70 ml.; agar, 15 g.; tap water, 1 l.; pH 6·5–7·0.

Nutrient agar with glucose. Nutrient agar with 1% (w/v) glucose.

Nutrient agar with glycerol. Nutrient agar with 70 ml. glycerol/l.

Soil extract glycerol agar. Peptone, 5 g.; beef extract, 3 g.; agar, 15 g.; soil extract, 250 ml.; tap water to 1 l.; pH 6·5–7·0. Soil extract was prepared by autoclaving 1 kg. air-dried garden soil with 2·4 l. tap water for 1 hr. at 121°, filtering through paper and autoclaving for storage.

Defined medium. Glycerol, 70 ml.; L-glutamic acid, 1·5 g.; L-arginine, 1·0 g.; K_2HPO_4, 1·0 g.; $MgSO_4.7H_2O$, 100 mg.; $CaCl_2$, 10 mg.; $ZnSO_4$, 10 mg.; $Fe_2(SO_4)_3.9H_2O$, 10 mg.; agar, 15 g.; distilled water, 1 l.; pH 6·5 to 7·0.

Photographs. Microphotographs were taken through a trinocular American Optical 'Microstar' Microscope with a 35 mm. photomicrography attachment. Kodak High Contrast Copy film was used. All microphotographs except Pl. 1, fig. 8, are of undisturbed plate cultures. Microphotographs were taken *in situ* through $\times 40$ or $\times 57$achromatic objectives. The microscope was equipped with a long focus condenser which gave adequate lighting even through thick layers of agar. Plate 1, fig. 8, was taken through a $\times 93$ fluorite objective.

Electron photomicrographs (Pl. 1, figs. 6, 7) were taken with an RCA EMU-1 electron microscope modified with Canalco equipment. Collodion films mounted on grids were touched to the surface of 5-day cultures grown at 37° on N-Z amine glycerol agar, portions of the mycelium becoming attached to the collodion membrane. The materials were not shadowed.

Examination of spore germination. Agar blocks from well-sporulating N-Z amine glycerol agar plate cultures were cut out aseptically and placed on sterile glass slides on the stage of a microscope. Spores were teased off the mycelium and spread over sterile portions of the agar blocks with a 5 μ sterile glass microhook, manoeuvred with a Cailloux micromanipulator. Slides were incubated in a humid chamber at 37° and examined periodically under the microscope. The number of germ tubes was counted after incubation for 18 hr.

RESULTS

Description of Micropolyspora *Lechevalier, Solotorovsky and McDurmont, gen. nov.*

Morphology. Fine mycelium (about 1 μ in diam.) which is differentiated into: (1) a substrate (primary) mycelium which grows into and forms a compact layer on top of agar media; (2) an aerial (secondary) mycelium which arises from the substrate mycelium and grows in the air away from the agar surface. Both the substrate hyphae and the aerial hyphae bear chains of conidia which are produced either directly on the mycelium or on sporophores which branch from the mycelium. Three strains were isolated: no. 1084, 1085 and 1086. The type species is based on the properties of no. 1084 and 1086. Isolate 1085, which is morphologically similar, has different cultural properties as indicated below.

Type species, Micropolyspora brevicatena, *Lechevalier, Solotorovsky and McDurmont, sp.nov.*

Substrate mycelium. About 1 μ in diameter. Filaments long, branching, penetrating the agar medium and forming compact colonies which are at first whitish on most media and become yellowish. Single spores or short chains of spores (2 to 10) are formed in the agar (Pl. 1, fig. 3) and on the surface of the agar where they are most easily observed (Pl. 1, figs. 1, 2).

Aerial mycelium. About 1 μ in diameter. Long branching hyphae, not abundant on most media. Short chains of spores formed (Pl. 1, figs. 4, 5). Often aerial hyphae will bend back in the agar after having grown in the air, forming solon-like structures (Pl. 1, fig. 9). Aerial hyphae will often aggregate to form long multi-filamentous strands which may link adjoining colonies. The tip of an aerial hypha, in young growing cultures, often curls up into a globose structure which is formed by the

tightly coiled filament (Pl. 1, fig. 4). These bodies have been observed to uncoil suddenly, with the resulting formation of a stolon. This sudden uncoiling was reminiscent of the casting of a fisherman's line.

Dome-shaped bodies. Hyphae often aggregate in a tough matty pseudo-tissue, which eventually forms a dome on the surface of the agar. Cutting through the cover of the dome reveals a hyaline gel which is rich in spores.

Spores. Spherical to oblong, sometimes pyriform, about 1·5 μ in diameter. Spores are borne terminally on sporophores (Pl. 1, fig. 7) which are sometimes branched (Pl. 1, fig. 6), as well as at the tip of main hyphae. They are also borne sessile on the side of hyphae. Spores are formed singly or in short chains (2 to 10 spores) and are most abundant and most easily observed on the surface of the agar. Isolate 1086 forms straight chains of spores, whereas the chains are slightly coiled or wavy in isolate 1084 (Pl. 1 figs. 1, 2). Spores are easily separated one from the other, but often stay and germinate in pairs. During germination, one to three germ tubes are formed. An incubation period of 5 to 7 days at 37° was optimal for the observation of the spores on the NZ-amine glycerol medium. The surface of the spores is very slightly warty (Pl. 1, figs. 6, 7).

Spore formation. A bud on a hypha grows to form a small branch, near the tip of which one septum appears; while the branch is still growing, a second septum is formed, then a third, etc. (Pl. 1, figs. 10, 11). The cells which have been walled off swell slightly, so that the spores are of a somewhat larger diameter than the hyphae, as indicated above (Pl. 1, figs. 3, 6, 7, 8). The mode of formation of the conidia is typically fungal in nature and is of the type illustrated in Fig. 251 A of Langeron & Vanbreuseghem (1952).

Fragmentation. Even though fragmentation is not apparent during the *in situ* examination of the mycelium, either with bright field or phase contrast microscopy, the mycelium is easily separated into numerous fragments in the process of making smears.

Diffusible pigments. Isolate 1084 forms a light brown pigment after prolonged incubation (2 weeks) on certain media, such as NZ-amine glycerol agar. Isolates 1085 and 1086, in our studies, did not form any diffusible pigment.

Appearance on various media. All cultures were incubated for 2 weeks at 37°. No growth took place on the following media: starch agar A, starch agar B, glucose asparagine agar, Czapek agar. In addition, no growth occurred in nitrate broth, carbon-free cellulose medium, or oatmeal agar (Lechevalier & Lechevalier, 1957).

Yeast extract agar. Isolate 1084: thin growth with powdery surface; pale orange (Séguy 200). Isolates 1085 and 1086: thin growth with powdery surface; pale orange-yellow (Séguy 250).

Rice-extract agar and Pablum-extract agar (Lechevalier & Lechevalier, 1957). Isolates 1084 and 1086: very little growth, powdery white. Isolate 1085: powdery white with a few light orange spots.

Potato plug. Isolate 1084: thin powdery growth, centre pale mauve (Séguy 239), edge pale orange-mauve (Séguy 180). Isolates 1085 and 1086: scarce thin powdery growth, pale mauve (Séguy 5).

NZ-amine glycerol agar. Isolate 1084: abundant, slightly wrinkled growth, pale orange (Séguy 200). Isolates 1085 and 1086: same as isolate 1084; pale orange-yellow (Séguy 250).

Defined medium. Isolate 1084: growth less abundant than on NZ-amine glycerol agar, pale orange (Séguy 199), white powdery surface. Isolate 1086: same, as 1084 pale orange-yellow (Séguy 250). Isolate 1085: no growth.

Nutrient agar. Isolate 1084: scarce, thin, buff growth. Isolate 1086: scarce, thin colourless growth. Isolate 1085: scarce, thin white growth.

Nutrient agar with glucose. Isolate 1084: powdery growth, thicker than on yeast extract agar, pale orange (Séguy 190). Isolate 1086: growth pale orange (Séguy 190) with a powdery white surface. Isolate 1085: thin powdery growth, pale orange-yellow (Séguy 250).

Nutrient agar with glycerol. Isolates 1084 and 1086: thick, shallowly wrinkled growth, pale orange-yellow (Séguy 249). Isolate 1085: thin powdery growth, pale orange-yellow (Séguy 250).

Litmus milk. Isolate 1084: slight powdery white pellicle, no effect on milk. Isolates 1085 and 1086: no growth.

Effect of pH and temperature on growth. Yeast-extract agar slopes were prepared at four different pH values (5·1, 6·3, 7·4, 8·4), inoculated with isolates 1084, 1085 and 1086, and incubated at 28°, 36° and 50°. Growth was best at pH 6·3 and 36°. No growth took place on any medium at 50°. No growth took place at pH 5·1 at any temperature.

Oxygen requirement. No growth took place on soil extract glycerol agar under a nitrogen atmosphere, or when the pyrogallol method (Fred & Waksman, 1928) of removing oxygen was used. Control aerobic cultures grew abundantly.

Defined medium for growth. In the presence of glucose, sucrose or glycerol, the following inorganic sources of nitrogen did not permit growth: NH_4NO_3, $NaNO_3$, $(NH_4)_2SO_4$. In presence of glycerol, DL-asparagine and DL-aspartic acid hardly permitted any growth, but L-glutamic acid was a good source of nitrogen for growth, even though sporulation was not abundant. Sporulation of isolates 1084 and 1086 was stimulated by L-arginine, which by itself was not satisfactory for growth and which prevented the growth of isolate 1085 in presence of L-glutamic acid. Good growth and sporulation of isolates 1084 and 1086 were obtained when magnesium sulphate, potassium phosphate, and calcium chloride were added to the L-arginine + L-glutamic acid + glycerol mixture. The addition of iron and zinc was not essential in this medium, which was obviously grossly contaminated with trace elements (see composition of the medium under METHODS).

Staining properties. The staining properties did not vary with the age of the culture (between 2 weeks and 2 months), but there was variation from one medium to another. On Lowenstein's and penicillin blood agar about 75 % of the organisms on smears were acid-fast, whereas on Sabouraud glucose and yeast-extract agar about 30 %. Less variation was observed with the Gram reaction, both organisms being about 50 % Gram-positive on all media.

Antibiotic properties. No activity by cross-streak test (Waksman, 1950) on yeast-extract glucose agar against *Aspergillus niger* 13, *Mucor rouxii* 80, *Candida albicans* 204, *Saccharomyces cerevisiae* 216, *Bacillus cereus* 8, *Escherichia coli* 54, or *Sarcina lutea* 14.

Sensitivity to antibiotics and antimicrobial agents. On yeast-extract agar, isolates 1084, 1085 and 1086 grew in the presence of pure candidin, 80 % pure candicidin, and pure cycloheximide at 100 μg./ml. Streptomycin at 100 μg./ml. inhibited

completely the growth of these isolates; chloramphenicol or neomycin at 5 μg./ml. had the same effect. Neomycin at 10 μg./ml. sterilized cultures of these isolates in 18 hr. at room temperature. The other antibiotics listed above had no microbicidal effect. On nutrient agar with glucose, the growth of the two cultures (no. 1804, 1806) was inhibited by isoniazid at 10 μg./ml. and by neomycin at 1 μg./ml.

Source. The two strains of *Micropolyspora brevicatena* were isolated from the sputa of two adult white males who had been treated for tuberculosis, but who did not have active tuberculosis at the time the sputa were collected. Isolate 1085 was isolated from the sputum of a white adult female who had no active tuberculosis but whose husband had.

Pathogenicity. Intravenous and subcutaneous injections with heavy suspensions of isolates 1084 and 1085 in white mice produced no apparent ill effect. After 15 days, the animals were sacrificed and examined for evidence of pathological changes. Spleens in the inoculated animals were slightly larger than those of the controls. All other organs appeared normal. Microscopic examination of the organs of the animals did not reveal any specific lesions. Guinea-pigs injected intraperitoneally with heavy suspensions of isolate 1084 were unaffected as far as could be seen from the examination of the growth curves over a period of 30 days and gross examination of organs 30 days after injection of the culture suspensions.

DISCUSSION

As pointed out previously, in Waksman's classification of actinomycetes the basic difference between the two families Actinomycetaceae and Streptomycetaceae is based on the assumption that the substrate mycelium of members of the Actinomycetaceae fragments into bacillary or coccoid elements, whereas the substrate mycelium of the members of Streptomycetaceae remains unfragmented. It is very doubtful whether such a system is sound, since variation in fragmentation can be observed between isolates obtained from the same strain of organisms presently called *Nocardia* and *Streptomyces*.

The fact that the distinction between the two families is unsound is further demonstrated by the organism here described. *Micropolyspora* fragments like Actinomycetaceae, and sporulates like Streptomycetaceae by forming chains of conidia on aerial hyphae. In addition it has a novel mode of sporulation, namely, the formation of chains of conidia on the substrate mycelium. These conidia are located in and on agar media. It would be most unwise to solve the dilemma by creating a new family to accommodate the genus *Micropolyspora*; instead, it is suggested that the name Streptomycetaceae be dropped and that the family Actinomycetaceae be enlarged as follows.

Family Actinomycetaceae Buchanan, 1918: Branching hyphae about 1 μ in diameter form a substrate mycelium composed of hyphae, growing in and on agar media and an aerial mycelium formed of hyphae growing away from agar media. The aerial mycelium is sometimes lacking. The mycelium may or may not fragment into short segments which are either coccoid or rod-shaped. Mycelium may form single conidia or chains of conidia. Spores not formed in sporangia.

The following key to the genera of the Actinomycetaceae is proposed:

I. Anaerobic, or microaerophilic, organisms forming no conidia

1. *Actinomyces* Harz

II. Aerobic organisms. Might form conidia singly or in chains.
  A. Conidia formed singly
    1. No aerial mycelium — 2. *Micromonospora* Ørskov
    2. Aerial mycelium formed — 3. *Thermoactinomyces* Tsiklinsky
  B. Conidia formed in longitudinal pairs on the aerial mycelium — 4. *Waksmania* Lechevalier & Lechevalier
  C. Conidia formed singly and in chains on the substrate and on the aerial mycelium — 5. *Micropolyspora* Lechevalier, Solotorovsky & McDurmont
  D. Conidia when formed are in chains on the aerial mycelium only
    1. Mycelium fragmenting, conidia not always formed — 6. *Nocardia* Trevisan
    2. Mycelium non-fragmenting, conidia always formed — 7. *Streptomyces* Waksman & Henrici

Fragmentation should not be confused with septation. Septa can be observed in the mycelium of some actinomycetes which do not have any tendency to fragment.

In the proposed key, attention has been paid mainly to morphological differences based on the types of conidia formed. It seems hardly proper to put morphologically similar organisms in different genera because one organism is mesophilic and the other thermophilic.

The distinction between *Nocardia* and *Streptomyces* is simple in certain cases. A typical *Streptomyces* sp. which formed a non-segmenting substrate mycelium and a well-developed aerial mycelium bearing chains of conidia is easily distinguished from an organism which forms a fragmenting substrate mycelium with poorly developed aerial hyphae which do not bear any conidia. Frequently, however, the distinction is hard to make, as shown by some recently published papers (Gordon & Mihm, 1958, 1961; Bradley, 1959).

In any event, *Micropolyspora brevicatena* represents a novel morphological type easily distinguishable from the previously described types that we have found in the literature and from those that we have studied up to now. One should note, however, that a thermophilic organism was described by Henssen (1957), under the name *Pseudonocardia thermophila*, which presumably forms chains of spores both on the substrate and aerial mycelium. The substrate mycelium of Pseudonocardia is septate and the chains of spores of this thermophilic organism seem to be fragments of the mycelium, quite different from the Waksmania-like conidia of Micropolyspora. Henssen's *Pseudonocardia thermophila*, judged on the basis of her description and photographs, seems to be a facultatively thermophilic Nocardia. We have examined two strains of *Pseudonocardia thermophila* (nos. 1179 and 1180) originally isolated by Henssen. We were unable to observe chains of conidia on the substrate mycelium.

A study of the ecology of strains of *Micropolyspora* would be of great interest. Such organisms have not been observed in soil. One might wonder if such organisms are to be found in healthy humans and animals, or, strangely, only in patients who have recovered from tuberculosis.

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EXPLANATION OF PLATE 1

Fig. 1. Isolate 1086; chains of spores on the surface of the agar (NZ amine glycerol agar, 6-day culture). ×660.

Fig. 2. Isolate 1804; chains of spores on the surface of the agar (yeast-extract agar, 3-week culture). ×730.

Fig. 3. Isolate 1084; chain of spores deep in the agar (soil-extract glycerol agar, 20-day culture). ×670.

Fig. 4. Isolate 1086; chains of spores and globose structure on aerial hyphae (defined medium, 6-day culture). ×670.

Fig. 5. Isolate 1084; chains of spores on aerial hyphae (soil-extract glycerol agar, 20-day culture). ×2000.

Fig. 6. Isolate 1086; electron microphotograph of a chain of spores, with a branched sporophore. ×10,000.

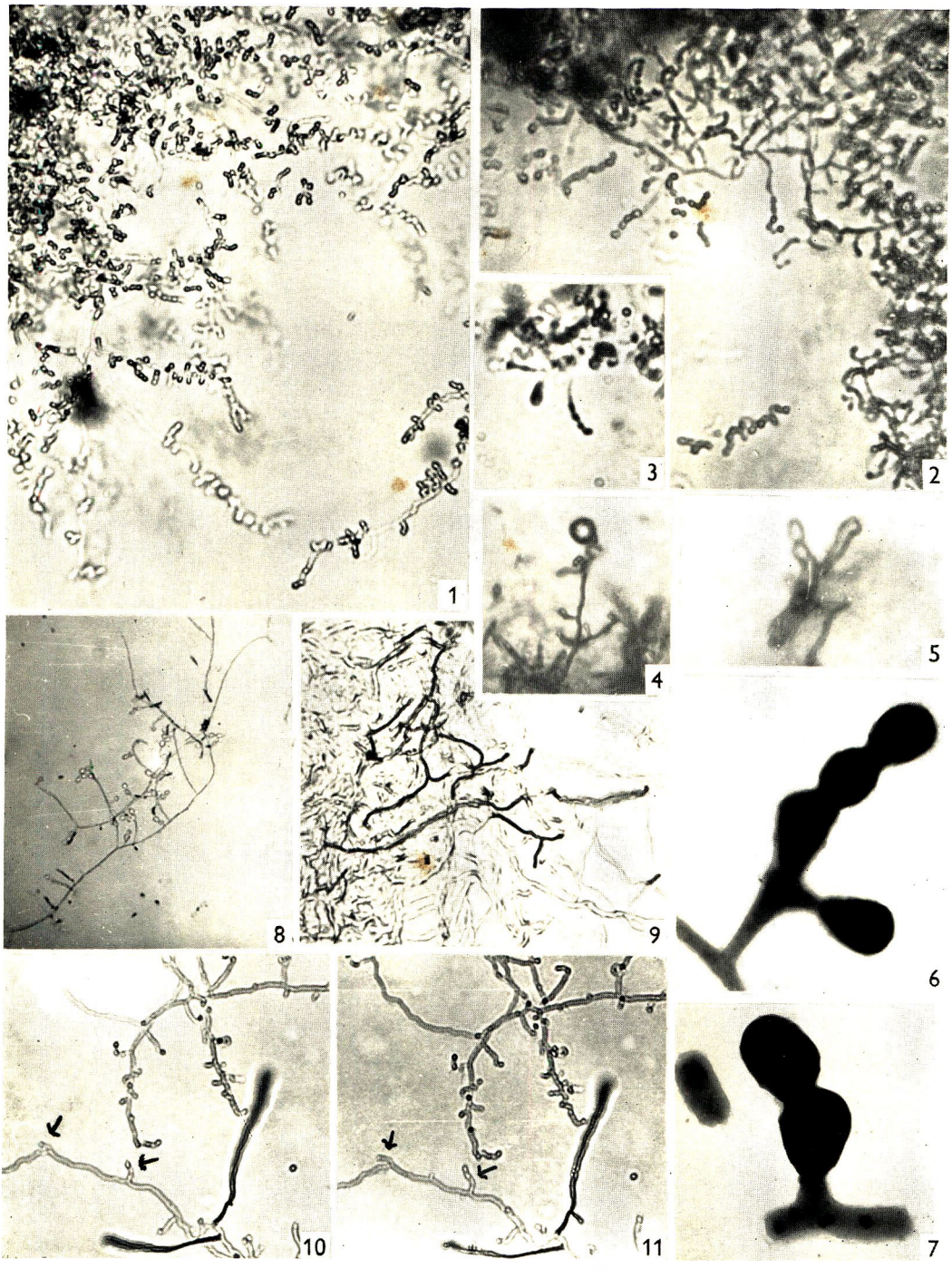
Fig. 7. Isolate 1086; electron micrograph of a chain of spores. ×10,000.

Fig. 8. Isolate 1085; smear showing chains of spores. Gram stain. ×600.

Fig. 9. Isolate 1086; stolon-like aerial hyphae (NZ-amine glycerol agar, 2-day culture). ×800.

Fig. 10. Isolate 1084; 4-day culture on nutrient agar with glycerol. ×800.

Fig. 11. Same culture 7 hr. later. Note formation of spores. ×800.



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Recombination in *Actinomyces aureofaciens*

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SUMMARY

Biochemical mutants of *Actinomyces aureofaciens* were obtained after treatment with ultraviolet irradiation or ethyleneimine. A specificity of the mutational process in a series of *A. aureofaciens* strains was observed, revealing itself mainly in formation of arginine-requiring mutants. Prototroph formation was shown to result from crosses of *A. aureofaciens* biochemical mutants with either the same or different amino acid requirements. Colonial morphology and antibacterial activity of some prototrophs were studied.

INTRODUCTION

The possibility of obtaining hybrids or heterozygous diploids in various species of imperfect fungi was shown for the first time by Pontecorvo (1956) and others. This was extended to antibiotic-producing actinomycetes by Sermonti & Spada-Sermonti (1955, 1956) and by Sermonti (1957). Later the methods developed by these authors were used by Alikhanian & Mindlin (1957) for obtaining recombinants in *Actinomyces rimosus*. The aim of this investigation was to study the possibility of hybridization in *A. aureofaciens* so as to use it for the purposes of selection.

METHODS

Biochemical mutants were obtained after ultraviolet (u.v.) irradiation and ethyleneimine treatment of *Actinomyces aureofaciens*. At the very beginning of our studies we found that this organism differed greatly from other actinomycetes in the extreme specificity of the mutational process in a number of strains, such as 536, II, Bd, BMK, B-16. These strains of *A. aureofaciens* differed from each other in their origin, colonial morphology and antibiotic-producing properties. The biochemical mutants were isolated on the following media: corn steep liquor (N12) medium, containing (w/v) 0·5 % corn steep liquor, 0·4 % $(NH_4)_2HPO_4$, 0·2 % KH_2PO_4, 0·29 % $MgSO_4$, 0·1 % $CaCO_3$, 2 % starch, 2·0 % agar; and two modifications of this medium, one with the addition of yeast extract and another with the addition of yeast extract and casein hydrolysate. The data on selection of biochemical mutants in *A. aureofaciens* are presented in Table 1.

As is evident from Table 1 our data do not conform to the regularities of biochemical mutant formation described in the literature in that from all strains, independent of the nutrient medium used, the occurrence of arginine-requiring (*arg*) mutants only was in general observed (99 cases), and only in two cases other amino acid requiring mutants (histidine (*his*) and isoleucine + valine (*isl* + *val*)) were detected.

The occurrence of arginine-requiring mutants in almost all cases could not but direct our attention to study hybridization in *Actinomyces aureofaciens*. The prototrophs were obtained by the method described by Alikhanian & Mindlin (1957).

Table 1. *The frequency of isolation of biochemical mutants from different strains of* Actinomyces aureofaciens *treated with various mutagenic factors*

Strain	Mutagenic factor	Medium	No. of biochemical mutants	Nutritional requirements	No. of isolates examined
BMK	u.v. radiation	N12	21	Arginine	1300
		N12 + yeast extract	7	Arginine	—
		N12 + yeast extract and casein	5	Arginine	—
	Ethyleneimine	N12	0	—	1075
		N12 + yeast extract	0	—	—
		N12 + yeast extract and casein	0	—	—
Bd	u.v. radiation	N12	19	Arginine	1211
		N12 + yeast extract and casein	4	Arginine	—
	Ethyleneimine	N12	0	—	1200
		N12 + yeast extract	2	Arginine	—
		N12 + yeast extract and casein	1	Arginine	—
536	u.v. radiation	N12	5	Isoleucine + valine Arginine (4)	3319
		N12 + yeast extract	10	Arginine	—
		N12 + yeast extract and casein	0		
	Ethyleneimine	N12	4	Arginine	1273
		N12 + yeast extract	7	Arginine	—
		N12 + yeast extract and casein	1	Arginine	—
B-16	u.v. radiation	N12	6	Arginine	2006
		N12 + yeast extract	3	Arginine	—
		N12 + yeast extract and casein	4	Histidine (1) Arginine (3)	—
	Ethyleneimine	N12	0	—	1740
		N12 + yeast extract	0	—	—
		N12 + yeast extract and casein	0	—	—
II	u.v. radiation	N12	1	Arginine	3071
		N12 + yeast extract	0	—	—
		N12 + yeast extract and casein	0	—	—
	Ethyleneimine	N12	0	—	1600
		N12 + yeast extract	0	—	—
		N12 + yeast extract and casein	0	—	—

RESULTS

It was first necessary to demonstrate the possibility of recombination in *Actinomyces aureofaciens* in order that crosses might be made between all arginine-requiring mutants to explain their genetic nature. For this purpose we used the isoleucine + valine-requiring mutant, the histidine-requiring mutant, and a number of arginine-requiring mutants. The data on the investigation of combinations in the three groups are presented in Table 2.

Table 2. *Biochemical mutant combinations and the frequency of prototroph formation from these combinations*

Combination group	Biochemical combination	Prototroph frequency (%)	Frequency biochemical mutant reversions
I	*arg*-1 × (*isl* + *val*)	0·13	0
	arg-2 × (*isl* + *val*)	0·06	0
	arg-3 × (*isl* + *val*)	0·0009	0
	arg-4 × (*isl* + *val*)	3·3	0
	arg-5 × (*isl* + *val*)	0·0004	0
	arg-6 × (*isl* + *val*)	0·13	0
	arg-7 × (*isl* + *val*)	0·01	0
	arg-8 × (*isl* + *val*)	0·03	0
	arg-9 × (*isl* + *val*)	0·006	0
	arg-10 × (*isl* + *val*)	0·0	0
	arg-11 × (*isl* + *val*)	0·004	0
	arg-12 × (*isl* + *val*)	0·02	0
	arg-13 × (*isl* + *val*)	0·0025	0
	arg-14 × (*isl* + *val*)	0·0003	0
II	*arg*-2 × *his*	0·0015	0
	arg-7 × *his*	0·0007	0
III	*his* × (*isl* + *val*)	0·9	0

Biochemical mutants *arg*-1 to *arg*-5 were derived from strain Bd; *arg*-6 to *arg*-14 from strain BMK; (*isl* + *val*) from strain 536; *his* from strain B-16.

It should be pointed out that these crosses gave rise to a comparatively high frequency of prototrophs, reaching 3·3 % in some combinations. This was higher than the frequencies observed in crosses of *Actinomyces rimosus*.

The prototrophs, obtained from the various combinations, independent of the starting strains, belonged morphologically to the classical wild-type strain of *A. aureofaciens* as for example strain 536 type. Such prototrophs formed flat colonies of mouse-grey or dark-grey coloration with aerial mycelium and buff-yellow substrate mycelium. Some prototrophs were characterized by a different rate of sporulation but this cannot serve as a distinguishing feature. Prototrophs from combination *arg*-7 × *his* may be regarded as an exception, since they were characterized by dingy-beige coloured spores and low rate of sporulation.

In contrast, the biochemical mutants from which the prototrophs were derived differed greatly in colonial morphology from the wild-type strains. Thus, all arginine-requiring mutants were characterized by flat colonies, not highly folded or having radial lines, asporogenic, with no aerial mycelium or specific pigment (Plate 1). The histidine-requiring mutant formed light-grey, flat, compact colonies, elevated above

the agar surface and characterized by a low rate of sporulation and limited growth. The mutant requiring isoleucine and valine differed from the wild-type strains only by the production of colonies with poor sporulation and a broad asporogenic periphery.

Segregation patterns of prototrophs

The prototrophic recombinants gave rise to various types of segregants with differing frequencies. In order to study in detail the various segregation patterns we used prototrophs from eleven combinations, eight combinations of group I (*arg*-1 to

Table 3. *Distribution of prototrophs from combinations according to the segregation types*

Combination group	Biochemical mutant combination	Prototrophs studied	Segregation type
I	*arg*-1 × (*isl*+*val*)	No. 1	I
		No. 2	I
		No. 3	I
		No. 4	II
		No. 5	VI
	arg-2 × (*isl*+*val*)	No. 1	I
		No. 2	I
		No. 3	I
		No. 4	IV
		No. 5	VI
		No. 6	VI
		No. 7	VI
	arg-3 × (*isl*+*val*)	No. 1	I
		No. 2	II
		No. 3	III
	arg-4 × (*isl*+*val*)	No. 1	I
		No. 2	I
	arg-5 × (*isl*+*val*)	No. 1	I
		No. 2	I
		No. 3	II
	arg-6 × (*isl*+*val*)	No. 1	I
		No. 2	I
		No. 3	I
		No. 4	IV
		No. 5	IV
	arg-7 × (*isl*+*val*)	No. 1	I
		No. 2	I
		No. 3	V
	arg-8 × (*isl*+*val*)	No. 1	IV
II	*arg*-2 × *his*	No. 1	I
		No. 2	II
		No. 3	VI
		No. 4	VI
		No. 5	VI
	arg-7 × *his*	No. 1	I
		No. 2	IV
III	*his* × (*isl*+*val*)	No. 1	III
		No. 2	VI
		No. 3	VI
		No. 4	VI
		No. 5	VI

arg-8 × (*isl*+*val*) and all combinations of group II (*arg*-2 × *his*; *arg*-7 × *his*) and group III (*his* × (*isl*+*val*)). As a result of this study the segregation patterns which are summarized in Table 3 were observed.

In all, the behaviour of 41 prototrophs from eleven combinations were studied. As far as their segregation pattern is concerned, six types were observed which were produced to a varying extent from each combination.

Segregation of type I. When plated out, the prototrophs of this type resembled the starting prototrophs, but segregated one of the starting biochemical mutants, in this instance the arginine-requiring mutant, but only in very small amounts (0·02–0·7 %).

The arginine-requiring segregant in its turn segregated in the first and sometimes in the second generation a few prototrophic colonies (0·5 %), morphologically similar to the arginine-requiring mutant. Further segregation of such colonies was not observed. It should be noted that the arginine-requiring mutants never segregated prototrophic or other auxotrophic forms. Neither were reverse spontaneous mutations observed.

Segregation of type II. A considerably smaller number of the prototrophs segregated the other parental form, that is the form similar to the isoleucine + valine-requiring mutant. The amount of such segregants was 1·5 to 0·14 %.

The isoleucine + valine-requiring segregant in further generations segregated no new forms.

Segregation of type III. A single prototroph (one prototroph among 41 prototrophs studied) which segregated both parental biochemical mutants (1·4 to 0·1 %).

Segregation of type IV. In this type of segregation, the prototroph segregated morphologically changed prototrophs. The number of such prototrophs was not high (0·15 %). In most cases, asporogenic forms similar to the arginine-requiring mutant were observed. Sometimes solid colonies with black substrate mycelium and various degrees of sporulation were encountered.

Table 4. *Quantitative distribution of prototrophs according to the segregation types*

Segregation type	No. of prototrophs	Segregation type	No. of prototrophs
I	18	IV	5
II	4	V	1
III	2	VI	11

Segregation of type V. In the first generation prototrophs belonging to this type segregated only morphologically-changed prototrophs, while in the second generation segregation of arginine-requiring variants was also observed.

Segregation of type VI. Prototrophs of type VI proved to be stable and segregated no new forms in a number of generations. The quantitative distribution of all 41 prototrophs according to these six types of segregation is presented in Table 4. As is evident from Table 4, the prototrophs belonging to type I, as well as stable prototrophs were the most common.

It should be pointed out that prototrophs segregating according to different types were detected from the same combination. Thus, five prototrophs obtained from the combination *arg*-7 × (*isl*+*val*) segregated according to three segregation types. Three of them belonged to type I, one to type II, and one to type VI.

Antibacterial activity of prototrophs

110 prototrophic colonies were studied with respect to the amount of chlortetracycline produced. The antibacterial activity was estimated on submerged cultures by the colorimetric assay method after acid hydrolysis of the culture fluid. Biochemical mutants and the wild-type strains, from which they were derived, served as the controls. The activity of the biochemical mutants was never greater than 100 μg./ml., the activity of the arginine-requiring mutants in most cases being equal to zero, while the activity of the wild-type strains was 850–1000 μg./ml. The activity of all prototrophs was many times higher than that of the biochemical mutants and reached the activity of the starting strains. Sometimes the activity of all the prototrophs from one combination was higher than that of the starting strains by 5–20 %. Examples of the antibacterial activity of prototrophs from three combinations (*arg*-1 × (*isl* + *val*); *arg*-4 × (*isl* + *val*); *arg*-3 × (*isl* + *val*)) are presented in Table 5 and the corresponding diagram (Fig. 1).

The activity of prototrophs from combinations *arg*-2 × (*isl* + *val*); *arg*-7 × (*isl* + *val*); *arg*-8 × (*isl* + *val*); *arg*-5 × (*isl* + *val*), *arg*-7 × *his*; *his* × (*isl* + *val*) was no higher than that of any of the wild-type strains similar to the activity of prototrophs from combination *arg*-4 × (*isl* + *val*). Some prototrophs from combinations *arg*-6 × (*isl* + *val*) and *arg*-2 × (*isl* + *val*) were superior with respect to their activity as compared with the wild-type strains, resembling those prototrophs from combination *arg*-1 × (*isl* + *val*). Combination *arg*-3 × (*isl* + *val*) was the only one among the eleven combinations studied, which produced prototrophs always superior in their chlortetracycline activity to the wild-type strains.

Table 5. *Chlortetracycline activity of the wild-type strains, biochemical mutants and prototrophs from combinations arg*-4 × (*isl* + *val*), *arg*-1 × (*isl* + *val*) *and arg*-3 × (*isl* + *val*)

Strain	Activity (μg./ml.)	Strain	Activity (μg./ml.)
Bd	990	*arg*-1	0
536	850	*arg*-3	0
arg-4	0	(*isl* + *val*)	50
Prototrophs from combination *arg*-4 × (*isl* + *val*)			
No. 1	351	No. 6	316
No. 2	814	No. 7	582
No. 3	732	No. 8	632
No. 4	707	No. 9	420
No. 5	802	No. 10	772
Prototrophs from combination *arg*-1 × (*isl* + *val*)			
No. 4	678	No. 9	1086
No. 5	256	No. 10	1143
No. 6	884	No. 11	1062
No. 7	241	No. 15	326
No. 8	466	No. 17	391
Prototrophs from combination *arg*-3 × (*isl* + *val*)			
No. 1	1199	No. 9	1288
No. 2	1038	No. 11	1223
No. 3	1152	No. 14	1080
No. 5	1288	No. 16	1109
No. 7	1203	No. 17	996

It is interesting to note that in the combinations *arg*-4 × (*isl*+*val*), *arg*-1 × (*isl*+*val*) and *arg*-3 × (*isl*+*val*), all of which produced prototrophs differing in antibacterial activity, one and the same isoleucine + valine requiring mutant, derived from strain 536, was used. As the other crossing component in these combinations, one of the arginine-requiring mutants, *arg*-4, *arg*-3 or *arg*-1 derived from strain Bd, was used. These mutants differed neither in colonial morphology, nor in antibacterial activity. In addition to the arginine deficiency, each of these mutants is likely to possess specific physiological properties which determine the difference of the

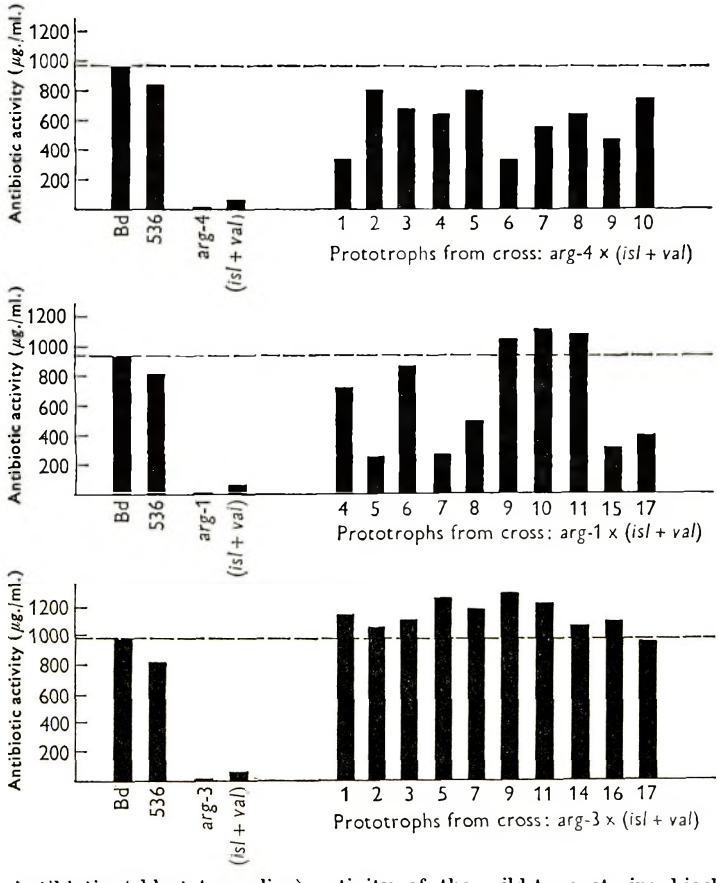

Fig. 1. Antibiotic (chlortetracycline) activity of the wild-type strains, biochemical mutants and prototrophs. The activity levels are presented in absolute values. The interrupted line indicates the highest activity level of the wild-type strains.

prototrophs from the above combinations with respect to their antibacterial activities.

One prototroph which proved to be the most active, that is prototroph no. 11 from combination *arg*-3 × (*isl*+*val*), was selected for studying the range of induced variation in comparison with the wild-type strains. Antibacterial activity of the prototrophic-segregants was also studied. Usually the activity of non-sporulating prototrophs similar in their morphological properties to the arginine-requiring

mutants was zero, while the activity of segregants, such as segregant no. 3 from combination *arg*-7 × (*isl* + *val*) reached 300 μg./ml. After these experiments, demonstrating recombinant formation in *Actinomyces aureofaciens*, combinations within the arginine-requiring mutants were studied.

In these combinations 56 arginine-requiring mutants of the 99 mutants isolated were used. All these strains were very similar in colonial morphology. They were characterized by asporogenic flat colonies, not highly folded nor having radial lines, and with no aerial mycelium or specific pigment.

Table 6. *Combinations of arginine-requiring mutants from which prototrophs were obtained*

No reversions of arginine-requiring strains were obtained

Combination	Biochemical mutant combination*	No. of prototrophs obtained
No. 24	*arg*-19 × *arg*-20	8
No. 25	*arg*-21 × *arg*-9	2
No. 52	*arg*-24 × *arg*-23	1
No. 1108	*arg*-15 × *arg*-1	1
No. 1073	*arg*-18 × *arg*-3	1
No. 1107	*arg*-15 × *arg*-16	1
No. 1176	*arg*-11 × *arg*-17	1
No. 958	*arg*-11 × *arg*-1	2
No. 1167	*arg*-7 × *arg*-22	1
No. 1155	*arg*-23 × *arg*-22	2
No. 306	*arg*-25 × *arg*-23	1

* Biochemical mutants *arg*-7; *arg*-9; *arg*-11; *arg*-15; *arg*-18; *arg*-19; *arg*-20; *arg*-21; *arg*-23; *arg*-24 were derived from strain BMK; *arg*-1; *arg*-3; *arg*-16; *arg*-17 and *arg*-25 from strain Bd; *arg*-22 from strain B-16.

The strains are very stable and segregate neither prototrophic, nor auxotrophic forms over a number of generations. An attempt was made to differentiate the mutants with respect to their requirements for intermediary products of arginine biosynthesis, that is for either glutamic acid, ornithine or citrulline; or according to an alternative way of synthesis, for urea or guanidine. It was found that all the arginine-requiring mutants isolated were similar in that the addition of any of the above intermediates to the medium did not restore the synthesis of arginine and permit growth. 1540 combinations of arginine-requiring mutants were studied. Prototrophs were obtained in eleven cases (Table 6). The frequency of prototrophs in these combinations is as low as 1 in 10^7.

The prototrophs obtained differed in colonial morphology from one another and from strain 536 typical of *Actinomyces aureofaciens*. Most of them formed well sporulating colonies of white to light-grey coloration, with sporophores in the form of spirals with 0·5–1·0 μ coils, which is characteristic of *A. aureofaciens*. Some prototrophs formed asporogenic colonies, morphologically similar to those produced by arginine-requiring mutants (Plate 1).

The study of these prototrophs with respect to their segregation pattern showed that the majority of them were stable and segregated no new forms in a number of generations. Only one prototroph, that is no. 1073, segregated a small amount (0·3 %) of non-sporulating prototrophs. Therefore we had no data, with respect to

direct segregation of the starting biochemical mutants, to indicate the deviation of these prototrophs by recombination. However, certain changes were found in the antibiotic-producing properties of these prototrophs as compared with the arginine requiring mutants (Table 7).

Table 7. *Antibacterial activity of the wild-type strains, arginine-requiring mutants, and prototrophs from combinations of these mutants*

Strain	Chlortetracycline activity (μg./ml.)	Strain	Chlortetracycline activity (μg./ml.)
	Starting strains		
BMK	950	B-16	1600
Bd	990		
	Arginine-requiring mutants		
arg-24	0	*arg*-7	20
arg-23	80	*arg*-22	0
arg-15	0	Prototrophs from	
arg-1	80	*arg*-23 × *arg*-24	0
arg-18	0	*arg*-1 × *arg*-15	0
arg-3	0	*arg*-18 × *arg*-3	0
arg-11	20	*arg*-11 × *arg*-17	0
arg-17	16	*arg*-7 × *arg*-22	0

DISCUSSION

As is evident from the data presented in Table 6, 16 arginine-requiring mutants out of 56, on crossing in definite combinations, produced the parental form, that is gave rise to prototrophs. It is possible to assume that they belong to different alleles, though probably not to different genes. All prototrophs from combinations of arginine-requiring mutants independent of the activity of the wild-type strains were unable to synthesize chlortetracycline. This was not observed in the other crossings of *Actinomyces aureofaciens* involving mutants having dissimilar biochemical requirements and many crossings of *A. rimosus*. When *A. aureofaciens* mutants with different nutritional requirements were crossed, even when the antibacterial activity of these mutants was either zero or as low as 50μg./ml, the prototrophs obtained were similar in their activity to the wild-type strains. On the basis of these data it is possible to assume that the arginine-producing locus is related to the chlortetracycline-producing locus. This assumption is confirmed by the specificity of biochemical mutant formation in *A. aureofaciens*, as well as by the fact that active hybrids are formed only in combinations, where one of the crossing components is a biochemical mutant requiring histidine or isoleucine + valine. It should be noted that the data presented are preliminary, and further studies on arginine-requiring mutants are needed.

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EXPLANATION OF PLATE

Colonies of arginine-requiring mutants and of prototrophs obtained from combinations of arginine-requiring mutants. Grown on medium N12 for 8 days.

Arginine-requiring mutants

Prototrophs obtained from combinations of arginine-requiring mutants

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The Habitat and Description of a New Genus of Sulphur Bacterium

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SUMMARY

A new species and genus of colourless sulphur bacteria, for which the name *Thiodendron mucosum* is proposed, is described from two habitats in Florida. Its morphology and physiology relate it to the Beggiatoaceae, but it is branching, non-filamentous and non-motile so it should not be included in that family and a new family should be created for it. Some characteristics of its environment are discussed and also the organisms common to such an environment. It is probable that the particular organisms are closely connected to the metabolism of sulphur, while their variety and biomass attribute an important role to sulphur in nature, especially in environments containing little or no oxygen.

INTRODUCTION

In January 1956 a collection of sulphur bacteria was made at Warm Mineral Spring near Venice in south-west Florida, U.S.A. One of these was considered to be new. Dr E. G. Pringsheim of the Botanische Austatem, The University of Göttingen, Germany, was visiting the laboratory at the time and he concurred in this view. Since that date the organism has been studied intensively, and has been found at only one other location, despite diligent search.

METHODS

The work reported here consisted of making field collections from sulphur springs and other environments, and studying the organisms in the laboratory under high magnifications. Physicochemical characteristics of the waters were also determined where possible, and some staining was done. The organisms stained very unevenly by Gram's method, overstained intensely with carbol-fuchsin and did not take up methylene blue. Apparently the large amount of jelly-like material interferes greatly with the stains used. Many laboratory culture methods were tried, but since none has been successful thus far, they are not described here.

The State of Florida, U.S.A., has seventeen first-magnitude springs (Ferguson, Lingham, Love & Vernon, 1947), as well as vast numbers of smaller ones. Many of these are sulphur springs, giving off H_2S and supporting white growths of sulphur bacteria, principally Beggiatoa and Thiothrix. These growths appear as a white coating on the spring bottom and sides and often extend far down the run. All the springs are constant in their characteristics, although differing sharply among themselves; for this reason Odum (1957) referred to them as excellent laboratories. Most

of the sulphur springs show a single dominant species of sulphur bacteria with others in smaller numbers. The white coating is due to the dominant organism. Table 1 lists the sulphur-accumulating bacteria belonging to the families Beggiatoaceae and Thiobacteriaceae which were abundant at some of the eleven stations studied.

Warm Mineral Spring is an exception to the single dominant condition because its huge population consists principally of six species of *Beggiatoa* and five species of *Thiothrix* and because dominance shifts. It is also the only spring whose salt content is so high; Table 2 gives its physicochemical characteristics. Its temperature gradient suggests that the water comes from Ocala limestone at a depth of about 800 ft., and Dr A. P. Brooks (private communication) suggested that the water is 50 % connate sea water and 50 % fresh water. There is virtually no fluctuation in any characteristic, except that of light, and this only to a depth of about 65 ft. The water is very clear in the early morning, but after sunrise it becomes cloudy because of a photochemical precipitation of sulphur. Sulphur granules are easily found free in the sand and water; this photochemical mechanism was not mentioned by Ivanov (1957) in addition to his account of the precipitation of sulphur by bacteria, although indeed he was working principally with sulphur in underground formations.

Table 1. *Occurrence of colourless sulphur-accumulating bacteria at eleven locations*

All these bacteria were identified during the years 1956–60 by direct microscopic examination, except *Thiobacillus denitrificans* which was cultured. The identification of *Thiobacterium bovista* might be questionable.

Locations

1. Warm Mineral Spring, Fla. Half salt.
2. Orange Springs, Fla. Fresh.
3. Welaka Mud Springs, Fla. Fresh.
4. Sarasota, Fla., Ringling Fountain. Fresh.
5. Green Cove Springs, Fla. Fresh.
6. Fort Myers, Fla. Fresh.
7. Sanibel Island, Fla. Fresh.
8. Hampton Springs, Fla. Fresh.
9. Lake Alice, Gainesville, Fla. Fresh.
10. Titusville, Fla., Inland Waterway. Salt.
11. Woods Hole, Mass., Salt Marshes.

	Location										
	1	2	3	4	5	6	7	8	9	10	11
Achromatium oxaliferum	X	.	.	.	.	.	.	.	X	.	X
A. volutans	X	.	.	.	.	.	.	.	.	X	X
Beggiatoa alba	X	X	X	X	X	.	X	X	X	X	X
B. arachnoidea	X	.	X	.	X	.	.	.	X	X	X
B. gigantea	X	.	.	.	.	.	.	.	.	X	X
B. leptomitiformis	X	.	X	.	X	.	X	X	X	.	X
B. minima	X	.	.	.	.	.	.	.	X	.	X
B. mirabilis	X	.	.	.	.	.	.	.	.	X	X
Thiodendron mucosum n.sp.	X	.	.	.	.	.	.	.	.	X	.
Thiospirillopsis floridana	X	.	.	.	.	.	.	.	.	.	.
Thiothrix annulata	.	.	.	.	.	.	.	.	X	.	X
T. marina	X	.	.	.	.	.	.	.	.	X	X
T. nivea	X	.	X	.	X	X	X	X	X	.	X
T. tenuis	X	.	X	X	X	.	.	X	X	.	.
T. tenuissima	.	.	.	.	.	.	.	.	X	.	X
Macromonas bipunctata	X	.	.	.	.	.	.	.	.	X	X
M. mobilis	.	.	.	.	.	.	.	.	X	.	X
Thiobacillus dentrificans	X	X	X	.	.	.	.	X	X	.	X
Thiobacterium bovista?	X	.	.	.	.	.	.	.	.	.	X
Thiospira winogradskyi	X	.	.	.	.	.	.	.	.	X	X
T. bipunctata	X	.	.	.	.	.	.	.	X	X	X
Thiovulum majus	X	.	.	.	.	X	.	X	X	X	X

X = present

Table 2. *Physicochemical characteristics of Warm Mineral Spring*

	Ferguson *et al.* (1947)	Morgan (1956)
Dissolved solids	17,812 p.p.m.	17,988 p.p.m.
Iron	0·12 p.p.m.	0·09 p.p.m.
Calcium	766 p.p.m.	596 p.p.m.
Magnesium	471 p.p.m.	567 p.p.m.
Silica (SiO_2)	18·00 p.p.m.	23·80 p.p.m.
Sodium, potassium	5,124 p.p.m.	.
Chloride	9,350 p.p.m.	.
Bicarbonate (HCO_3)	.	167 p.p.m.
Total hardness ($CaCO_3$)	3,846 p.p.m.	.
Temperature	.	84° F. ±2°
pH value	.	7·2±0·2
Volatile solids (600° for 30 min.)	.	17·1 %
Nitrate	.	0·05 p.p.m.
Dissolved PO_4	.	0·0016 p.p.m.
Total PO_4	.	0·0037 p.p.m.
C.O.D. (dichromate)	.	813 p.p.m.
Dissolved oxygen*	.	0·0 p.p.m.
H_2S	.	0·162 p.p.m.
HS	.	0·078 p.p.m.
SO_4	.	1704 p.p.m.

* Odum says that interference accounts for this value; if the interference is removed, a value of 1·0 is obtained.

RESULTS

Occurrence of the new bacterium

The first specimens of the new organism were found in January 1956. Collections of the dense growths of sulphur bacteria were brought to the laboratory for studies of the widths of the various types. Among these were arboroid colonies densely packed with what appeared to be sulphur granules. Most of these were attached to the blue-green alga Lyngbya. Some of the colonies had a spread of over 2 mm. They were examined alive, with a Zeiss Optovar microscope at magnifications of 100–1600 diameters. Some were set aside in small moist chambers (Petri dishes containing filter paper saturated with salt water) for subsequent examination. It was concluded that the new organisms represented a free-living sulphur bacterium of a new type. A second trip to the spring 3 weeks later yielded no specimens, but it has since been found many times in this spring, and as far as half a mile down the run. Many other sulphur springs in Florida have been investigated without finding the new organism nor was it found in H_2S-containing water at La Jolla, California, at Woods Hole, Massachusetts, in the desert around Las Vegas, Nevada, and in aquaria containing good natural growths of Beggiatoaceae in the Narragansett Marine Laboratory, Rhode Island. However, in February 1959 a heavy coating of sulphur bacteria was found in shallow water along the intracoastal waterway at Titusville, Florida. Examination revealed many colonies of the new bacterium, and it has been found at all subsequent visits to this location. These sulphur bacteria (Beggiatoales) were in a grossly polluted situation behind a crab-meat packing plant. There should be no pollution at all in Warm Mineral Spring (now being exploited as a spa), but it should be noted that the volatile solids are very high for such a situation.

Description of the organism

This description is based primarily upon microscopic examination of unkilled, unfixed material. Two small blobs of viscous grease were put on a slide, a drop of water containing the organism was added between the bits of grease, and a no. 1 thickness cover glass was added. This permitted brightfield and darkfield examination at magnifications up to 1600 diameters.

Fig. 1. Drawing of a portion of a living colony of *Thiodendron mucosum* to show distribution of sulphur and cells within the mucus. × 1600.

The organism, which will be referred to as Thiodendron, consists of rod-shaped bacteria 3–9 μ long and 1·0–2·5 μ in diameter, forming dendroid colonies up to 3000 μ across, in a jelly-like matrix (Pl. 1, figs. 1, 2, 4; Fig. 3). In this jelly, the individual organisms are distributed at random, but tend to be most numerous near the ends of the colony. Most of the organisms are of similar size (3–5 μ long × 1·5 μ diameter), but often the organisms at the swollen ends of the branches are three times this size (Fig. 3). These large forms appear to be typical of older colonies, because quite young colonies, although they may have swollen tips, normally

exhibit only smaller forms. The smaller forms usually have 3 to 8 sulphur granules which vary in size, but which are generally arranged along the linear axis. The organisms in the larger tips may have as many as 20 sulphur granules which vary in size, but which are generally arranged along the linear axis, rather crowded, and when a tip is crowded with organisms it is much darker than the distal part of the colony (Fig. 3).

Evidence that the granules are indeed sulphur is based upon the type of the inclusion, round bodies with uniform black edges (the 'droplets' of Winogradsky), and upon the recovery of sulphur on evaporating extracts of the colonies with carbon disulphide. The colony does not show branching until it is at least several cell-lengths long. While there is no definite pattern for the arrangement of the bacteria within the common jelly, most of the bacteria have their long axis parallel to the filament axis.

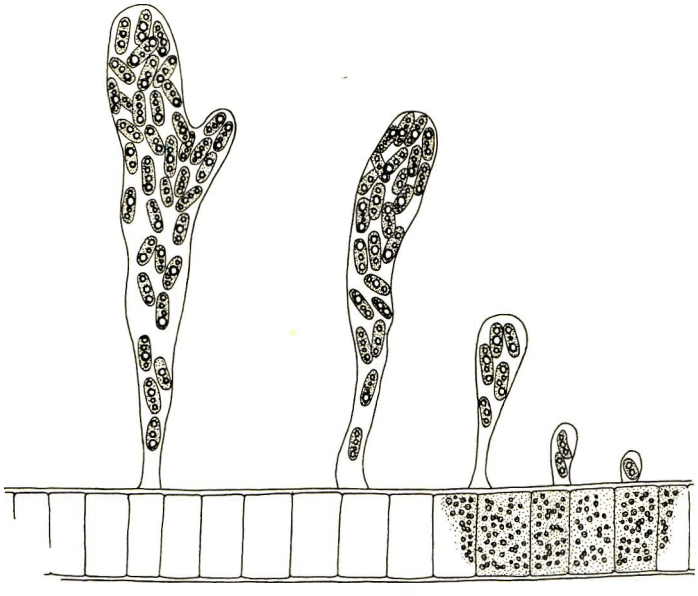

Fig. 2. Drawing of five young living colonies of *Thiodendron mucosum* attached to a filament of Lyngbya. × 1600.

Outlines of the bacterial forms are very difficult to see. In fact, their shape was first suggested by the linear clumps of sulphur granules (Fig. 1). Their internal make-up is quite homogeneous, with very nearly the same optical density as the jelly. One gets the impression of branching jelly crowded with sulphur granules. However, the bases of the mucous fingers are frequently almost devoid of bacteria. Also, when colonies are left under a cover glass in a moist chamber, after 24 hr. they begin to lose their sulphur, and pale ghosts of the bacteria become visible. Sometimes bacterial forms showing a constriction may be seen, as if they were undergoing binary fission. The bacterial forms stain very poorly, if at all, by Gram's method. Acids cause swelling and disintegration of the mucilage, and distort it. The bacterial forms are not coloured by iodine.

Older colonies are quite arboroid in habit (Pl. 1, figs. 1, 2, 4). Superficially they resemble *Zooglea ramigera*. A given colony tends toward uniformity in the size of its club-shaped parts (Fig. 3). Colonies over 2 mm. across have been found on many occasions. No holdfasts have ever been detected, but the attachment is strong. A colony brushed by a rotifer or ciliate bends, but is not detached. Usually attachment is to a filament of Lyngbya (Fig. 2), but occasionally it is to some other surface. Plate 1, fig. 2, shows a colony attached to the stalk of a colonial vorticellid. Colonies

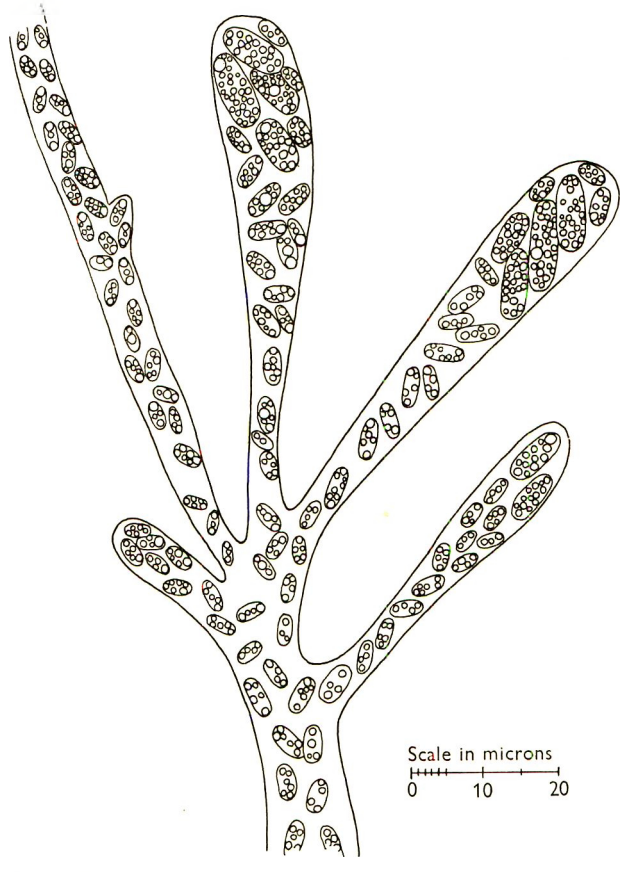

Fig. 3. Portion of a mature living colony of *Thiodendron mucosum* to show the larger bacterial forms near the swollen ends of branches. × 1600.

evidently start from a single motile organism which settles down and grows out. Colonies with as few as three bacterial forms (Fig. 2) may be seen, already becoming club-shaped. Bacterial forms leaving the jelly of a colony have not been seen.

The organism has not been successfully cultivated in the laboratory. Colonies remain intact, but with no growth for several days, in a moist chamber. During this time, the sulphur granules gradually disappear. The temperature of Warm Mineral Spring is a uniform 84° F. (29° C.), and that of the intracoastal waterway at a time when the organism was taken there was 55° F. (13° C.).

Colonies have been kept apparently thriving for 3 months at 72° F. (20° C.) in

jars of Warm Mineral Spring water, but colonies picked out and transferred to various liquid culture media invariably died within a few days. No increase in visible sulphur content, nor in viability resulted when such colonies were exposed to 0·3–3·0 % of hydrogen sulphide. This species did not show an ability to oxidize hydrogen sulphide under the conditions in the laboratory, being different from Sphaerotilus in this respect, as reported by Skerman, Dementjeva & Carey (1957), and by Waitz & Lackey (1959). Scotten (1951) also reported that Beggiatoa did well when small amounts of hydrogen sulphide were present. More recently Faust & Wolfe (1961) secured pure cultures of Beggiatoa without adding hydrogen sulphide, and we normally obtain massive cultures of Beggiatoa with other organisms in the same manner. However, every time the organism Thiodendron has been taken, enough H_2S was present in its environment to be detectable by its odour. Hydrogen sulphide is present constantly in the Warm Mineral Spring. Sulphide determinations show amounts varying around 0·1 p.p.m.; calculations show H_2S at about 0·16 p.p.m., and HS about 0·08 p.p.m. These are manifestly considerably below limiting factors for many organisms, certainly for Thiodendron.

The organism Thiodendron is another addition to the long list of parallel forms found in the lower plants. The organism essentially is a collection of rod-shaped bacterial forms in a common jelly, apparently not organically connected to each other. The same is true of *Zooglea ramigera*, whose cells are smaller and without sulphur. Even the dendroid habits of the two organisms are similar. Except for the branching of the jelly, almost the same comparison could be made with the blue-green algae Aphanothece and Bacillosiphon. Pringsheim (1949) discussed possible relationships between bacteria and Myxophyceae, and this new organism adds material for that discussion.

Taxonomic position of Thiodendron

The organism occurs in sufficient abundance and is sufficiently distinctive to represent a valid species. Its resemblance to Zooglea is superficial, and the latter never accumulates sulphur, although a number of experiments has been made in attempts to induce this. The new organism does not accumulate iron in its sheath and does not resemble other jelly-inhabiting bacteria such as Nevskia or Leuconostoc. Devidé (1952) described as new colourless sulphur-accumulating bacteria, Thioglea, which live within jelly, but these are non-branching, oval or elongate masses. They would seem to be the closest relatives of Thiodendron even to the detail that cell outlines are difficult to make out. However, Devide' thinks they might belong to the Achromatiaceae, which Thiodendron obviously does not.

The accumulation of sulphur granules within the cell is the most striking characteristic of Thiodendron. This is true of Beggiatoa, Thiothrix, Thioploca and Thiospirillopsis. Thiothrix is attached; the others move about freely. The cells of each of these (if colonial; Thiospirillopsis is not) are in organic apposition, whereas Thiodendron cells may be far removed from each other, in older colonies. The family Beggiatoaceae, then, shows considerable variation among its respective genera, and this new genus is certainly a related one. In Achromatium there are free sulphur granules, but also large calcium carbonate inclusions and the cells are without jelly and freely motile. It does not seem that Thiodendron is closely related to this genus. Neither does it seem related to the families Vitreoscillaceae and Leucotrichaceae.

The only other possible relative seems to be *Thiobacterium bovista* of the family Thiobacteriaceae. This species is rod-shaped, about the same size and is described as having one to four sulphur granules within each cell. Furthermore it is colonial, but the colonies are bladder-like. Thiodendron in some respects is quite similar. But it has few similarities to Thiospira or Thiobacillus, the other members of the family Thiobacteriaceae.

Thiodendron never shows colour and cannot be mistaken for a blue-green alga or any of the Thiorhodaceae. It is therefore considered a new genus and species for which the name *Thiodendron mucosum* is proposed. This is from the Greek nouns thion, sulphur, and dendron, tree (a sulphur tree) and the Latin adjective mucosus, slimy. Thiodendron is neuter. Pringsheim, when he saw the organism, suggested Thiobrachys for it, but this proved to be etymologically unacceptable. The taxonomic position of many of the sulphur bacteria seems a matter of diverse opinion, as shown by Bisset & Grace (1954). This one seems completely unrelated morphologically to many Beggiatoales and to the Achromatiaceae; in addition, no movement of the bacterial forms within the jelly, or of the colony as a whole, has been observed. Its lack of colour is enough to exclude it from the Thiorhodaceae or the Athiorhodaceae, even though its sulphur relationships are still not understood. It seems preferable to place it in the order Beggiatoales despite its apparent lack of motility in vegetative cells. However, a new family, Thiodendraceae, is proposed and it is hoped that the organism may become better known.

Habitat and associations

The occurrence of a unique organism in a unique environment usually brings the question 'What factors are responsible for its presence?' It was at first thought that the half sea-water concentration, H_2S without pollution, and the temperature in Warm Mineral Spring were responsible for the occurrence of Thiodendron, but then it was found at Titusville. No analysis of the water there is available, but its salinity was found to be about 17,000 p.p.m. The water temperature at the time of collection was 55° F. (12·5° C.) and there were ample visual evidences of organic pollution. In June 1959 Pagosa Hot Spring in Colorado was examined; this spring is hot to warm, and has a high salt content. *Beggiatoa* and *Thiothrix* species occurred there, but no Thiodendron in the samples taken. At present physicochemical factors do not explain its occurrence.

However, Warm Mineral Spring has a unique flora and fauna; it seems ideal for sulphur bacteria. All species of *Beggiatoa* are abundant; *B. gigantea*, which has not been recorded from fresh water and which we have not found elsewhere in dense aggregates, may be scooped up there in great handfuls. Symploca has not been found, but many other groups are (see Table 3). *Thiospirillopsis floridanus*, described by Uphof (1927) from Welaka, Florida, 200 miles north, has been found once in some thirty trips to Warm Mineral Spring. It is thus recorded now (the second record?) from brackish as well as fresh water.

In Warm Mineral Springs the blue-green algae are unusual, so are the diatoms, green algae and flagellates; few filamentous green algae occur and only an occasional green euglenid has been taken. The dominant green flagellate is *Trentonia flagellata*, which apparently had not been seen until the present work since it was described by Stokes (1886); Hollande (1952) called it a synonym of Vacuolaria, but the original

description is valid. Incidentally, it was also a dominant near the bottom of Green Hill Pond, a Rhode Island salt pond of low salinity, in July 1960. The rhizopods are few, but include two Foraminifera. The ciliata are mostly salt-water types, and include several undescribed species. Little work has been done with other organisms, but some flatworms are new and a new nereid was described by Hartman (1959), while two of the three microcrustacea are species which Dr H. C. Yeatman (personal communication, 1959) believes to be distinct undescribed species. Many other groups are represented by few species, or are absent. The whole picture is of a very unusual environment, which has produced unusual organisms.

Table 3. *The number of genera or species of various organism groups found at Warm Mineral Spring*

About 5 % have not been identified and there are some which are new. All identifications were made upon living organisms by direct microscopic examination

Sulphur bacteria	34	Dinoflagellata	6
Blue-green algae	27	Bacillarieae (Diatoms)	20
Green algae and Charales	6	Zooflagellata	24
Euglenida	24	Rhizopoda	26
Chloromonadida	1	Ciliata	99
Cryptomonadida	3	Metazoa	32

DISCUSSION

It seems reasonable to suppose that an organism is a product of its environment; on this score a highly specialized environment such as Warm Mineral Spring might reasonably be supposed to have the very special or rare organisms such as Thiodendron appears to be. Factors common to Titusville and Warm Mineral Spring appear to include degree of salinity, presence of H_2S and some few related characteristics. But the environment at Titusville is not constant, so there the comparison ends. Thiodendron is such a striking organism that were it not greatly restricted in occurrence it would have been reported long before this.

In this case, the particular organisms present in abundance, and constantly, emphasize the importance of sulphur, especially hydrogen sulphide, in the energy relationships of a given locale. These energy relationships may be expressed by the formula: $H_2S \rightarrow S + 2H + 2e$. The free energy liberated by this reaction is $-7{\cdot}89$ Cal. Lamanna & Malette (1953) state that the Beggiatoaceae use as an energy source the oxidation of H_2S. Since H_2S oxidizes readily in the presence of minute quantities of metals, Warm Minerals Spring with a flow of about 9,000,000 gallons daily, and an H_2S content of 0·162 mg./l. provides a large source of energy to those microorganisms able to obtain it. Some of the organisms found in Warm Mineral Spring are ubiquitous; others are characteristic of an environment containing H_2S. When a jar containing a large variety of Warm Mineral Spring organisms is tightly closed, most of the organisms die within 24 hr. Table 4 gives a list of the resistant organisms.

There are many organisms which use soluble sulphur directly in their metabolism. But there are also many which ingest either the free sulphur granules, or sulphur bacteria. With some ciliates, ingested bacteria may be clearly observed in food vacuoles. Frontonia ingests some of the smaller Beggiatoales; others ingest Thiovulum, Chromatium or *Macromonas bipunctata*. This ingestion suggests a mechanism for the transfer of S from H_2S to the soil as elementary sulphur. Beggiatoa (and

similar bacteria, perhaps including Thiodendron) oxidize H_2S to S: this may be oxidized in turn to H_2SO_4, but in an alkaline medium (the sea, Warm Mineral Spring) the acid is neutralized. When the bacteria are ingested, there may be a direct deposition of their contained elementary S in the soil. Indeed, free S is readily found in bottom deposits at Warm Mineral Spring. The water there contains adequate carbonates, and at least 0·05 p.p.m. NO_3, also CO_2, so that respiration is no problem, yet dissolved oxygen remains low. There is a high organic content in Warm Mineral Spring water which evidently serves as food for the micro-organisms. Considerable quantities of Chara and four genera of blue-green algae, *Chroococcus*, *Gomphosphaeria*,

Table 4. *Organisms alive after* 48 *hr. saturation of Warm Mineral Spring water with hydrogen sulphide*

All identifications based upon direct microscopic examination.

1. *Beggiatoa alba*
2. *B. arachnoidea*
3. *B. gigantea*
4. *B. leptomitiformis*
5. *B. minima*
6. *B. mirabilis*
7. *Chromatium* spp.
8. *Chroococcus turgidus*
9. *Gomphosphaeria aponina*
10. *Lyngbya* sp.
11. *Oscillatoria* spp.
12. *Spirulina major*
13. *S. princeps*
14. *Amphiprora* sp.
15. *Amphora ovalis*
16. *Biddulphia* sp.
17. *Navicula* spp.
18. *Petalomonas* sp.
19. *Gymnodinium* sp.
20. *Cyathomonas truncata*
21. *Bicoeca lacustris*
22. *Bodo reniformis*
23. *Monas vulgaris*
24. *Spirochaeta* spp.
25. *Amoeba* sp.
26. *Cryptopharynx* sp.
27. *Cyclidium* spp.
28. *Epalxis exiguua*
29. *Metopus es*
30. *M. intercedens*
31. *M. vestitus*
32. *Parablepharisma pellitum*

Aphanocapsa and *Oscillatoria* grow in the shallow areas, but can hardly act as primary producers. Instead, the sulphur bacteria whose biomass is much greater probably use soluble organic matter of unknown nature and become primary producers. We have recently found that ^{14}C is taken up from urea and glucose present in a medium containing living Thiothrix. The low dissolved oxygen in Warm Mineral Spring and its run is easily compensated for by the presence of oxidized sulphur and oxygen-containing organic compounds as long as there is biological action which is easily demonstrated.

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Fig. 1

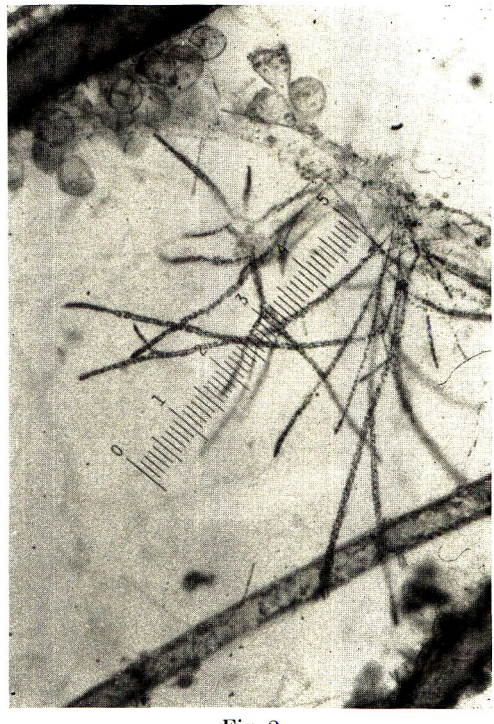

Fig. 2

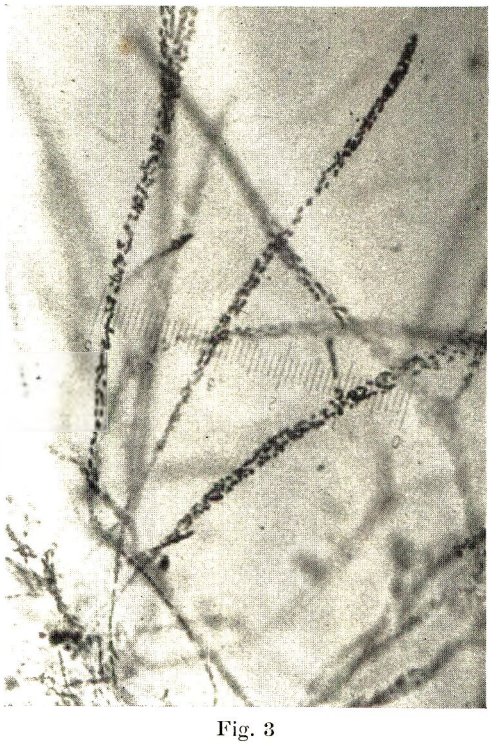

Fig. 3

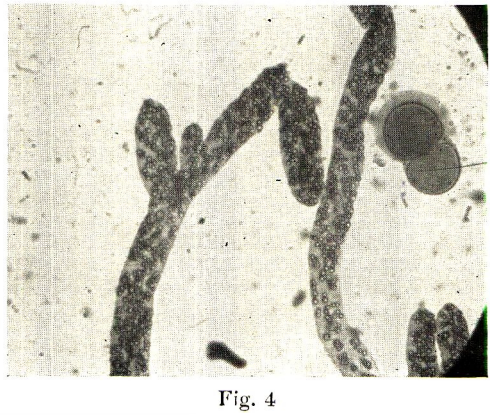

Fig. 4

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EXPLANATION OF PLATE

Fig. 1. Portion of a mature colony of *Thiodendron mucosum* to show branching. Colony stained while living with aqueous safranin. ×400. Photographed with Kodak 682C, $\frac{1}{20}$ sec., at f. 4.5.

Fig. 2. Photograph of an old colony of *Thiodendron mucosum* attached to the stalk of a colonial vorticellid. ×400. Photographed with Zeiss Contaflex IV using Dupont SX-Pan film, ASA-4000, $\frac{1}{250}$ sec. at f. 2.8.

Fig. 3. Portion of a mature colony of *Thiodendron mucosum* to show the distribution of sulphur in the colony fingers. Note the frequent linear aggregates, which indicate the long axes of unstained individual cells. The black pointer at the left ends on a *Chroococcus turgidus* colony (Blue green alga). ×750. Photographed with Kodak 682C, $\frac{1}{20}$ sec., at 4.5.

Fig. 4. Photograph of a mature living colony of *Thiodendron mucosum*, to show its arboroid appearance. ×400. Photographed with Speed Graphic, Century, $2\frac{1}{4} \times 3\frac{1}{4}$ using Ansco Super Hypan film, $\frac{1}{25}$ sec., at f. 4.5.

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Torulopsis castellii sp.nov., a Yeast Isolated from a Finnish Soil

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(*Received* 10 *January* 1961)

SUMMARY

A new species of *Torulopsis* is described; it was isolated from a Finnish soil. This species is named *T. castellii* in honour of Professor Tommaso Castelli of the Agricultural University of Perugia, Italy.

INTRODUCTION

In a microbiological study of 32 samples of Finnish soil taken in August 1957, 22 species of yeasts were isolated; 15 of them were referred to an already described species, while 7 had to be considered as new species or varieties. The present paper describes a new species belonging to the genus *Torulopsis* for which the author proposes the name *Torulopsis castellii* in honour of Professor Tommaso Castelli, Director of the Istituto di Microbiologia Agraria e Tecnica of Perugia University, Italy.

METHODS

Origin of strains. Seven strains were studied, all isolated from a soil sample from Hyytiala in the Province of Tavastia Australis, Finland. The soil was from a pine bog with underbrush taken at a depth of 2–10 cm. It was tan coloured, humiferous, without $CaCO_3$ and at pH 3·7. The methods used to study the characters of the yeasts were those of Lodder & Kreger-van Rij (1952). In examining the utilization of sugars, the boiled and washed agar method described by Capriotti (1955) was used.

RESULTS

Growth in grape must. After 3 days at 25°, the cells oval; 1·8–3·5 × 2·2–4 μ; single or in pairs or in chains of 3–10 cells. After 1 month at 17°: a thin ring, more or less limpid liquid, and sediment well developed.

Growth in malt extract. After 3 days at 25°, the cells oval; 2–3·5 × 2·3–4·3 μ; single or in pairs or in chains of 7–16 cells. After 1 month at 17°: a trace of ring, limpid liquid, and sediment well developed (Fig. 1).

Growth on malt agar. After 3 days at 25°, the cells oval, elliptical to long narrow 1–3·5 × 2–5 μ, single or in pairs. Streak culture white gray, glistening, smooth, flat, waxy, rather thin, with smooth margin. After one month at 17°, streak culture white-yellowish, glistening, smooth, rather flat, not well developed, with smooth margin.

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Grape must+gelatine stab. After 60 days, at 18–20°, superficial growth only of colony, white-ochraceous, expanded, flat; gas bubbles formed; no liquefaction.

Sporulation. Ascospores not formed.

Giant colony on grape must+gelatine. After 1 month at 18°, the giant colony appeared round with large and smooth cavity, and slightly lobate crown.

Pseudomycelium. In slide culture, pseudomycelium not formed.

Fermentation. Glucose well fermented; galactose, maltose, sucrose, lactose, trehalose, raffinose, inulin, dextrin not fermented.

Assimilation of carbon compounds. Glucose, trehalose (slowly), D-ribose (very slowly), glycerol well assimilated. Not assimilated: galactose, maltose, sucrose, raffinose, lactose, L-sorbose, cellobiose, melibiose, melezitose, inulin, soluble starch, D-xylose, L-arabinose, D-arabinose, L-rhamnose, D-glucosamine HCl, i-erythritol, adonitol, mannitol, D-sorbitol, α-methylglucoside, salicin, potassium-D-gluconate, potassium-5-keto-D-gluconate, potassium sodium-saccharate, pyruvic acid, D-lactic acid, succinic acid, citric acid, ethyl acetoacetate, i-inositol.

Assimilation of nitrogen compounds. Ammonium sulphate assimilated; potassium nitrate not assimilated.

Splitting of arbutin. Negative.

Fermentative power in grape must. Ethanol produced 5·3–7·8 % (v/v).

DISCUSSION

The isolated strains are referred to the genus *Torulopsis* because the cells are oval and form no spores, pseudomycelium or carotinoid pigments. Lodder & Kreger-van Rij (1952) reported in the genus *Torulopsis* only one species with good fermentation and assimilation of glucose, namely, *T. glabrata* (Anderson) Lodder & De Vries, which was originally isolated by Anderson from human faeces, with the name of *Cryptococcus glabratus*. However, this species also fermented trehalose. More recently other species have been described which ferment only glucose; but these species are different from *T. castellii* in the following characters: *T. pintolopesii* Van Uden (1952) assimilates only glucose, does not assimilate glycerol, D-ribose or trehalose; does not grow at 24°; *T. nitratophila* Shifrine & Phaff (1956) ferments glucose (latent); assimilates glucose, galactose, sorbose, trehalose, xylose, L-arabinose, D-arabinose, mannitol, sorbitol, adonitol, glycerol; does not split arbutin; *T. wickerhamii* Capriotti (1958) ferments glucose well; assimilates glucose, galactose, potassium nitrate; splits arbutin.

Two strains of *Torulopsis castellii* sp.nov. have been sent to the Centraalbureau voor Schimmelcultures, Delft, Netherlands; 7 strains have been put in the yeast collection of Istituto di Microbiologia Agraria e Tecnica dell'Università di Perugia, Italy.

TORULOPSIS CASTELLII SP.NOV.

Maltato in musto cellulae ovales 2–3·5 × 2·3–4·3 μ, singulae, binae. Ruente mense (17°) sedimen, medium nitidum et annulum.

Maltato in agaro trium dierum (25°) cellulae ovatae, ellipticae etiam angustae 1–3·5 × 2–5 μ, singulae aut binae. Elapso mense fit patina albaflava, cerea, lucida, laevigata, non copiosa. Nulla sporificatio observatur. Pseudomycelium deest.

Glucosum fermentatur. Assimilantur glucosum, glycerina, trehalosus (lente) et D-ribosum (lente). Nitras kalicus non assimilatur.

Minerali in medio cum alcohole aetilico ut unum carbonii pabulum, non augescit Alcohole aetilicus gignit 5·3–7·8 %. Arbutinum non finditur. E terra Finnica se.-junta fuit. Typus: cultura 211 iacens in C.B.S., Delft, Holland.

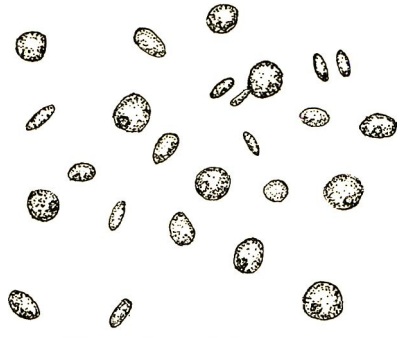

Fig. 1. *Torulopsis castellii* nov.spec. (×700) (after 3 days on malt extract).

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A Growth Medium without Blood Cells for *Pasteurella tularensis*

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SUMMARY

A modification of glucose cysteine blood agar (GCBA) is described for the cultivation of *Pasteurella tularensis* in which the blood is replaced by plasma and catalase. It has the advantage for colony counting of being clear, and is at least as good as GCBA for enumerating fastidious aged *P. tularensis* suspensions and aerosols. The liquid medium supports the growth of very small inocula.

INTRODUCTION

Glucose cysteine blood agar (GCBA) was described by Downs, Coriell, Chapman & Klauber (1947) as a suitable medium for the enumeration of *Pasteurella tularensis* by a plate colony counting method. The medium gives satisfactory counts in experienced hands but the need to use blood presents two problems: control of its quality is difficult, and it renders the medium opaque and therefore less satisfactory for colony counting. Won (1958) described a blood-free medium, but we have found it unable to promote full growth of aged organisms from stored suspensions or aerosols. This paper describes a clear medium which promotes the growth of *P. tularensis* as well as GCBA and gives consistent results.

METHODS

Glucose cysteine blood agar

GCBA was used throughout the work as the control medium against which variants of it were tested. The formulation was changed somewhat from that of Downs *et al.* (1947). The basal medium contained (g./100 ml.): Lab-Lemco (Oxoid), 0·3; peptone (Evans), 0·5–3·0; sodium chloride (Analar), 0·5; agar (Davis), 1·25; distilled water to 100 ml. The medium was completed by the addition of separately autoclaved solutions of L-cysteine HCl (10%) 1 ml.; L-histidine HCl (10%) 1 ml.; glucose (50%) 5 ml.; and 4 ml. of whole human blood (citrated).

The optimum amount of peptone was found to vary from batch to batch. The histidine decreased the required incubation time by several hours.

The basal medium was prepared by dissolving the Lab-Lemco, peptone and salt and adjusting to pH 7·6–8·4 (according to peptone batch and concentration) with sodium hydroxide so that the medium when completed was at pH 6·8–7·0. The agar was then added and, after steaming to melt it, the basal medium was autoclaved in convenient amounts for 15 min. at 120°. No deterioration of the basal medium was found to occur for at least 1 year when stored in screw-capped bottles in the dark at room temperature.

The medium was completed by melting the basal portion, cooling to 50–55°, and adding the remaining ingredients glucose, cysteine, histidine and finally blood. Penicillin to 10 units/ml. was frequently added; this was desirable to inhibit contaminants in certain uses of the medium, and had no detectable effect on the growth of *Pasteurella tularensis*. Petri plates were poured and 'dried' in the usual way for about 2 hr. at 37°.

Organism. Small-scale preparations were made on a laboratory shaker, using a modified casein partial hydrolysate medium. Strain Schu D was used for most of the work, and four other strains in confirmatory tests. The suspensions were stored at 0 to +4°.

Viable counts. Viable counts were made by the drop technique of Miles & Misra (1938). Dilutions were chosen to give about 150 colonies/plate on the control medium; six plates of each medium under test were counted. Colonies were counted when they reached 1–2 mm. diam. which was usually after 2 days at 37°; with some media longer incubation was necessary.

Use of 'aged' organisms. In the early stages of the work it was found that suspensions which had been stored longer than 3 or 4 weeks, and also samples from some 'aged' aerosols, were much more exacting in their requirements for growth than were 'young' suspensions or aerosols of high viability. Media were therefore tested with the more fastidious aged suspensions and sometimes also with aerosol samples. This was important, since media which were satisfactory in all other respects sometimes failed in this test.

RESULTS

Plate counts on the control GCBA medium were scaled to 100, and counts on test media in the range 85–115 were not considered significantly different from the control. A few typical results are shown in Table 1.

During preliminary work it was found that growth was completely inhibited when the glucose was autoclaved in the basal medium; this inhibition was partly annulled when the cysteine also was autoclaved in the basal medium. No inhibition was found when cysteine alone was autoclaved in the basal medium. Nevertheless, because of its instability it was decided to add it separately to the medium when required. A 10% solution of L-cysteine autoclaved 15 min. at 120° in a screw-capped bottle lost 8% of its cysteine. This loss could be safely ignored since it was found that the cysteine at 1000μg./ml. in the medium was about 100 times minimal requirement: suspensions of *Pasteurella tularensis* up to 33 weeks old and aerosol samples of less than 0·5% viability gave plate counts equal to the control with cysteine down to 10μg./ml. Cysteine was found to promote better growth than cystine in plain (i.e. no blood) agar, confirming the work of Downs *et al.* (1947). No difference could be demonstrated between them, however, when used at concentrations 1–1000μg./ml. in the medium with blood. It was apparent that not only did blood eliminate the difference between growth promotion of the amino acids but it also stimulated the growth of aged *P. tularensis* which failed to grow on plain cysteine or cystine agar.

Attention was then turned to the blood. We usually used citrated whole human blood. Neither the cells nor the plasma would alone support full growth of aged *P. tularensis*.

Lysis of erythrocytes takes place in a relatively short time even in cold storage,

and it was found that lysed blood was inferior to non-lysed blood when tested with aged *Pasteurella tularensis*. Erythrocytes were lysed in distilled water and washed by centrifugation until there were no intact cells in the deposit and the supernatant fluid was colourless. The deposit was used in the same proportion as the stroma content of whole blood in the control medium, and with 4% (v/v) human plasma it gave growth equal to the control; with 2% (v/v), the count was about 75% of control. The evidence suggests the presence of inhibitors in the erythrocytes rather than loss of growth factors when the erythrocytes were lysed.

Table 1. *Growth of* Pasteurella tularensis *on glucose cysteine agar with various additions*

Addition	Age of suspension (weeks)		
	6	15	20
	% growth		
None	0	0	0
4% human blood (control)	(100)	(100)	(100)
Human blood cell stroma (from 4% human blood)	101	55	25
Human blood cell stroma and 2% human plasma	92	74	74
Human blood cell stroma and 4% human plasma	102	90	90
4% human plasma and 10 μg./ml. catalase (crude)*	101	68	45
4% human plasma and 100 μg./ml. catalase	107	75	67
4% human plasma and 300 μg./ml. catalase	90	107	106
4% horse plasma and 300 μg./ml. catalase	105	—	97
300 μg./ml. catalase + 4% reconstituted freeze-dried human plasma	98	93	95
300 μg./ml. catalase + 4% dialysed human plasma	93	101	94

* About 1 *Kat. f.*/mg.

The growth-promoting action of the stroma seemed likely to be enzymic, and as it was found to have retained some catalase action preparations of this enzyme were tested. An impure beef-liver catalase extract of activity about 1 *Kat. f.*/mg. was Seitz-filtered in 1% (w/v) solution in phosphate buffer (pH 7·4) and added to the medium, together with human plasma, in place of whole blood. With this catalase preparation at 300 μg./ml. + 4% (v/v) plasma, the *Pasteurella tularensis* counts were equal to that of the control. The activity of the catalase required was about equal to that of 4% (v/v) of whole blood and much more than that of the separated stroma. The function of the stroma therefore cannot be attributed solely to the catalase content. Catalase was, however, capable of completely replacing the stroma. It was shown by tests with pure catalase that it was the enzymic activity that was responsible. Pure crystalline catalase (about 100 *Kat. f.*/mg.) was equal in effect, at 3 μg./ml., to the crude catalase (about 1 *Kat. f.*/mg.) at 300 μg./ml.

In the first tests with the 'blood-free' medium, citrated human plasma (about 4%, v/v) was used + 300 μg./ml. crude catalase preparation. This plasma could be replaced by reconstituted freeze-dried human plasma and by human plasma dialysed against physiological saline. Ox plasma or horse plasma were also suitable but the pH value was rather critical at about pH 6·7, in contrast to human plasma which was satisfactory within the range pH 6·5–7·0.

Recommended medium

The composition of the medium finally adopted was as follows (g./100 ml.): Lab-Lemco (Oxoid), 0·3; peptone (Evans), 0·5–3; sodium chloride, 0·5; agar (Davis), 1·25; distilled water to 100 ml. The following ingredients were added separately as sterile solutions: glucose, 5 ml. (50 % solution); L-cysteine HCl, 1 ml. (10 % solution); L-histidine HCl, 1 ml. (10 % solution); catalase (pure), 0·1 ml. (1 % solution); plasma (human, ox, horse), 5 ml., pH 6·6–6·8. The method of preparation for this medium was as for GCBA. It should be noted that the catalase content given is about three times the adequate concentration of 3μg./ml.; this margin of safety may not be necessary. Poured plates of this medium keep about as well as GCBA when stored at 4°. For routine use plates kept for 2 weeks were satisfactory, but for extremely sensitive aged organisms 1 week was the limit.

The medium was tested with *Pasteurella tularensis* aerosols which had been held until the viability was considerably decreased (as determined on GCBA by the method of Harper, Hood & Morton, 1958). The bacteria remaining viable in such aerosols are in a sensitive state, and make demands on the culture medium that are similarly exacting to those of aged suspensions. In a series of five experiments, 12,376 colonies were counted on the new medium and 11,215 on the control, the ratio of 1·1 showing a small (possibly not significant) advantage in favour of the medium described here. The new medium was tested with aged suspensions of four other strains of *P. tularensis* (Fam, Jap, 403, and LV), and the results confirmed the performance with Schu D.

The success of the solid medium in growing *Pasteurella tularensis* suggested trial of the liquid medium, without agar, in shaken cultures. The yields of organisms obtained were similar to those from the casein hydrolysate medium (about 4×10^{10} organisms/ml.). Unlike the casein hydrolysate medium the new medium supported growth from small inocula of less than 10 organisms/ml.

My thanks are due to Mr I. H. Silver for helpful criticism, Mr L. Flower for technical assistance, and Dr H. T. Eigelsbach (Fort Detrick, Frederick, Maryland, U.S.A.) for supplying strains of *Pasteurella tularensis*.

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Purification of Factor I and Recognition of a Third Factor of the Anthrax Toxin

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With a note by H. Smith, K. Sargeant and J. L. Stanley about serological precipitation in gels as a criterion of purity of antigens

(*Received* 1 *February* 1961)

SUMMARY

Factor I of the anthrax toxin was isolated and showed one major component in the ultracentrifuge and on paper electrophoresis; it contained less than 5·5% of extraneous antigens detectable by serological precipitation in gels. The final preparation contained all the usual amino acids (N = 10·1%) and some carbohydrate (6%, calculated as glucose) and phosphorus (0·7%). The most striking aspects of its analysis were a high ash (10–13%) and a light absorption at 260 mμ. The high ash was not due to one element but to a highly variable metal content (mainly Ca, Mg, Ni, Cu) indicating a powerful and indiscriminate chelating action of factor I. This chelating action might have been due to the chemical entity which absorbed light at 260 mμ and which was not RNA or DNA.

The final preparation of factor I was not toxic when injected alone but when mixed with purified factor II it evoked oedema in the skin of a rabbit and killed mice. However, the concentration of this mixture which killed mice formed a much larger skin reaction in rabbits than a comparable dose (based on mouse LD50) of either crude toxin or a mixture of crude factors I and II. An investigation of this fact led to the demonstration and partial purification of a third factor (III) of the anthrax toxin which: (1) was different serologically from factors I and II; (2) was present in anthrax toxin produced *in vivo*; (3) was non-toxic when injected alone; (4) was lethal for mice when mixed with factor II but not with factor I; (5) increased the lethality of mixtures of factors I and II for mice and decreased their capacity to produce oedema in the skin of rabbits. A mixture of factors I, II and III showed synergic action in toxicity tests in mice; the mixture killed guinea pigs which showed signs of oligaemic secondary shock (as did guinea pigs killed by anthrax infection).

INTRODUCTION

The specific lethal and oedema-forming toxin of *Bacillus anthracis* was first recognized *in vivo* and then produced *in vitro*; it consisted of at least two components —factors I and II—which acted synergistically (Smith, Keppie & Stanley, 1955*a*; Smith *et al.* 1956; Harris-Smith, Smith & Keppie, 1958; Thorne, Molnar & Strange, 1960; Stanley, Sargeant & Smith, 1960; Sargeant, Stanley & Smith, 1960). This paper describes the purification, biological action and chemical properties of factor I of anthrax toxin.

A mixture of the final preparation of factor I + purified factor II resembled crude toxin in producing oedema in the skin of a rabbit and in killing mice, but its behaviour was not identical with that of the original toxin. The concentration of the mixture of purified preparations which killed mice formed a much larger skin reaction in rabbits than a comparable dose (based on mouse LD 50) of either crude toxin or a mixture of crude factors I and II. An investigation of this result led to the recognition of a third factor of the anthrax toxin which is described in this paper.

METHODS

Assay for factor I of the anthrax toxin. This was as described by Stanley *et al.* (1960).

Tests for immunogenicity. These were carried out in guinea pigs and rabbits as described by Smith & Gallop (1956) and Strange & Thorne (1958).

Tests for lethality to mice and guinea pigs. These were as described by Smith *et al.* (1955*a*).

Tests for oedema production in rabbit skin. These were as described by Smith *et al.* (1955*a*) for guinea pigs, including the nomenclature used to describe the size of the skin lesion.

Serological precipitation in gels. Unless stated otherwise the anthrax antisera ('spore' H. 533; 'antigen' H25) and methods used were as described by Sargeant *et al.* (1960).

Diethylaminoethyl cellulose (DEAE–C, Peterson & Sober, 1956). This was bought from Eastman Kodak Ltd.

Analytical ultracentrifugation. We are indebted to our colleagues Dr K. Cammack and Mr K. Grinstead for these observations in the Spinco Ultracentrifuge Model E.

Paper electrophoresis. Samples were electrophoresed at two pH values (0·2 μ barbitone buffer pH 8·6; 0·2 μ acetate buffer pH 5) on Whatman no. 3 paper for 18 hr. with a potential gradient of 10 V./cm. and in a cooled apparatus; the papers were stained with naphthalene black.

Nitrogen. This was determined by the Kjeldahl (Kj.) and Dumas (D) methods.

Total carbohydrate. This was estimated by the orcinol method of Sørensen & Haugaard (1933).

Phosphorus. This was measured by the method of Fiske & SubbaRow (1925).

Lipid. Samples were hydrolysed with N-HCl for 1 hr. at 100° and evaporated to dryness at room temperature over P_2O_5 and NaOH. The residue was extracted by the method of Folch, Lees & Sloane Stanley (1954); the material soluble in chloroform was weighed.

Hexosamine. This was determined by the Elson & Morgan method.

Ash. This was sulphated.

Protein. This was estimated for chromatographic purposes by the method of Lowry, Rosebrough, Farr & Randall (1951) with ovalbumin as a standard.

Amino acids. A hydrolysate (16 hr., 100°, 6N-HCl) was examined for amino acids by two-dimensional paper chromatography, with phenol and collidine as solvents.

Absorption of ultraviolet (u.v.) radiation. A recording spectrophotometer was used (Type CF4DR, Optica U.K., Gateshead-on-Tyne, England).

Emission spectroscopy. We are indebted to our colleagues Mr L. C. Thomas and Mr J. L. Clipson for these determinations.

Infrared spectroscopy. We are indebted to our colleague Dr K. Norris for this.

Haematocrit, plasma protein and plasma inorganic phosphate. These were determined by the methods described by Smith *et al.* (1955*b*).

Crude anthrax toxin produced in vivo. This was as described by Smith *et al.* (1955*a*).

Crude anthrax toxin produced in vitro. This was prepared by the method of Thorne, Molnar & Strange (1960) and horse serum (10%, v/v) was added before filtration.

Crude factor I from toxin produced in vitro. A modification of the method of Thorne *et al.* (1960) was used. A culture (1 l.) containing crude antigen of *Bacillus anthracis* (Thorne & Belton, 1957; Strange & Thorne 1958) was passed through a sintered-glass bacterial filter (6 cm. diameter; 5/3 porosity), which was washed with 0·05 M-phosphate buffer (pH 7; 4 × 20 ml.) at 0–2°. On extraction of the filter for 1 hr. with ice-cold saturated Na_2CO_3 solution (4 ml.) followed by neutralization of the extract with HCl a solution of crude factor I was obtained. One batch of culture contained 40–100 l. and 40–100 filters were washed and extracted within 2 hr. of filtration of the original culture.

Crude factor II from the toxin produced in vitro. This was precipitated from the filtrate of the culture described above, by ammonium sulphate as described by Strange & Thorne (1958) and dialysed for 6 hr. at 0–2° against buffered saline (0·01 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris buffer) in saline; pH 7·4).

Purified preparation of factor II of the anthrax toxin produced in vitro. This was the preparation of Strange & Thorne (1958) described by Sargeant *et al.* (1960).

RESULTS

Purification and properties of factor I of the anthrax toxin

Purification of factor I on diethylaminoethyl cellulose (DEAE–C). A solution of crude factor I (200 ml. ≡ 50 l. original culture; factor I null point 1/500; protein content about 0·06%) at pH 7·4 was diluted with 0·005 μ phosphate buffer (1800 ml.; pH 7·4) and applied to a column (4·5 cm. diameter, 10 cm. length) of DEAE–C (25 g.) which had been packed at 4 lb./sq.in. pressure and equilibrated with 0·1 μ phosphate buffer (pH 7·4). The flow rate was adjusted to 20 ml./min. by application of a slight positive pressure. The effluent (2000 ml.) had negligible activity and the column was eluted successively with quantities (450 ml.) of 0·1, 0·12, 0·15, 0·17 and 0·2 μ phosphate buffers (pH 7·4). In preliminary experiments each application of buffer was collected as a single fraction (450 ml.); the bulk (60–90%) of the factor I activity was eluted by the 0·15 and 0·17 μ buffers.

In subsequent experiments the eluates from the applications of the 0·15 and 0·17 μ buffers were each collected in 6 × 75 ml. fractions. Each fraction was purified and concentrated in the following manner. Each fraction (75 ml.) was diluted with distilled water (225 ml.) and applied to a column (2 cm. diameter, 1 cm. length) of DEAE–C (0·2 g.) equilibrated with 0·1 μ phosphate buffer (pH 7·4). No pressure was applied and the flow rate was 5–10 ml./min. The effluent (300 ml.) contained only a small amount of factor I activity but over 75% of the original material reacting as protein in the method of Lowry *et al.* (1951). The column was washed

once with 0·1 μ phosphate buffer (6 ml.; pH 7·4) and the active material eluted in 0·2 μ phosphate buffer (pH 7·2) containing M/5 NaCl (4 ml.).

Before combining any active fractions for further processing the 12 concentrated fractions from the eluates with 0·15 and 0·17 μ buffers were examined separately on serological diffusion plates for the presence of impurities. Preliminary work showed that factor I was associated with a line formed on serological precipitation plates against 'spore' anthrax antiserum (H. 533), whereas it formed no line against 'antigen' antiserum (H25) (see Sargeant *et al.* 1960). On serological diffusion plates the 12 concentrated fractions formed against anthrax antisera faint extraneous lines in a pattern which varied slightly from batch to batch but was usually as indicated in Table 1.

Table 1. *Pattern of lines formed on serological diffusion plates of toxin fractions from DEAE–C columns with anthrax antiserum*

Vertical braces enclose fractions usually bulked for final concentration.

Buffer used for elution	Fraction*	Intensity of line associated with factor I†	Intensities of lines (1) (2) and (3) formed by extraneous antigens†				
			Antiserum H533		Antiserum H25		
			(1)	(2)	(1)	(2)	(3)
0·15 μ phosphate (pH 7·4)	1	1	1	.	1	1	.
	2	3	1	.	1	1	.
0·17 μ phosphate (pH 7·4)	3	5	1	.	1	1	.
	4	4	.	.	.	1	.
	5	4	.	.	.	1	.
	6	4	.	.	.	1	.
	7	3	.	.	.	1	.
	8	3	.	.	.	1	.
	9	3	.	.	.	1	.
	10	3	.	1	.	1	1
	11	3	.	1	.	1	1
	12	2	.	1	.	1	1

* 75 ml. concentrated to 4 ml. on a 2nd DEAE–C column (see text).

† Numbers 1–5 indicate approximately the intensity of lines on serological diffusion plates (high number: high intensity).

Appropriate fractions (usually 3–8, but in each experiment the inclusion of fractions 2, 9 and 10 or exclusion of fractions 3, 7 and 8, depending on the particular pattern of lines formed by the fractions on serological diffusion plates) from this and a second similarly fractionated batch (200 ml.) of crude factor I, were combined, dialysed overnight at 0–2° against 0·02 μ phosphate buffer (pH 7·4) and applied to a column (0·5 cm. diameter, 3 cm. long) of DEAE-C (80 mg.) equilibrated with 0·02 μ phosphate buffer (pH 7·4). The effluent (about 50 ml.) contained negligible activity. The column was eluted with 0·2 μ phosphate buffer (pH 7·2) containing M/5 NaCl. The first 0·4 ml. of eluate, which contained negligible activity, was discarded. The next 0·8 ml. constituted the final preparation of factor I which was kept at −20° until required.

Yield and recovery of activity. In five similar experiments, 0·8 ml. of final product having an average factor I null point of 1/64,000, containing about 0·4–0·7 % of

non-diffusible material and 0·3–0·5 % of 'protein' (see Methods), was obtained from 400 ml. of crude factor I having an average factor I null point 1/500 and containing 0·06 % of protein (see Methods). Hence about 25 % of the original factor I activity was recovered in the final product which had been considerably purified from other protein. There was no extensive loss of activity during the process since, in all the fractions taken therefrom, 80 % of the original activity was recovered.

For most experiments, the final product was used as prepared in solution and stored at −20°. For chemical analysis the solution was dialysed for 7 days at 0–2° against frequent changes of distilled water until free from salt and then freeze-dried. After this prolonged dialysis 50 % of the original activity was lost; after freeze-drying, the material was not completely soluble.

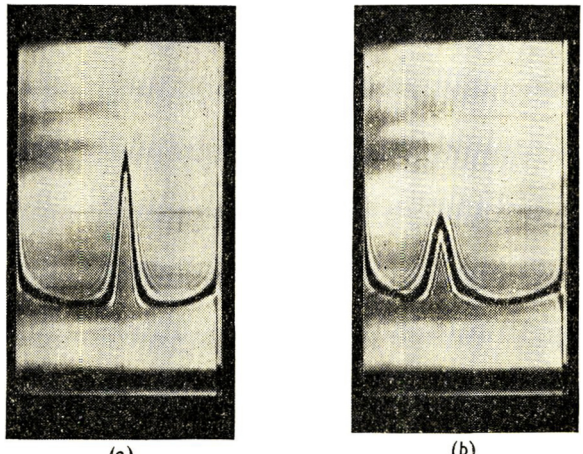

Fig. 1. Ultracentrifuge diagrams of the final preparation of anthrax toxin factor I. Concentration about 0·75 % (w/v), 0·2μ phosphate buffer (pH 7·4); field 100,000 g. Pictures taken (*a*) 8 min. and (*b*) 32 min. after sedimentation in synthetic boundary cell (sedimentation right to left).

Criteria of purity

Ultracentrifugation. The final preparation (0·7 %) showed one major component when examined in a synthetic boundary cell at pH 7·4 in 0·2μ phosphate buffer (see Fig. 1).

Paper electrophoresis. The final preparation was concentrated by dialysis against carbowax. The material (0·3 mg.) showed no evidence of heterogeneity when examined in 0·2μ barbitone buffer (pH 8·6) and 0·2μ acetate buffer (pH 5); it was almost stationary but it did move from the origin, towards the anode at pH 8·6 and towards the cathode at pH 5.

Serological precipitation in gel diffusion plates. Experiments described in an addendum to this paper indicated that two extraneous serologically-reacting materials were present in the final product, but that the degree of contamination was not more than 0·5 % of one material and 5 % of the other.

Biological properties

Oedema production. In the assay for factor I activity about 0·02 μg. produced an oedematous reaction when mixed with the standard quantity of factor II, but 6 μg. produced no reaction when injected alone. The material was stable at $-20°$ at pH 7 for 1–2 months, but at 0–2° 50 % of factor I activity disappeared after 7 days at pH 7, and 75 % was lost after 7 days at pH 5 and 9. The material (in 0·2 μ phosphate buffer, pH 7·4) lost 50 % of its activity in 2 hr. at 37°, 75 % of its activity when shaken with glass beads for 1 hr. at 0° and all of its activity on standing for 24 hr. at 0° with cysteine (0·2 %), sodium metabisulphite (0·1 %) and potassium periodate (0·05 %).

Lethality to mice (see below)

Serological precipitation in gels. The behaviour of the final preparation of factor I was described by Sargeant *et al.* (1960). The line associated with factor I activity was just formed by 0·5 μg. of the final preparation in serological diffusion plates against 'spore' anthrax antiserum (H 533); no line was formed against 'antigen' anthrax antiserum (H25) unless the material was examined at high concentrations (see above).

Immunizing activity. In the assay for immunizing activity in guinea pigs 120 μg. of the final preparation showed no significant activity. Similarly, 40 μg. or 100 μg. of the final preparation did not immunize rabbits (10 rabbits were used in each batch and the material was mixed with horse serum before injection).

Table 2. *Chemical analysis of the final preparation of anthrax toxin factor I*

	%
N	12·0 (Kj), 12·0 (D)
Carbohydrate	6·4 (as glucose)
Lipid	Less than 3
Hexosamine	Less than 1
P	0·7
Ash (SO_4)	10·1*
Amino acids	All usual ones and no evidence of extra ones

* After reheating with hydrofluoric acid, 10·3 %.

Chemical properties

Table 2 summarizes the chemical analyses of the final product. Fig. 2 shows its infrared spectrum and Fig. 3 (curve A) its ultraviolet absorption spectrum in neutral solution. The material was not solely protein, although it contained much amino acid material; it had no significant amount of lipid and some carbohydrate residues. The most striking aspect of its chemical analysis was a high metal content as shown by its high ash, and an apparent nucleic acid content (about 6–8 % from its absorption at 260 mμ) and its P content. These aspects were investigated in more detail.

The metal content. The high ash of the final material seemed to be a specific property and not due to the fractionation process. Thus, ovalbumin, rabbit γ-globulin, human serum albumin and two samples of factor II of anthrax toxin had 1·3, 2·7, 0·9, 0·6 and 1·2 % sulphate dashes, respectively, after being subjected to the same

fractionation process as that described above for factor I. The high ash was not due to the presence of SiO_2 (or B_2O_3) since heating with HF did not decrease it.

The nature of the metal content was investigated by spectroscopic analysis. Mg, Ca, Ni and Cu were present in appreciable amounts in the four batches of material examined; only traces of other metals were present. The estimated contents (which were subject to an error of $\pm 100\%$ due to the small amount of material examined and the necessity of estimating all metals on one sample) of the four batches fell in the ranges (%): Mg, 0·001–0·1; Ca, 0·003–0·8; Ni, 0·3–0·75; Cu, 0·1–1·3; but the relative proportions were not consistent from batch to batch.

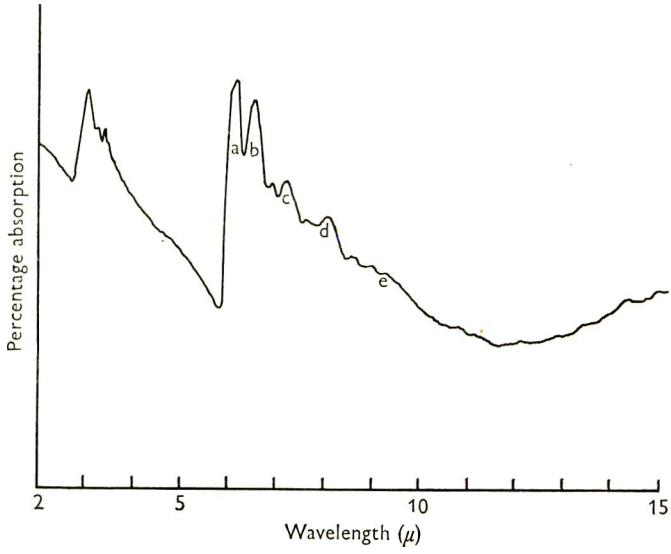

Fig. 2. Infrared spectrum of the final preparation of anthrax toxin factor I; 0·8 mg. on AgCl. Peaks *a* and *b* indicate protein; peak *c*, —CH_2— and —CH_3 groups; peak *d*, P=O groups; the absorption *e* a small amount of carbohydrate.

In attempts to remove the metal content or at least to obtain a consistent pattern, four batches of active material were prepared as described above but with the use of deionized water throughout the process and DEAE–C columns which had been pretreated with ethylenediaminetetraacetic acid (EDTA) to remove metals as follows: 100 g. DEAE–C were treated with 4 l. 0·1 % EDTA overnight, decanted, re-treated with a further 4 l. 0·1 % EDTA and washed with 3×4 l. 0·1 μ phosphate buffer (pH 7·4). The sulphated ash of the final material (11·3 and 12·6 % on two of the batches) was not decreased by these precautions, although the nature of the metal content changed to some extent; the Ni and Cu content appeared to be decreased (range Ni 0·12–0·19 %; Cu 0·1–0·5 %) and the Mg and Ca content increased (range Mg 0·17–1·1 %; Ca 0·3–1·0 %). It appeared that the preparation of factor I had a chelating action on most metals and that no single metal was characteristic of the preparation.

The absence of RNA and DNA. The following observations were carried out in collaboration with our colleague Mr H. E. Wade. (1) Adequate control experiments showed that the absorption of u.v. radiation was not due to the high Ni and Cu content. (2) Although in neutral solution the u.v. absorption spectrum of the

final preparation of factor I (Fig. 3, curve A) resembled the spectrum of a mixture of 10% yeast RNA in ovalbumin (Fig. 3, curve B), the shifts in their spectra (Fig. 3, curves C and D, respectively) in 0·3 N-KOH were different. The absorption of the experimental sample at 260 mμ was less than that at neutral pH, whereas the absorption of the artificial mixture of nucleic acid + protein was greater than at neutral pH. Furthermore, in alkali the experimental sample showed a characteristic plateau of absorption at 270–295 mμ which was not shown by the control mixture. (3) The final preparation of factor I (2 mg.) and the control mixture (2 mg.) were treated separately as follows. A solution in 0·3 N-KOH (3 ml.) was left at 37° for 18 hr. and cooled to room temperature. Perchloric acid solution (0·1 ml., 72%, w/v)

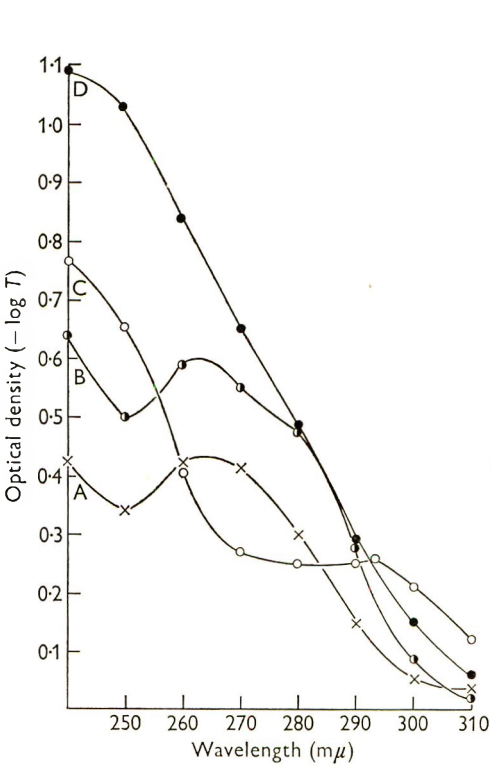

Fig. 3

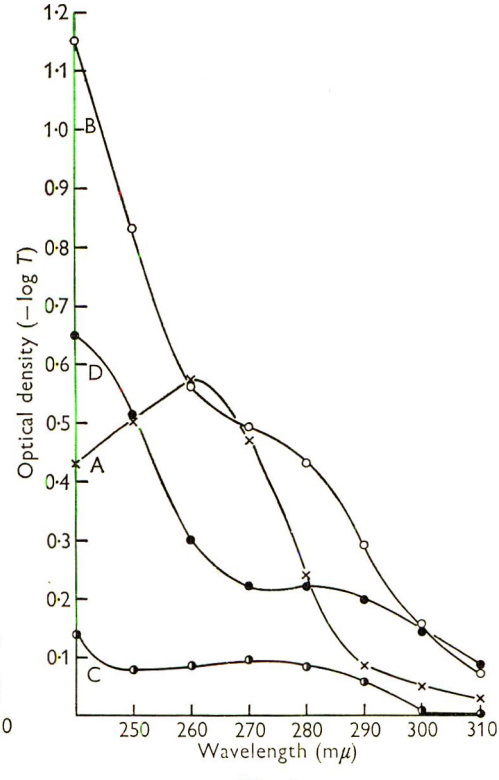

Fig. 4

Fig. 3. Ultraviolet absorption spectra of the final preparation of anthrax toxin factor I and ovalbumin containing 10% (w/w) yeast RNA. Adsorption determinations were made in a 0·5 cm. cell. Curve A: 0·05% final preparation of factor I at pH 7·4. Curve C. 0·05% final preparation of factor I in 0·3 N-KOH. Curve B: 0·05% of a mixture of ovalbumin with 10% yeast RNA at pH 7·4. Curve D: 0·05% of a mixture of ovalbumin with 10% yeast RNA in 0·3 N-KOH.

Fig. 4. Ultraviolet absorption spectra of the products of hydrolysis of the final preparation of factor I. Adsorption determinations were made in a 1 cm. cell (compare Fig. 3). Original material (2 mg.) was hydrolysed in a volume of 3 ml. (see text); all materials were adjusted to this volume for examination. Curve A: extract from mild alkaline hydrolysis examined in acid solution (0·1 N-$HClO_4$). Curve B: extract from mild alkaline hydrolysis examined in alkaline solution (0·1 N-KOH). Curve C: extract prepared by acid hydrolysis of the residue left after alkaline hydrolysis; examined in acid solution. Curve D: residue left after alkaline and acid hydrolysis; examined in neutral solution.

was added and the precipitate removed by centrifugation and reserved (see below); the extract was clarified by adding ovalbumin solution (0·1 ml., 0·2 %) and recentrifugation. The spectra of the extracts, which should contain all the ribonucleotides from RNA if present, were examined under acid (as prepared) and alkaline (after adding 20 N-KOH to make the solution 0·1 N-KOH and removing the $KClO_4$ by centrifugation) conditions. The spectra of the extracts from the control mixture containing RNA were as expected; practically all the original material absorbing at 260 mμ was soluble in acid after alkaline hydrolysis and the extinction ($-\log T$) of the extract at 240 mμ was less than that at 260 mμ under both acid and alkaline conditions. In contrast, the spectra of the extract from the experimental sample [Fig. 4, curves A (acid conditions) and B (alkaline conditions)] showed that much of the material in the original sample which absorbed at 260 mμ had not been liberated by alkaline hydrolysis (compare Fig. 3, curve C with Fig. 4, curve B) and that the extracted material was not the usual mixture of ribonucleotides, i.e. there was a large increase of absorption at 240 mμ (relative to that at 260 mμ) when the conditions changed from acid to alkaline. The absence of ribonucleotides in this extracted material was confirmed by paper ionophoresis (Wade, 1961). The hydrolysate was neutralized with $HClO_4$ and concentrated in a desiccator before applying to the paper. A comparison with a similar extract from the control mixture of RNA + protein indicated that the original sample of factor I contained less than 0·6 % RNA. (4) The absence of DNA was indicated by the following hydrolytic studies. The material precipitated by $HClO_4$ after alkaline hydrolysis of the final preparation of factor I, including the deposit centrifuged after clarification with ovalbumin, was suspended in water (3 ml.) and heated with $HClO_4$ solution (0·15 ml., 72 %, w/v) for 15 min. at 90°. A deposit was centrifuged from the cooled product and the spectrum of the supernatant fluid examined (Fig. 4, curve C); it showed no evidence for the presence of hydrolytic products of DNA. On the other hand, when the deposit from this acid hydrolysis was dissolved in water (3 ml.) at pH 7 (addition of 5 N-NaOH), its spectrum showed that it still retained material absorbing at 260 and 240 mμ (Fig. 4, curve D). (5) In a micro-adaptation of the diphenylamine method for deoxysugars (Volkin & Cohn, 1954) no colour was formed by the final preparation of factor I (90 μg.), whereas 2·5 μg. of DNA (thymus) produced a colour.

Recognition of a third factor of the anthrax toxin

The low toxicity for mice of oedema-producing mixtures of purified factors I and II. The results in the top half of Table 3 show that the final preparation of factor I and a purified preparation of factor II produced no oedema in rabbits and did not kill mice when large amounts were injected alone, but that smaller quantities injected together produced large skin reactions in rabbits and killed some mice. However, the lethality of these mixtures for mice was much smaller than that warranted by their capacity for oedema production when compared with results of similar experiments with crude preparations (see bottom half Table 3). Thus, crude unseparated anthrax toxin produced *in vivo* and *in vitro* killed more mice at concentrations which produced a relatively smaller amount of oedema in rabbits; and the same type of result was obtained by injecting mixtures of crude factors I and II which, like the purified factors, did not when injected alone produce oedema in rabbits and were almost non-toxic for mice. The finding that some factor which con-

tributed to lethality for mice was present in either crude factor I or II and absent from the purified preparation, was indicated by the results shown in the last two lines of Table 3. In contrast to mixtures of purified I and II, mixtures of either crude I + purified II or purified I + crude II killed mice, although they produced a relatively small skin reaction in rabbits.

Table 3. *Oedema production and lethality for mice of mixtures of crude and purified factors I and II of the anthrax toxin*

Results are the average or aggregate of similar results on several batches of material. In all cases the production of oedema and the killing of mice were completely neutralized by mixing the solutions with 'spore' (H533) anthrax antiserum (1/3 vol.).

Material injected	Approx. amount* (μg.) in 0·5 ml. Factor I	Factor II	Size of skin reaction† (0·2 ml.)	Lethality for mice (0·5 ml.) dead/total
A. Final prep. of factor I	16	—	Nil	0/21
B. Purified factor II	—	320	Nil	0/14
A + B	8	80	17:75†	19/31
A + B	4	40	15:62	5/86
A + B	2	20	15:60	0/15
A + B	1	10	14:40	0/5
A + B	0·5	5	11:40	0/5
Crude anthrax toxin (*in vivo*)	—	—	9:40	64/75
Crude anthrax toxin (*in vitro*)	—	—	10:40	15/30
C. Crude factor I	240	—	Nil	0/22
D. Crude factor II	—	600	Nil	4/14
	—	300	Nil	3/25
	—	150	Nil	1/20
	—	75	Nil	0/10
C + D	60	75	8:35	32/35
A + D	4	75	9:40	17/27
C + B	60	40	9:37	25/30

* The amounts of materials equiv. about 500 times the quantities corresponding to the null points in the assays for factors I and II (see methods) were (1) final preparations of factor I 4μg. (2) purified factor II 80μg.; (3) crude factor I 60μg. ('protein'); (4) crude factor II 150μg. ('protein').

† A skin reaction of size 17:75 means that the oedematous lesion had a fold thickness of 17 mm. and a diameter of 75 mm. Significant differences were 2 mm. in fold thickness or 10 mm. in diameter; these differences were produced by 2–4-fold differences in toxin concentrations.

Enhancement of the lethality for mice and oedema production in rabbits of mixtures of purified factors I and II by adding serum and other proteins. The possibility that the effect of the constituents of the crude preparations described above was a non-specific action of extraneous protein (e.g. possibly by protecting the toxin against destruction in the mouse) was investigated. The results in Table 4 show that the specific lethality for mice of mixtures of the final preparation of factor I + purified factor II was significantly increased by adding various sera and proteins. However, the addition of this extraneous protein also increased the skin reactions; the increase was about 2–4-fold when compared by the more sensitive null point assay, and the anomaly described above regarding size and skin reaction and lethality for mice

remained. This effect of serum and other proteins only occurred with mixtures of the purified factors; in similar experiments with crude preparations extraneous proteins had little or no effect on oedema production or on lethality for mice.

Fractionation of a third factor of the anthrax toxin. A factor was sought which, when added to a mixture of purified factors I and II, would form a preparation which killed mice at concentrations that produced in rabbits a relatively small skin reaction. Preliminary experiments showed that such a factor was present in fractions collected from the chromatography of crude factor I on DEAE–C when the column was eluted with 0·2μ phosphate buffer after factor I had been removed with more dilute buffers (see previously). The factor was also present in those fractions discarded during the purification of factor II by the method of Strange & Thorne (1958). A preparation of this factor III was produced for the experiments described below by the following method.

Table 4. *Effect of serum and other proteins on oedema production and lethal effect of mixtures of purified anthrax toxin factors I and II*

The lethal and oedema-producing effects were completely neutralized when horse anthrax antiserum (spore H533) was used instead of the other sera.

Material in 0·5 ml.					
Purified factor I (μg.)	Purified factor II (μg.)	Serum or other protein (0·2 ml.)	Lethality for mice (0·5 ml.): dead/total	Skin reactions in rabbits (0·2 ml.) Size†	Null point‡
4	40	Saline	5/86	15:62	1/320
4	40	Horse serum	22/25	17:62	1/1280
4	40	Rabbit serum	18/30	18:60	1/1280
4	40	Guinea-pig serum	8/10	17:65	1/1280
4	40	Ovalbumin (5 %)	4/5	16:58	1/640
4	40	Sodium polyglutamate (1 %)	4/5	15:64	1/640
2	20	Saline	0/15	—	—
2	20	Horse serum	4/25	—	—
2	20	Rabbit serum	2/5	—	—
2	20	Guinea-pig serum	3/10	—	—
2	20	Ovalbumin (5 %)	0/5	—	—
2	20	Sodium polyglutamate (1 %)	0/5	—	—

* When injected alone these preparations did not kill mice (0·5 ml.) or evoke oedema (0·2 ml.) in rabbits. † See Table 3.

‡ The null point was the first dilution of two fold descending dilutions which did not form an oedematous skin reaction.

Filtrate (5 l.) from a culture containing crude anthrax toxin (see Methods) was applied to a column (4·5 cm. diam.; 4 cm. long) of DEAE–C (5 g.) which had been equilibrated with 0·1μ phosphate buffer (pH 7·4). No pressure was applied and the flow rate was about 30 ml./min. Factor III was retained on the column and most of the factor II was not.

The column was eluted with: (1) 0·15μ phosphate buffer (60 ml.; pH 7·4); (2) 0·2μ phosphate buffer (60 ml.; pH 7·4); (3) 0·2μ phosphate buffer containing 0·05 M-NaCl (60 ml.; pH 7·4); (4) 0·2μ phosphate containing 0·2 M-NaCl (180 ml.; pH 7·2). Factor III was eluted by buffer (4); 15 ml. fractions of this were collected, assayed for protein and appropriate fractions corresponding with the elution peak

were concentrated in the following manner. The bulked fractions from 5 batches of filtrate were dialysed against 0·1 μ phosphate buffer (pH 7·4, 0–2°, overnight) and applied to a column (2 cm. diam.; 1 cm. length) of DEAE–C (0·2 g.) which had been equilibrated with 0·1 μ phosphate buffer (pH 7·4). No pressure was applied and the flow rate was 5–10 ml./min. Factor III was eluted with 0·2 μ phosphate buffer containing 0·2 M-NaCl (8 ml.). This preparation of factor III contained 0·15 % protein. In gel diffusion plates against 'spore' antiserum (H 533) it (10 μg.) formed two lines which were also formed by crude anthrax toxin produced *in vivo* and by an impure preparation of factor I from this source (see Stanley *et al.* 1960); these lines were different from those formed by the final preparations of factor I (see above) and of purified factor II (see Sargeant *et al.* 1960). Against 'antigen' (H 25) antiserum the preparation of factor III formed a line which very easily dispersed and dissolved in antigen excess, indicating a low content of factor III antibody in this antiserum.

Table 5. *Effect of partially purified third factor (III) of the anthrax toxin on oedema production and lethal effect of mixtures of factors I and II*

Material in 0·5 ml.				
Purified factor I (μg.)	Purified factor II (μg.)	Partially purified factor III* (μg.)	Lethality for mice (0·5 ml.): dead/total	Size of skin reaction in rabbits† (0·2 ml.)
8	80	—	19/31‡	17:75‡
4	40	—	5/86	15:62
2	20	—	0/15	15:60
16	—	16	0/10	Nil
4	—	8	0/10	Nil
4	—	4	0/10	Nil
4	—	2	0/20	Nil
2	—	8	0/20	Nil
—	160	16	14/19	Nil
—	40	16	28/30	Nil
—	40	8	28/69‡	Nil
—	40	4	6/49	Nil
—	40	2	0/30	Nil
—	20	8	3/20	Nil
4	40	8	48/70‡	8:35‡
4	40	4	19/50	10:45
4	40	2	4/30	15:60
2	20	8	8/15	6:27

* When injected alone partially purified factor III—64, 32, 16 and 8 μg—killed 0/10, 0/10, 0/15, 0/10 mice respectively and produced no oedema in the skin of rabbits.

† See Table 3.

‡ These lethal and oedema producing effects were completely neutralized in adequate batches (10–20) of animals by admixture with anthrax antiserum ('spore' H 533)–$\frac{1}{3}$rd vol.

The results shown in Table 5 indicate that: (1) In common with factors I and II, factor III was non-lethal for mice and produced no oedema in rabbits when injected alone in large amount. (2) A mixture of factors I and III at high concentrations was non-lethal for mice and formed no skin reactions in rabbits. (3) In common with a mixture of factors I and III, a mixture of factors II and III showed synergic

action and was toxic for mice. On the other hand, it did not produce oedema in the skin of rabbits as did the I+III mixture. (4) A mixture of factors I, II and III showed synergic action in toxicity tests in mice and produced a greater effect than expected from the addition of the effects of the pairs of factors described in (3). Table 6 shows the statistical analysis of the cogent results, for which we are indebted to our colleague Mr S. Peto. (5) The addition of factor III to a mixture of factors I and II decreased the size of the oedematous skin reactions produced in rabbits. (6) All these toxic effects were neutralized by anthrax antiserum.

Injection of a mixture of factors I, II and III (about 10 and 20 times the amounts which killed mice) killed guinea pigs (see Table 7). This toxicity was neutralized by anthrax antiserum.

Table 6. *Synergism of factors I, II and III of the anthrax toxin in mice*

Doses* (μg.)			Dead (%)* obtained when submitted to				Dead (%) expected assuming independent action	Significance of difference		
I	II	III	I+III	I+II	II+III	I+II+III		*t*	Prob. %	Comment
4	40	8	Nil	5·8	40·6	68·6 (5·5)†	52·0 (6·8)†	3·0	0·3	Highly sign.
4	40	4	Nil	5·8	12·2	38·0 (6·9)	17·3 (4·9)	2·4	1·5	Sign.
4	40	2	Nil	5·8	1·5‡	13·3 (6·2)	7·2 (3·2)	0·9	37	Not sign.§
2	20	8	Nil	2·5‡	15	53·3 (12·9)	17·1 (8·3)	2·3	1·9	Sign.

* Obtained from data in Table 5. † Standard deviations. ‡ Assuming 1/2 animal died.
§ Note small sample sizes.

Table 7. *The toxicity of a mixture of anthrax toxin factors I, II and III in guinea pigs* (250 g.)

Doses (μg.) of factors				
I	II	III	Antiserum*	Dead/total
80	600	160	Nil	2/2
40	300	80	Nil	5/6
40	300	80	0·8 ml.	0/5

Injections (2·5 ml.) were made into the saphenous vein (Smith *et al.* 1955*a*).

* 'Spore' anthrax antiserum (H533).

Observations were made during the terminal phase on two of the guinea pigs (250–300 g.) killed by the mixtures of factors I, II and III. For comparison corresponding observations were made on a few normal animals; these were the same as those described by Smith *et al.* (1955*a*, *b*). The two animals died with the clinical signs of shock; their bleeding volumes (Smith *et al.* 1955*a*, *b*) were low (about 4 ml.) and their plasma inorganic phosphate and non-protein-N contents were high (PO_4^{---}, 10·5, 15·5; non-protein-N, 100, 85 mg./100 ml.). Evidence that oligaemia contributed to the shock syndrome in these guinea pigs (compare guinea pigs dying of anthrax and from injection of crude anthrax toxin produced *in vivo*; see Smith *et al.* 1955*a*, *b*) was provided by the fact that their haematocrit values were high (about 50%) and their plasma protein contents low (500 and 475 mg. N/100 ml.). One guinea pig had a gross subcutaneous oedema comparable with that seen when some but not all guinea pigs die from anthrax.

DISCUSSION

In any comparison between the final preparations of factor I of the anthrax toxin produced *in vitro* (as described here) and that produced *in vivo* (Stanley *et al.* 1960, and subsequent unpublished observations), it must be remembered that whereas the former preparation was relatively pure, the latter was known to contain some constituents of guinea pig plasma (about 15 %) and to be serologically heterogeneous in gel diffusion against anthrax antisera. Bearing this in mind, the two preparations resembled each other in being unstable, having a high ash content, absorbing light at 260 mμ, being almost stationary when electrophoresed on paper at pH 8·6, containing much protein but having a relatively low N content, and having a small carbohydrate and phosphorus content (the latter might conceivably be due to a strong binding of phosphate from the buffers used in the process). The two preparations were quite different in three respects, however: (1) The preparation from the *in vivo* source tended to aggregate and sediment more rapidly in the ultracentrifuge than that from the *in vitro* source. (2) The preparation from the *in vivo* source contained much lipid (probably serum lipid) whereas the other preparation did not. (3) The preparation from the *in vivo* source immunized animals, whereas the present preparation did not.

The most striking chemical property of factor I of the anthrax toxin was its high and complete metal content, the nature of which was not consistent from batch to batch. Factor I was not an acid, binding the metals by a purely ionic linkage, because it did not migrate rapidly when electrophoresed on paper at pH 8·6. It appeared therefore that factor I had a strong and indiscriminate chelating action on the metals which were present in the original culture medium (this contained $CaCl_2$, $MgSO_4$ and 'Casamino Acids' with a high ash) and in materials (e.g. DEAE–C) used for purification. The chelating action might be due to the presence of the chemical group which absorbed light at 260 mμ and which was not RNA or DNA; this might be the same group that produced in alkaline solution a characteristic 'blip' in the u.v. absorption at about 293 mμ. The nature of this group is at present unknown; the main difficulty in its investigation is the very small amount of material available for experiment. The metal-binding activity of factor I may be the reason for its biological action but this is not proven; a 0·1 % (w/v) solution of EDTA did not replace factor I in the biological assays.

A comparison of the nature of the biological properties of a mixture of final preparation of factor I + purified factor II with those of crude materials led to the demonstration of a third factor of the anthrax toxin. This factor was partially purified from crude factor II produced *in vitro*, and shown to be different from factors I and II and present in the anthrax toxin produced *in vivo* by serological methods. Table 8 summarizes the biological relationship between the three factors. The reason for the decrease in skin reaction when factors I and II were mixed with factor III is not clear. Possibly factor III helps in the dispersion of the other factors and leads to a smaller local action. In contrast to the action of factor III, the addition of serum to factors I and II increased their capacity for local oedema production as well as the mouse lethality, probably by a non-specific protective effect. Although it was shown that mixtures of factors I, II and III showed synergism in mouse toxicity tests, it was impossible to obtain the optimal proportions of the three

factors because of the large amount of material and the number of mice needed for a comprehensive titration. For the same reason, although it was shown that guinea pigs were killed specifically by injecting a mixture of the three factors, the determination of their optimal proportions and the demonstration of synergism in guinea pigs was precluded. The guinea pigs killed by the mixtures of the three factors died with some of the symptoms of oligaemic secondary shock as did guinea pigs dying of infection (Smith *et al.* 1955*b*).

Table 8. *Relationship in toxicity tests of the I, II and III factors of the anthrax toxin*

All toxic effects were neutralized by anthrax antiserum

Factors	Oedema production in skin of rabbits	Lethality for mice
I	Nil	Nil
II	Nil	Nil
III	Nil	Nil
I+II	++++	+
I+III	Nil	Nil
II+III	Nil	++
I+II+III	++	++++
Crude toxin	++	++++

The existence of a third factor of the anthrax toxin possessing the properties described above explains an apparent anomaly in previous results (see Smith *et al.* 1956, Table 2, line 3) when the injection of a mixture of crude *in vitro* antigen + crude factor II prepared *in vivo* killed mice, but did not produce a large skin reaction. The demonstration of three factors of the anthrax toxin leads to speculation as to whether at one time they were joined, at least in a loose complex, and to whether mixtures of the factors would immunize animals better than any individual factor.

Our thanks are due to Mr F. C. Belton for producing the anthrax toxin *in vitro* and to Mr R. Blake for excellent technical assistance.

NOTE ON SEROLOGICAL PRECIPITATION IN GELS AS A CRITERION OF PURITY OF ANTIGENS

By H. Smith, K. Sargeant and J. L. Stanley

In the purification of factors I and II of the toxin of *Bacillus anthracis* (Stanley *et al.* 1960; Sargeant *et al.* 1960) serological precipitation in gels was used as a criterion of purity of antigens. This focused our attention on a problem which we think is evaded in similar studies on bacterial and other products and which warrants some discussion.

A purified product may be obtained which shows no evidence of heterogeneity when examined ultracentrifugally or electrophoretically in the conventional manner. At an arbitrarily chosen concentration, the product may form one major non-

composite (cf. Sargeant *et al.* 1960) line in serological diffusion plates against a strong antiserum. However, this same antiserum may detect many antigenic impurities since it is prepared by hyperimmunizing animals with either the original mixture of antigens, or with the appropriate live organism. Hence, when higher (and occasionally lower) concentrations of the purified product are diffused against this antiserum further faint lines may appear, indicating the presence of antigens in addition to the one forming the major line; these additional antigens can usually be demonstrated in other fractions discarded during the separation of the final product. The difficulty is to know how one can be reasonably certain that the antigens which produce the additional lines are not present in the purified product in large amounts. The fact that, at the concentration examined, these additional lines are fainter than the major line does not necessarily mean that the antigens producing them do not predominate in the product under examination. The antigens in question might be present in large amounts but precipitated feebly in the test system, e.g. they might be poor antigens and hence the serum relatively deficient with their precipitating antibodies. When the serological behaviour of products on gel-diffusion plates is described there is usually no mention of the concentrations at which the material first formed one line and then more lines. Furthermore, if one line were formed, no assessment is made of the purity of the product on the basis of relative weights of the precipitating antigens.

We propose a practical working rule for studies of this kind which, although not foolproof, sets a standard of assessment of impurity on a weight basis comparable with the conventional methods of analytical electrophoresis and ultracentrifugation. Usually in the latter procedures a full examination of the product does not take place because of the large effort involved and especially because of the lack of sufficient material. As a compromise, the material is generally examined at one or two pH values at concentrations near 1 %, w/v. Such examinations can indicate that the product is not grossly heterogeneous but they will not detect less than 5 % of impurity. The object of the following procedure with the gel-diffusion method is to make reasonably certain that extraneous antigens, which are usually more easily detected by this method than by other methods, do not exceed about 5 % of the final product.

The final product is examined in one of the conventional systems of gel diffusion. This should be accurately described and the antiserum used should be the one which will detect the largest number of antigenic impurities. Concentrations of 0·5–1 % to 0·001 % are examined for the production of extraneous lines (cf. Sargeant *et al.* 1960) and then the importance of these lines is assessed by titrations. It is assumed that the line-forming ability of any antigen does not vary in different preparations.

The determination by titration in gel-diffusion plates of the amount of extraneous antigens in a purified product would be a simple matter if pure preparations of these antigens were available for comparison; but usually this is not so, since the extraneous antigens are unknown and may be of no importance except as impurities in the final product. However, if we are aiming at the standard of assessment of impurities described above, quite crude samples of extraneous antigens are suitable for comparative titrations. The aim is to obtain a discarded fraction from the purification process, which in a gel-diffusion system forms a line at a concentration about

20 times less than the lowest concentration of purified preparation which forms this same line against the same antiserum in the same system. If this can be achieved, it means the purified product contains less than about 5% of the extraneous antigen. This contamination may be far less than about 5%, if the sample of extraneous antigen used for comparative titrations contained little of the active material. If at first this 20-fold difference in the titrations cannot be obtained, it might be more profitable to spend time purifying the extraneous antigen sufficiently to attain the desired difference in titrations, rather than to attempt to remove from the purified product what may be a relatively small contamination with extraneous antigen. When the final product, which passes this criterion of purity, is examined chemically, it would be advisable to check that any peculiar chemical attribute is not possessed by the (impure) samples of extraneous antigens.

As an example, the final preparation of factor I of the anthrax toxin produced *in vitro*—described above—was examined by the above procedure. In addition to the line associated with factor I activity, which did not appear to be composite, the final preparation (0·5%, w/v) formed one faint line against 'spore' antiserum (H 533). Against 'antigen' antiserum (H 25) it (0·5%, w/v) formed two faint lines. The faint line formed with antiserum H 533 corresponded with one of the two lines formed with antiserum H 25 and both lines were due to extraneous antigens, since they were formed by smaller amounts of relatively inactive fractions discarded during fractionation of factor I. A short purification of these inactive fractions on diethylaminoethyl cellulose columns produced samples of each extraneous antigen; these were used in comparative titrations with the final preparation of factor I on gel diffusion plates against both types of anthrax antiserum. These titrations indicated that the final preparation of factor I was contaminated with not more than 0·5% of one extraneous serologically-reacting material and not more than 5% of the other.

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Oxidation of *meso*-α,ϵ-Diaminopimelic Acid by Certain Sporulating Species of Bacteria

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SUMMARY

Investigations were made of the transformations undergone by the stereoisomers of α,ϵ-diaminopimelic acid in suspensions of acetone-dried organisms of two species of sporulating bacteria, *Sporosarcina ureae* and *Bacillus sphaericus*, both of which contain diaminopimelic acid in their spores but not in their vegetative cells. *Meso*-diaminopimelic acid was rapidly decarboxylated by vegetative organisms of both species; it was also utilized by some other unidentified anaerobic reaction. The vegetative organisms also oxidized *meso*-diaminopimelic acid with release of ammonia. L-Lysine was oxidized by *S. ureae*, but not by *B. sphaericus*. Neither LL- nor DD-diaminopimelic acid was attacked by either organism.

Disintegrated spores of *Bacillus sphaericus* did not oxidize *meso*-diaminopimelic acid, but decarboxylated it and also utilized it by the unidentified anaerobic reaction. The decarboxylation, but not the oxidation, of diaminopimelic acid by *Sporosarcina ureae* was greatly stimulated by pyridoxal phosphate; both reactions were inhibited by the same compounds. Study of the oxidation was complicated by the side reactions which occurred with *S. ureae*, but a simpler system was provided by an asporogenous variant of *B. sphaericus* which did not decarboxylate diaminopimelic acid without added pyridoxal phosphate. Only one equivalent of ammonia was produced, a small amount of CO_2 was evolved and two equivalents of oxygen were utilized; no oxidation product was identified. The methods of attacking diaminopimelic acid by these two atypical species are compared and discussed in relation to other species in their respective families.

INTRODUCTION

α,ϵ-Diaminopimelic acid differs from the majority of the common natural amino acids in being confined almost exclusively to bacteria where it occurs mainly in the cell walls (Work & Dewey, 1953; Work, 1957*a*, 1961). However, certain of its stereoisomers undergo enzymic reactions resembling those of other amino acids: thus, in many types of bacteria the *meso*-isomer is decarboxylated to L-lysine (Dewey, Hoare & Work, 1954) and is also racemized to the LL-isomer (Antia, Hoare & Work, 1957); all three isomers can transaminate (Meadow & Work, 1958*a*) while L-amino acid oxidases of Neurospora and snake venoms oxidize *meso*- and LL-diaminopimelic acid (Work, 1955). Studies on the decarboxylation of *meso*-diamino-

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pimelic acid by acetone-dried bacteria (Dewey *et al.* 1954) have not so far suggested that many of these preparations can carry out other types of reaction with the amino acid. Thus, the rate of decarboxylation was not usually affected by the presence of oxygen, and the volume of gas evolved, both aerobically and anaerobically, corresponded to that expected from the release of one mole CO_2/mole *meso*-diaminopimelic acid added. In the two species now used, *Sporosarcina ureae* and *Bacillus sphaericus*, neither of these conditions was found; the rate of CO_2 evolution from *meso*-diaminopimelic acid by acetone-dried organisms was apparently lowered by the presence of air, and the amount of CO_2 produced was less than stoichiometric even under anaerobic conditions.

Certain relationships between bacterial classification and the distribution of diaminopimelic acid and of its decarboxylase and racemase have been established in a few families (Work & Dewey, 1953; Antia *et al.* 1957; Dewey, 1954; Hoare & Work, 1957; Cummins & Harris, 1956*a*, *b*; Cummins, 1956). As a consequence, the species studied in this communication can be differentiated from other members of their families by their content of diaminopimelic acid and of enzymes attacking it (Table 2). *Sporosarcina ureae* is a spore-forming member of the family Micrococcaceae. Typical organisms in this family show diaminopimelic acid decarboxylase and racemase activities, but do not contain the amino acid itself (Antia *et al.* 1957; Cummins & Harris, 1956*b*); *S. ureae* has an active decarboxylase but no racemase. As described in this paper it has diaminopimelic acid only in its spores and not in its vegetative cells. *Bacillus sphaericus* is classified with the other aerobic spore-forming bacilli, most members of which contain diaminopimelic acid and its racemase but no decarboxylase. No diaminopimelic acid racemase was found by Powell & Strange (1957) in *B. sphaericus*, but there was a very active decarboxylase; diaminopimelic acid itself was not present in vegetative cells, but was present in the spores.

This paper describes the reactions undergone by diaminopimelic acid in suspensions of acetone-dried vegetative cells of these two species of bacteria which are atypical with respect to diaminopimelic acid content and metabolism. The anomalies of aerobic decarboxylation were due to oxidation, which has not been hitherto observed in bacteria.

METHODS

Organisms. The strain of *Sporosarcina ureae* studied originated from the Microbiological Laboratory, Technical High School, Delft. Another strain from the Edinburgh and East of Scotland College of Agriculture was examined briefly. The organism was subcultured weekly in a medium (called hereafter urea medium) containing urea 0·5% (w/v), Lab-Lemco 1% (w/v), peptone (Oxoid) 1% (w/v), adjusted to pH 7·0 with NaOH and filtered. The organism was grown at room temperature (18°) and cultures were stored at 0°. Other media used for this organism were nutrient broth fortified with tryptic digest of casein (0·5 g. nitrogen/100 ml.) called TMB broth, the CCY medium of Gladstone & Fildes (1940), and the meat extract-peptone of Tarr (1933). Preparations of the vegetative form were grown with shaking at 18°; for each 100 ml. of medium, 1 ml. of a 48 hr. culture in the urea medium was used as inoculum after adjusting to a standard optical density. After 24 or 48 hr. growth, the organisms were harvested by centrifugation,

washed twice with 0·9 % (w/v) NaCl solution and acetone-dried in the cold. They were stored at −15°.

Bacillus sphaericus was var. *fusiformis* NCTC 7582, and its asporogenous variant; these strains were studied by the late Mrs J. F. Powell and her colleagues (Powell & Strange, 1957; Powell 1958; Powell & Hunter, 1955). The organisms were grown at 37° by Mrs Powell in potato-extract medium enriched with CCY (Meadow & Work, 1958*b*); for vegetative preparations the growth period was 10 hr.; for spore preparations it was 48 hr. The harvested organisms were washed three times with water, and either acetone-dried or freeze-dried and stored at −15°. The spores were disintegrated mechanically in a Mickle disintegrator for 40 min. at 0° (20 mg. dry wt./ml.) in the presence of thiolacetic acid (mM) with octyl alcohol as antifoaming agent.

Other organisms. The strains and growth conditions were described by Antia *et al.* (1957).

Enzymic reactions. Decarboxylation and oxidation were measured in Warburg manometers at 37°. In both cases 0·1 M-phosphate buffer (pH 6·8) was used with 20–40 mg. dried organism suspended in a total volume of 2·5 ml. Unless otherwise stated, the substrates (tipped from side arm) were either *meso*-diaminopimelic acid (2 mg.; final concentrations of 4·2 mM) or L-lysine (15 mM); pyridoxal phosphate, when present, was 10 μM. Decarboxylation was carried out in an atmosphere of N_2; when gas evolution had ceased, M-citric acid (0·1 ml.) was tipped in from a second side arm to release CO_2 from solution (Hoare & Work, 1955). Oxidation was carried out in air with 20 % KOH (0·2 ml.) and filter paper in the centre well. Control flasks without substrate were included, and were corrected for when calculating the final reaction rates. Carbon dioxide output (Q_{CO_2}) was expressed as μl. CO_2 produced/mg./hr. (not corrected for gas retention), and oxygen uptake (Q_{CO_2}) as μl. O_2 taken up/mg./hr.

The manometric balance experiments were carried out essentially according to the method described under 'direct method for estimation of CO_2' (Umbreit, Burris & Stauffer, 1957). Pairs of flasks were set up, aerobically and anaerobically, containing either KOH or water in the centre well; this was done in the presence and in absence of synthetic diaminopimelic acid (8 mg./2·8 ml.). By this means the amounts of CO_2 evolved by oxidation and of O_2 absorbed were calculated. At the same time, the suspensions were incubated in open flasks with identical proportions of diaminopimelic acid, and samples were withdrawn at intervals for estimation of ammonia and diaminopimelic acid.

Examination of reaction mixtures. When not investigated immediately, the final reaction mixtures from the Warburg flasks were frozen rapidly and stored at −15°. Measured amounts of the thawed mixtures were examined as required.

Paper chromatography. The reaction mixtures were usually deproteinized and freed from organisms by treatment with 2 vol. ethanol and centrifugation before paper chromatography without preliminary hydrolysis; under certain circumstances the mixture was used after removal of the organisms by centrifugation only. The equivalent of 0·1 ml. of reaction mixture was examined by two-dimensional chromatography on Whatman no. 4 paper using as solvents aqueous phenol (NH_3 atmosphere) and *n*-butanol (4)+acetic acid (1)+water (5). Alternatively, 33 μl. was examined for diaminopimelic acid on one-dimensional chromatograms on

no. 1 paper with the solvent methanol (80) + pyridine (10) + 10 N-HCl (2·5) + water (17·5) (Hoare & Work, 1955). Amino acids were revealed by dipping the chromatograms in ninhydrin in acetone (0·1 %, w/v) and heating at 102°. When required, hydrolysis of reaction mixtures or of bacteria was carried out with 6 N-HCl for 24 hr.

Estimation of diaminopimelic acid. Ninhydrin in strong acetic acid was used to estimate diaminopimelic acid colorimetrically (Work, 1957*b*). For the balance experiments, synthetic diaminopimelic acid (mixture of *meso*, LL- and DD-isomers) was used, in preference to the *meso* isomer (the only form decarboxylated), in order to decrease the amount of reaction mixture necessary to give a measurable colour, and so to avoid high blank values due to the intracellular amino acids. Two flasks were used, one contained diaminopimelic acid, the other was a control. The mixtures were shaken at 37°, and at intervals samples were deproteinized with an equal volume of acetic acid and centrifuged after coagulation was complete; 0·1 ml. of the supernatant solution was mixed with water (0·4 ml.), acetic acid (0·5 ml.) and ninhydrin reagent (0·5 ml.). Ninhydrin reagent b was used (Work, 1957*b*); it consisted of ninhydrin (A.R. Grade) 250 mg., acetic acid 6 ml., 0·6 M-phosphoric acid 4 ml. The solutions were heated at 100° for 2 min., cooled and diluted with acetic acid (3·5 ml.); the optical density at 440 mμ was read against the mixture from the control flask. A standard curve was constructed by adding known amounts of synthetic diaminopimelic acid to the deproteinized contents of the control flask. The short heating time of 2 min. was used to minimize colour formation from intracellular amino acids and also from lysine formed by decarboxylation of *meso*-diaminopimelic acid. Estimations on other reaction mixtures were carried out by essentially the same method, with slight differences introduced according to conditions.

Estimation of ammonia. The reaction mixture (0·5 ml.) was aerated for 1 hr. at room temperature in the presence of 4 ml. water + 1 ml. saturated K_2CO_3 + 1 drop triamylcitrate. The ammonia carried over was trapped in 15 ml. of 0·04 N-H_2SO_4 and measured colorimetrically after treating 5 ml. of the distillate (diluted with an equal volume of water) with Nessler's reagent (0·5 ml.). The optical density at 450 mμ, read within 2 min. against that of a reagent blank, was compared against that of a standard curve previously constructed with solutions from known amounts of ammonium sulphate treated identically. The ammonia contents of the control suspensions incubated without added substrate were subtracted from those of the test solutions.

Qualitative examination for keto-acids. Keto-acids were examined in the reaction mixtures by a modification of the method of el Hawary & Thompson (1953). Some of the yellow dinitrophenylhydrazones of keto-acids were apparently unstable in the K_2CO_3 solution used to extract them from ethyl acetate, therefore the alkaline extractions were carried out at 0° and the extracts neutralized immediately with cold 3 N-HCl. After re-extraction into ethyl acetate and drying over anhydrous K_2CO_3, the extracts were examined by ascending paper chromatography in *n*-butanol (70) + ethanol (10) + 0·5 N-ammonia (20).

Examination for dipicolinic acid. This was carried out as suggested by Powell & Strange (1957); after deproteinization of the reaction mixtures with perchloric acid and dilution with 50 volumes of buffer (pH 7·3) the light absorption between 240 and 280 mμ was measured in the Beckmann spectrophotometer.

RESULTS

Sporosarcina ureae

Diaminopimelic acid in Sporosarcina ureae. Both strains of *S. ureae*, examined shortly after their arrival in the laboratory, yielded organisms which contained small amounts of diaminopimelic acid: subsequently, no diaminopimelic acid was found in whole or fractionated organisms grown in liquid culture. One culture, grown on solid medium, contained a trace of diaminopimelic acid and was found to consist of a mixture of vegetative organisms and spores. All further attempts in our laboratory to produce sporulation of either strain failed, but later Powell & Hunter (private communication) succeeded once in obtaining spores from the Edinburgh strain. These spores contained *meso*-diaminopimelic acid, the vegetative organisms had none.

Table 1. *Degradation of* meso-*diaminopimelic acid and* L-*lysine by acetone-dried* Sporosarcina ureae

Experiments were carried out in Warburg manometers with 40 mg. dried organism in total volume of 2·5 ml. 0·1 M-phosphate buffer (pH 6·8). Anaerobic experiments in nitrogen, aerobic in air with KOH in centre cup. Pyridoxal phosphate when present was 10 μM. Other conditions as in Methods.

					Manometric experiment							
	Culture				*meso*-Diaminopimelic acid						L- Lysine	
	Growth conditions				Anaerobic			Aerobic			Aerobic	
Expt. no.	Medium	Time (hr.)	Yield (g. dry wt./l.)	Pyridoxal phosphate	Q_{CO_2}	$V^*_{CO_2}$	Lag (min.)	Q_{O_2}	$V^*_{O_2}$	$V^*_{H_3}$	Q_{O_2}	$V^*_{O_2}$
1	Urea	24	1·46	+	9·3	92	.	.	.	.	3·0	87
				0	3·4	.	.	.	.	.	3·0	87
1	Urea	48	1·45	+	8·7	89	15	3·8	.	.	5·5	87
				0	4·5	.	.	.	.	.	5·5	94
2	Urea	48	1·10	+	6·9	77	16	2·3	106	.	.	.
				0	5·4	56	16	2·0	117	38	.	.
3	CCY	24	1·01	+	8·8	87	30	1·3	>142	.	0·6	.
3	CCY	48	1·60	+	8·6	94	19	1·6	>165	36	0·6	.
4	CCY	48	1·92	+	9·3	78	18	2·6	154	50	3·7	.
				0	7·4	63	20	2·5	157	50	.	.
5	TMB broth	48	1·48	+	7·7	85	37	0·8	32	21	.	.
				0	3·1	40	15	0·3	.	.	.	.
6	Tarr spore broth	48	0·73	+	7·0	86	27	0·7	.	.	.	.
				0	4·3	54	24	0·7	.	.	.	.

* Volume of reactants used or produced expressed as % of theoretical, based on 1 equivalent/mole amino acid present.

Q_{CO_2} = μl. CO_2 evolved/hr./mg. dry wt. Q_{CO_2} = μl. oxygen absorbed/hr./mg. dry wt.

Anaerobic transformations of diaminopimelic acid. Acetone-dried organisms from both strains of *Sporosarcina ureae* grown on urea medium decarboxylated *meso*-diaminopimelic acid; the rate with the Delft strain was about double that with the Edinburgh strain. Thereafter all experiments were carried out with the Delft strain. The rate of decarboxylation did not vary greatly with change in growth conditions

(Table 1); the highest activities (Q_{CO_2} = 9·3) were observed with organisms grown either on urea medium or on CCY medium. Omission of pyridoxal phosphate from the Warburg flasks lowered the decarboxylation rate. There was no decarboxylation reaction with LL-diaminopimelic acid, indicating the absence of diaminopimelic racemase (Antia *et al.* 1957).

The identifiable products of reaction were CO_2 and lysine. The organisms contained no lysine decarboxylase; an equimolar volume of CO_2 should therefore have been liberated from *meso*-diaminopimelic acid, but this was seldom the case (Table 1). Gas evolution stopped at 80–90 % of the theoretical value when pyridoxal phosphate was added, and at 40–80 % of theoretical without added pyridoxal phosphate. In spite of this, only 1–5 % of the original *meso*-diaminopimelic acid remained in the final reaction mixtures. Additional *meso*-diaminopimelic acid caused resumption of gas evolution, even in the absence of added pyridoxal phosphate. These results suggested that, in addition to decarboxylation, *meso*-diaminopimelic acid was undergoing some anaerobic reaction which was not stimulated by pyridoxal phosphate. Paper chromatography showed that the diaminopimelic acid unaccounted for had not been incorporated as a peptide or other form liberated by hydrolysis, either in the soluble or the insoluble portions of the reaction mixtures.

Aerobic breakdown of diaminopimelic acid. When the decarboxylation of *meso*-diaminopimelic acid by acetone-dried *Sporosarcina ureae* was carried out in air, the gas output was slower and lower than under anaerobic conditions (Fig. 1, curves A and B). In spite of this diminished CO_2 production, both diaminopimelic acid and lysine disappeared completely from the aerobic reaction mixture, while no other amino acids were produced. When the CO_2 evolved was absorbed by KOH, a steady uptake of gas occurred after a short lag entailing a slight gas output (curve D). A gas uptake also occurred with L-lysine (curve E and Table 1), but there was no lag. No gas uptake occurred with LL-diaminopimelic acid (curve C), DD-diaminopimelic acid, D-lysine, L-alanine, L-glutamic acid or glucose. It was evident that oxidative reactions had occurred with both *meso*-diaminopimelic acid and L-lysine. In the case of diaminopimelic acid, simultaneous oxidation and decarboxylation could result in curves such as B and D; in curve B where no KOH was present, oxygen uptake would produce a decrease in overall gas evolution. The preliminary lag in curve D can be attributed to the inability of KOH to absorb all the CO_2 released by decarboxylation in the first few minutes; when KOH was present during anaerobic decarboxylation of *meso*-diaminopimelic acid, there was also a considerable delay in the absorption of CO_2, but finally there was no over-all change in volume. There is, however, a possibility that only lysine was oxidized by the preparation, and that the lag in oxygen uptake with diaminopimelic acid was due to preliminary decarboxylation to lysine. The reactions were therefore studied further. Fresh suspensions or broken-cell suspensions could not be used because of their high endogenous respiration rate; in acetone-dried organisms this was not unduly high (Q_{O_2} = 1·1–1·8 for organisms grown on CCY medium, 0·5–0·8 for other organisms), and was allowed for in calculating oxidation rates of the various substrates.

The rate of oxidation of *meso*-diaminopimelic acid was raised only slightly by the addition of pyridoxal phosphate (Table 1), that of lysine was unaffected. The rates

and extents of oxygen consumption varied with the conditions of growth, particularly in the case of L-lysine, but they also varied from one preparation to another of organisms grown under the same conditions. The total oxygen consumed was usually between 80 and 100% of the theoretical value calculated for one atom of oxygen taken up by one molecule of either amino acid; but in some cases, particularly when the organisms were grown on CCY medium, the oxygen consumed

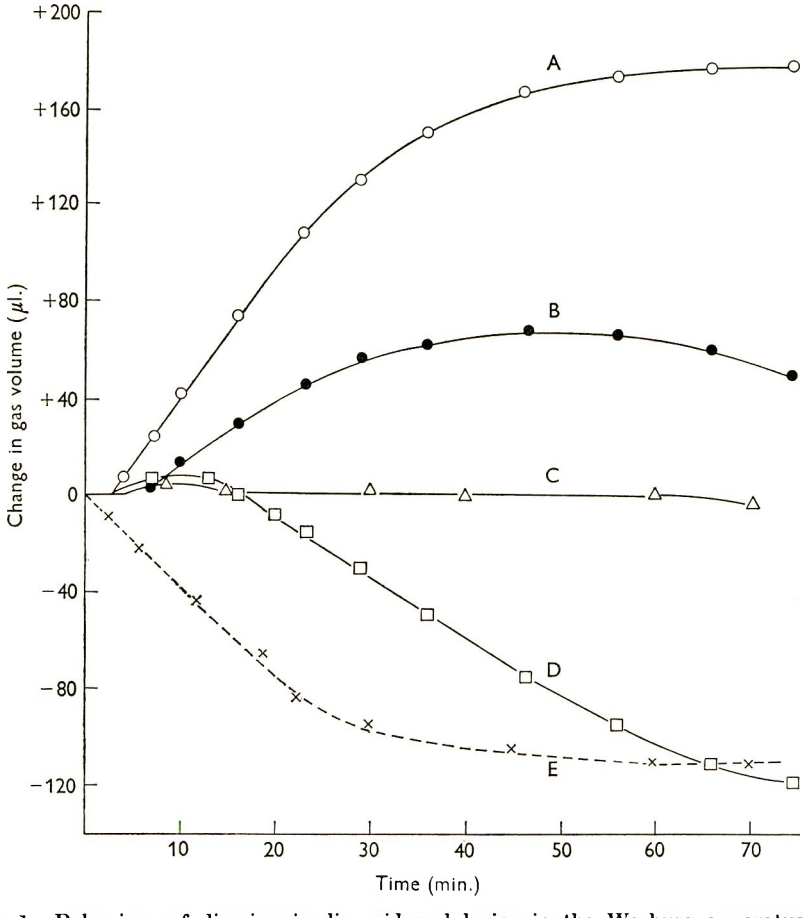


Fig. 1. Behaviour of diaminopimelic acid and lysine in the Warburg apparatus in presence of acetone-dried *Sporosarcina ureae*. Each flask contained 40 mg. dried organism (grown 48 hr. in urea medium) in 0·1 M-phosphate buffer (pH 6·8); total volume 2·5 ml.; present 10μM pyridoxal phosphate. Substrates tipped from side arms were as follows: curves A, B and D, *meso*-diaminopimelic acid (4·2 mM); curve C, LL-diaminopimelic acid; curve E, L-lysine (5·5 mM). Curve A, atmosphere N_2; curve B, atmosphere air, no KOH present; curves C, D, E, atmosphere air, KOH present. Changes in volume were corrected for those in control flasks without substrate.

by *meso*-diaminopimelic acid exceeded this value. There was no constant relation between the rates of oxidation of L-lysine and the decarboxylation of *meso*-diaminopimelic acid by different batches of organisms. The minimum concentrations of *meso*-diaminopimelic acid and L-lysine required to produce maximum oxidation rates were 2 and 15 mM, respectively.

The effects of inhibitors on the decarboxylation and oxidation of *meso*-diaminopimelic acid were investigated in the hope that decarboxylation might be inhibited specifically. Compounds which bind thiol or carbonyl groups inhibit diaminopimelic acid decarboxylase from *Aerobacter aerogenes* (Dewey *et al.* 1954; Hoare, 1956). Both oxidation and decarboxylation by *Sporosarcina ureae* were inhibited to the same extent by any one of these inhibitors.

Ammonia was always produced by these aerobic reactions, whereas none was produced anaerobically. Ammonia could only be estimated reliably in the products from *meso*-diaminopimelic acid oxidation; in the case of L-lysine, duplicate estimations did not agree, suggesting that a reaction product was unstable under the

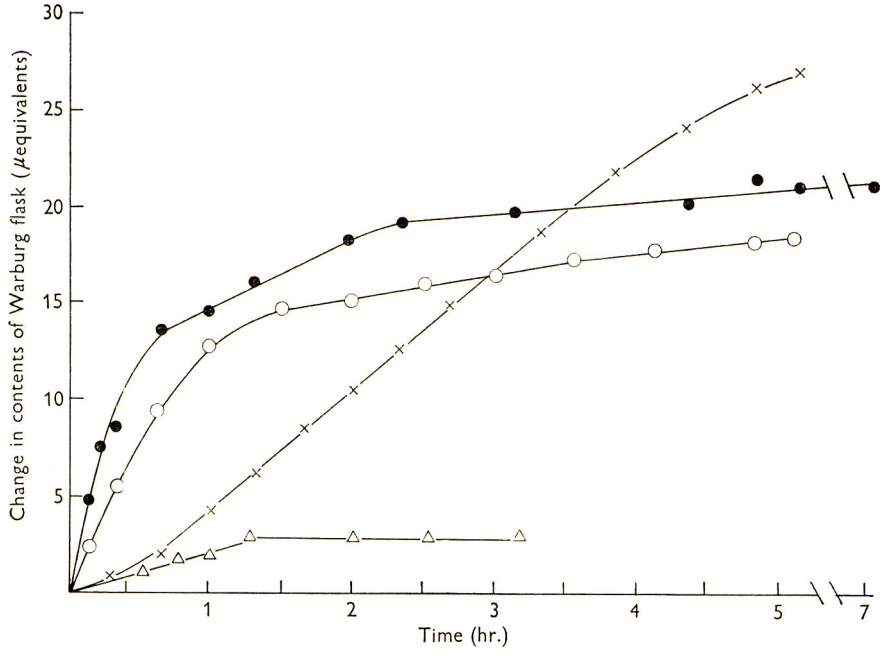


Fig. 2. Balance experiment showing aerobic decomposition of *meso*-diaminopimelic acid by acetone-dried *Sporosarcina ureae*. Final reaction mixture contained dried organism (grown CCY medium for 48 hr.) 1·6 mg./ml. in 0·1 M-phosphate buffer (pH 6·8) containing 10 μM pyridoxal phosphate. Pairs of Warburg flasks were used containing total volumes of 2·8 ml.: in one flask of each pair there was 8 mg. (42 μmole) of synthetic diaminopimelic acid, the other contained no substrate. In two pairs the reaction was carried out aerobically; KOH (0·2 ml. of 20 %) was in the centre well of only one pair. In a third pair of flasks KOH was present but the atmosphere was N_2. The same suspension was also incubated in two open flasks, with and without diaminopimelic acid, at the same concentration as in the Warburg flasks; samples were withdrawn at intervals for estimation of ammonia and diaminopimelic acid. Changes in gas volumes were calculated as in Umbreit *et al.* (1957). Results are expressed as change in amounts of reactants in a Warburg flask. ×——× = oxygen taken up; ○——○ = CO_2 evolved (not corrected for retention in solution); ●——● = diaminopimelic acid disappearing; △——△ = ammonia formed.

alkaline conditions of the ammonia distillation. The amount of ammonia produced during oxidation of diaminopimelic acid bore no constant relation to the oxygen uptake or to the amount of diaminopimelic acid utilized, but the molar ratio of

oxygen or diaminopimelic acid consumed to ammonia produced was always greater than unity (Table 1).

When the reaction products from diaminopimelic acid oxidation were examined by chromatography in methanol + water + pyridine + HCl and revealed with ninhydrin, a yellow spot having a pink fluorescence in ultraviolet radiation was often observed just behind the solvent front. The substance giving this spot has not been identified; it was not dipicolinic acid or piperidine-2:6-dicarboxylic acid, but it might have been a keto-acid, as suggested by the colour and fluorescence of its ninhydrin reaction product on paper (Rabson & Tolbert, 1958). Examination of the keto-acid dinitrophenyl hydrazones in the reaction mixtures showed very small amounts of oxoglutaric and pyruvic acids and also of an unidentified keto-acid with R (pyruvate) = 0·7; these were insufficient in amount to account for the diaminopimelic acid used.

Balance experiments were carried out, as described in methods, with synthetic diaminopimelic acid. One such experiment is illustrated in Fig. 2. During the first 40 min., in which rapid decarboxylation took place, diaminopimelic acid disappeared rapidly; after this it was utilized more slowly, finally reaching a constant value representing 50 % of the original amount present (this is the probable proportion of LL- and DD-isomers in a synthetic mixture). A steady uptake of oxygen continued even after utilization of diaminopimelic acid had stopped; it had not appreciably slowed by the end of the experiment (5 hr.), when 1·25 atom of oxygen had been used per mole of diaminopimelic acid consumed. This high oxygen consumption, typical of organisms grown on CCY medium, showed that in these cells, at any rate, the oxygen uptake was due in part to a secondary reaction. Ammonia production had only reached 2·6 μmole at 3 hr. when 20 μmole diaminopimelic acid had disappeared.

Degradation of diaminopimelic acid by dried cells of other species of bacteria

In the family Micrococcaceae, the marked stimulation by pyridoxal phosphate of anaerobic decarboxylation of *meso*-diaminopimelic acid was not peculiar to *Sporosarcina ureae*, although the low output of CO_2 was more specific. For example, with *Sarcina lutea*, although the decarboxylation rate was doubled by added pyridoxal phosphate, the CO_2 output was 97 % of theoretical even in the absence of added pyridoxal phosphate. Aerobic experiments were difficult to carry out on most Micrococcaceae, because of their high endogenous respiration, even after acetone-drying. With acetone-dried *Sarcina lutea*, this rate was not altered by synthetic diaminopimelic acid or L-lysine; *Staphylococcus citreus* had $Q_{O_2} = 8{\cdot}0$ with no added substrate or with L-lysine, and in the presence of *meso*-diaminopimelic acid $Q_{O_2} = 8{\cdot}3$.

The majority of Bacillaceae do not decarboxylate *meso*-diaminopimelic acid (Antia *et al.* 1957); the known exceptions being *Clostridium tetani* and *Bacillus sphaericus*. *C. tetani* decarboxylated *meso*-diaminopimelic acid faster under anaerobic conditions ($Q^{N_2}_{CO_2} = 3{\cdot}0$) than in presence of air ($Q^{air}_{CO_2} = 1{\cdot}4$). Even anaerobically, the gas output stopped after only 50 % of the theoretical amount of CO_2 had been evolved. This suggests that *C. tetani* utilized diaminopimelic acid by the unidentified anaerobic reaction and by oxidation, but no further work was done with this organism.

Bacillus sphaericus. The metabolism of diaminopimelic acid by *B. sphaericus* has been studied in detail, and is described here and elsewhere (Powell & Strange, 1957; Meadow & Work, 1958*b*). Diaminopimelic acid decarboxylase activity was very high in freeze-dried and acetone-dried vegetative *B. sphaericus*, and was greatly stimulated by pyridoxal phosphate, rates of the order of $Q_{CO_2}^{N_2} = 30$ being found under optimal conditions. Anaerobic decarboxylation of *meso*-diaminopimelic acid evolved suboptimal amounts of CO_2, especially in the absence of pyridoxal phosphate when only about 60% of the theoretical volume was produced (Fig. 3A, curve 1).

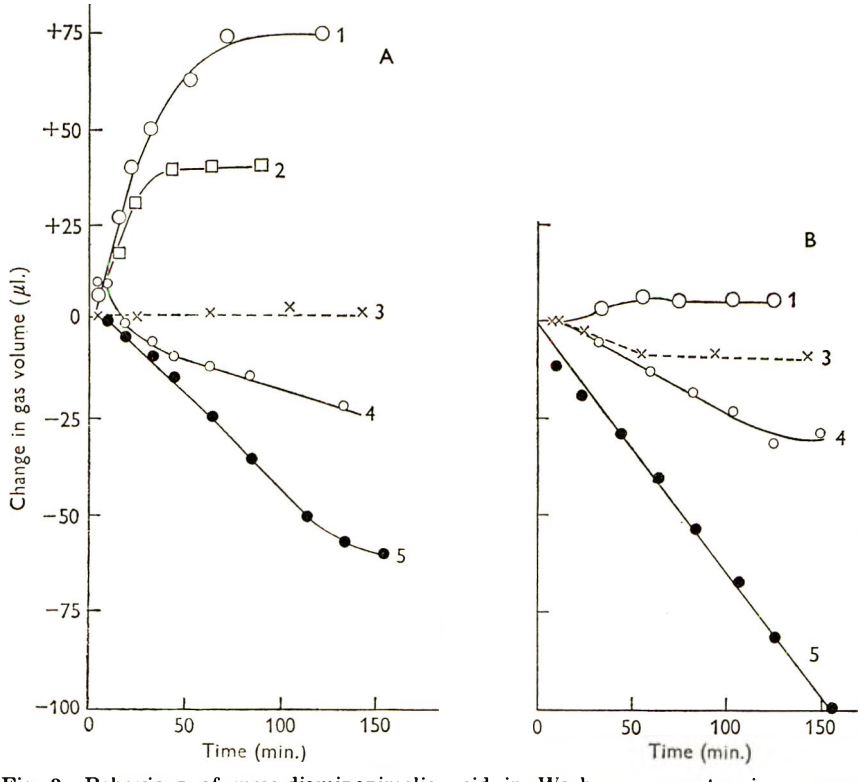


Fig. 3. Behaviour of *meso*-diaminopimelic acid in Warburg apparatus in presence of acetone-dried *Bacillus sphaericus*. Each flask contained a suspension of dried vegetative organisms (20 mg.) in 0·1 M-phosphate buffer (pH 6·8); final volume 2·5 ml. A; *B. sphaericus*, var. *fusiformis* NCTC 7582, normal strain. B; Asporogenous variant of this strain. All solid curves represent substrate *meso*-diaminopimelic acid (2 mg.), ×---× = substrate L-lysine (6 mg.). Curve 1, decarboxylation, atmosphere N_2, no KOH; curve 2, atmosphere air, no KOH; curves 3 and 5, oxidation, atmosphere air, KOH present; curves 1, 2, 3 and 5, no pyridoxal phosphate added. Curve 4, oxidation, atmosphere air, KOH present, pyridoxal phosphate (10 μM) added.

The rate of gas evolution was almost unaffected by the presence of air, but in this case the volume of gas evolved was lower (Fig. 3A, curve 2). In all experiments, 95–100% of the added diaminopimelic acid disappeared; with the exception of lysine no other amino acids were formed. No evidence for binding of diaminopimelic acid was obtained. Aerobic experiments in the presence of KOH resulted in slow gas uptakes with *meso*-diaminopimelic acid (Fig. 3A, curves 4 and 5). These

results resembled those found in acetone-dried *Sporosarcina ureae*, and suggested that in *B. sphaericus meso*-diaminopimelic acid was subjected to both the unidentified anaerobic reaction and oxidation. However, owing to very rapid decarboxylation, even in the absence of pyridoxal phosphate, the direct observation of oxidation of diaminopimelic acid was even more difficult than in the case of *S. ureae*. On the other hand, *B. sphaericus* did not oxidize L-lysine (Fig. 3A, curve 3), so that any observed oxygen uptake could be attributed directly to diaminopimelic acid even if decarboxylation were occurring at the same time. The oxygen uptakes in the absence of substrate were negligible with acetone-dried organisms. Disintegrated suspensions of fresh spores of *B. sphaericus* showed lower values for diaminopimelic acid decarboxylase ($Q_{CO_2} = 4{\cdot}1$) than did the vegetative organisms. Neither the rate nor the extent of decarboxylation by spores was affected by the presence of oxygen or of pyridoxal phosphate; the volume of CO_2 evolved was always about 60% if theoretical. Diaminopimelic acid was evidently not oxidized by spores, but the decarboxylase was active and fully saturated with pyridoxal phosphate; the unidentified anaerobic reaction also occurred.

The asporogenous variant of *Bacillus sphaericus* is known to have no diaminopimelic acid decarboxylase activity (even when acetone-dried) unless pyridoxal phosphate is added (Meadow & Work, 1958*b*). In the absence of this coenzyme, the unknown anaerobic reaction evidently also does not occur, since *meso*-diaminopimelic acid was unchanged in concentration after anaerobic incubation for 5 hr. with acetone-dried cell suspensions (Work, 1957*b*). However, *meso*-diaminopimelic acid was oxidized (Q_{O_2} between 2·0 and 3·0), whereas L-lysine and the other isomers of diaminopimelic acid were not attacked (Fig. 3B). The gas uptake with *meso*-diaminopimelic acid started immediately, without a preliminary lag (Fig. 3B, curve 5). Since pyridoxal phosphate had already been found to have little effect on the oxidation of diaminopimelic acid by *Sporosarcina ureae*, it was decided that the asporogenous variant of *B. sphaericus* would be a good material for a study of the reaction.

The pH optimum for the oxidation of *meso*-diaminopimelic acid by a suspension of acetone-dried *Bacillus sphaericus* (asporogenous) lay between 6·8 and 7·4, outside this range a rapid fall in reaction rate was noted (at pH 6·0 and 7·8 the rates were only 14% of the maximum).

Balance experiments showed that the reaction with asporogenous *Bacillus sphaericus* was more straightforward than in the case of *Sporosarcina ureae*, as the amounts of ammonia produced and oxygen taken up were proportional to the diaminopimelic acid utilized. For example, in an oxidation, not carried to completion, of 20·6 μmole synthetic diaminopimelic acid by 30 mg. acetone powder, in 165 min. 5·5 μmole ammonia were produced, 5·8 μmole of diaminopimelic acid disappeared and 13 μequivalents of oxygen were taken up. In another experiment, in 45 min., 2 μ equivalents of oxygen were used by 20 mg. of acetone powder and 0·6 μmole CO_2 produced; during the same period, only 0·2 μmole CO_2 was evolved anaerobically. No dipicolinic acid was formed during the aerobic reaction. There were trace amounts of the keto acid with a dinitrophenyl hydrazone having a mobility of R (pyruvate) = 0·7 on paper chromatograms.

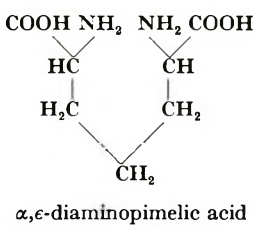
These experiments indicate that the reaction involved in *meso*-diaminopimelic acid oxidation by the asporogenous strain of *Bacillus sphaericus* is probably oxidative removal of one amino group, followed perhaps by oxidative splitting of the

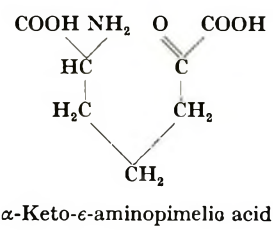
carbon chain or by decarboxylation. Another batch of asporogenous cells was grown, but the preparation showed very weak oxidase activity, although it still retained an active apodecarboxylase. Since this organism has been found to change very much in successive subculture (Meadow & Work, 1958*b*), it was not considered to be suitable for further study of diaminopimelic acid oxidation. The work has been temporarily abandoned.

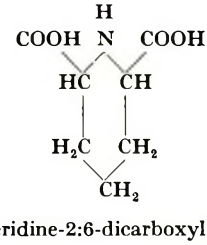
DISCUSSION

The experiments described showed that *meso*-diaminopimelic acid, but not the LL- or DD-isomers, is oxidized by acetone-dried vegetative organisms of two species of sporulating bacteria which are atypical in their contents of diaminopimelic acid and in their methods of utilizing this substance (summarized in Table 2). This stereochemical specificity of the oxidation, easily demonstrable owing to the unusual absence of diaminopimelic acid racemase from the organisms in question, distinguishes the reaction from the less stereospecific oxidations which both the LL- and *meso*-isomers undergo with L-amino acid oxidases from Neurospora or snake venoms (Work, 1955). The reaction probably involves a deamination, but whether this is the primary reaction is not yet known. For example, a transamination, catalysed by small amounts of keto acids found in the organisms, could remove one amino group, and the resulting α-keto-ε-aminopimelic acid might then be oxidized with or without loss of its amino group. The stereochemical specificity of the oxidation does not support this theory, since both LL- and *meso*-diaminopimelic acid are transaminated by the organisms in question (see Meadow & Work, 1958*a*, for transamination by *Bacillus sphaericus*). The virtual independence of added pyridoxal phosphate of the oxidative reaction by *Sporosarcina ureae* also suggests that it was not connected with transamination, which requires high concentrations (40 μM) of this coenzyme in acetone-dried organisms. The neutral pH value at which the oxidation occurs also does not favour transamination.

The failure to find significant amounts of keto acids in the reaction mixtures is not contra-indicative of an oxidative deamination, since α-keto-ε-aminopimelic acid would be expected to cyclize spontaneously by condensation of the α-keto and ε-amino groups, as in the case of α-keto acid resulting from oxidation of lysine (Meister, 1954). α-Keto-ε-aminopimelic acid is known to be an intermediate in the biosynthesis of LL-diaminopimelic acid in *Escherichia coli*, but in this case it is present as the *N*-succinyl derivative and is thereby protected against cyclization (Gilvarg, 1960).


α,ε-diaminopimelic acid


α-Keto-ε-aminopimelic acid


Piperidine-2:6-dicarboxylic acid

Neither piperidine-2:6-dicarboxylic acid, a reduced product of ring closure, nor dipicolinic acid, the fully unsaturated derivative which occurs in bacterial spores (Powell, 1953), was identified among the reaction products.

Although *Sporosarcina ureae* and *Bacillus sphaericus* are organisms belonging to different families, their methods of utilizing diaminopimelic acid (Table 2) are sufficiently similar to enable them to be compared and to be differentiated from other organisms in their respective families. *Clostridium tetani* may be a similar exception: it is the only other member of the Bacillaceae known to decarboxylate diaminopimelic acid and sometimes to lack it in its vegetative cells.

Table 2. *Comparison of certain characteristics in typical and atypical members of families* micrococcaceae *and* bacillaceae

	Micrococcaceae		Bacillaceae		
Characteristic	Typical species	*Sporosarcina ureae*	Typical species	*Bacillus sphaericus*	*Clostridium tetani*
DAP* in vegetative cells	−	−	+	−	Not invariably
DAP in spores	No spores	+	+	+	?
DAP decarboxylase†	+	+	−	+	+
Alternative anaerobic reaction†	−	+	?	+	+
DAP racemase†	+	−	+	−	Slight
DAP oxidation†	−	+	−	+	Possible

* Diaminopimelic acid. † Reactions investigated in acetone-dried vegetative organisms.

Diaminopimelic acid is an important constituent of the mucopeptide of the cell walls of many Gram-positive bacteria such as Bacillaceae; whenever it is absent, as in the Micro coccaceae, it is replaced in the wall by lysine (Cummins & Harris, 1956*b*). In the case of *Bacillus sphaericus*, Powell & Strange (1957) found that the soluble mucopeptides obtained by enzymic degradation of walls of vegetative organisms and spores had similar compositions except for the presence of lysine in the former and diaminopimelic acid in the latter. They also showed variations in the cellular activities of diaminopimelic acid decarboxylase throughout the growth and sporulation cycle, finding a marked decrease in activity in ageing cultures coincident with the appearance of spores and soluble mucopeptides containing diaminopimelic acid. We found that, in contrast to the vegetative cells, spores of this organism did not oxidize diaminopimelic acid.

It is possible that diaminopimelic acid is synthesized in vegetative cells of both *Bacillus sphaericus* and *Sporosarcina ureae*, but that before it can be inserted into the walls it is degraded by the three types of enzymes present in these cells. It is notable that the activities of decarboxylase found in normal dried vegetative cells of *B. sphaericus* and of *S. ureae*, are respectively, 10 and 3 times higher than those found in most other bacteria (Antia *et al.* 1957); however, considerably lower quantities were present in the asporogenous variant of *B. sphaericus*, which also has lysine in its cell wall. Nothing is known of the metabolic determinant which causes the change from a vegetative cell containing lysine in its wall mucopeptides to a spore containing diaminopimelic acid.

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Interaction of an Endotoxin with Cationic Macromolecules

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SUMMARY

The interaction of the anionic lipopolysaccharide complex isolated from a Gram-negative bacterium (*Serratia marcescens*) was studied in aqueous and saline solution with several cationic macromolecules, with the object of selectively inhibiting certain of the biological activities of this polysaccharide endotoxin. Incubation with lysozyme decreased the pyrogenic activity, while the tumour-damaging ability of this polysaccharide remained high. This was in contrast to interaction with RNase and with polymyxin B, in which cases the tumour-damaging activity was decreased while the pyrogenic activity was not affected. Ultracentrifuge experiments indicated that both the tumour-necrotic and especially the fever-producing fractions of this bacterial polysaccharide preparation are of much higher molecular weight ($> 10S$) than the major component ($3{\cdot}4S$), which is either inactive or of low activity. The highest molecular weight fever-producing components appeared to be broken down in an enzymic type of process by the lysozyme.

INTRODUCTION

The endotoxins from Gram-negative bacteria are high molecular weight lipopolysaccharide complexes which exhibit a whole array of biologic properties when injected into animals in microgram quantities: they are toxic and antigenic, produce fever, cause localized haemorrhage and necrosis in prepared skin sites and in tumours, etc. (cf. Burrows, 1951; Thomas, 1954). There is no *a priori* reason why all of these different biological properties should be caused by the same morphological or physico-chemical entity. For example, it might be that those components or molecular sites in the complex which cause the tumour-damaging activity are not the ones which cause the fever. However, prior attempts to separate the physico-chemical entities responsible for these different biological activities have all resulted in failure and no fractionation or other method has been found to be effective (Rathgeb & Sylven, 1954; Malmgren, 1954; Creech, Koehler, Havas, Peck & Andre, 1954). The general opinion developed that the biological activities are just different manifestations of host reactions to the same physico-chemical entity. When hydrolysis or some other means of splitting the endotoxic lipopolysaccharide complex was tried it led to a total loss of the activities (Rathgeb & Sylven, 1954; Ikawa, Koepfli, Mudd & Niemann, 1952, 1954; Merler, Perrault, Saroff & Mora, to be published). Only when a certain macromolecular size and complexity was maintained, after gentler treatment, were the biological activities retained (Fromme, Luderitz, Nowotny & Westphal 1958; Westphal *et al.* 1958; Ribi *et al.* 1960).

It occurred to us that selective or preferential macromolecular interaction with different components, or sites, of the bacterial polysaccharide preparations might

separate some of these biological properties, while maintaining the necessary macromolecular size.

We reported previously on the blocking of enzyme activity and of toxicity through interaction of oppositely charged macromolecules (Mora & Young, 1958; Mora, Young & Shear, 1959). This approach has been extended to the study of the interaction of a bacterial lipopolysaccharide preparation from *Serratia marcescens* of negative net charge near neutral pH, with positive (cationic) macromolecules. The cationic macromolecules used were polymyxin B, ribonuclease, lysozyme and protamine. Changes in pyrogenic potency and tumour damaging activity were then examined. There was previous evidence that cationic macromolecules affect some of the biological properties of endotoxin preparations (Fisher, 1959; Neter *et al.* 1958). We also studied the possibility of reversal of some of these changes of pyrogenic and tumour damaging activities, when a strong anionic synthetic polysaccharide preparation (polyglucose sulphate) was also added. This was expected to break up the interaction of the macromolecules by preferentially forming a complex with the cationic macromolecules and to restore the biological activity of the anionic bacterial polysaccharide. We found that interaction with lysozyme was qualitatively different from interaction with the other cationic macromolecules; there also was indication of a simultaneous enzymic action.

METHODS

Materials

The endotoxic polysaccharide complex from *Serratia marcescens* (referred to from now on as 'bacterial polysaccharide') was preparation P-25 of A. Perrault, obtained by a modification of the method of Shear & Turner (1943). Fractionation and chemical experiments (Rathgeb & Sylven, 1954*a*, *b*), electrophoretic and ultracentrifuge studies (Malmgren, 1954) have been carried out previously on this preparation, as well as extensive biological studies.

The cationic macromolecules used were: polymyxin B (Pfizer no. 5×070), crystalline ribonuclease (Armour no. 381–059), protamine sulphate (Nutritional Biochemicals Corp. no. 9193), and lysozyme. Lysozyme was used either as obtained commercially (Nutritional Biochemicals Corp. nos. 1417 and 7545) which gave a neutral solution or, when so specified, in a form which we will designate 'basic lysozyme'. This 'basic lysozyme' was obtained from the commercial preparation by bringing to pH 11·4 with dilute NaOH in an aqueous solution and then dialysing this solution in heat-treated cellophan and freeze-drying the residue; this 'basic lysozyme' upon re-solution in water gave a pH of 9.

The anionic polysaccharide derivative was a sulphated polyglucose prepared from a chemically synthesized polyglucose (number average mol. wt. about 20,000) by treatment by chlorosulphonic acid (Prep. H; Wood & Mora, 1958). The polyglucose sulphate contained three sulphate groups per anhydroglucose unit and had intrinsic viscosity (η) = 0·04. The sodium salt or the acid form was used.

Interaction

Interaction of the cationic macromolecules with the bacterial polysaccharide was achieved by mixing dilute aqueous solutions followed by incubation for different

lengths of time. A representative example was as follows: 100 μg. bacterial polysaccharide plus the appropriate amount of basic protein was incubated in 5 ml. pyrogen-free water at 37° for 16 hr. The solution was then diluted with pyrogen-free water for assay of tumour-damaging potency. In the reversal experiments, a concentrated aqueous solution of polyglucose sulphate was added to the incubated aqueous mixture of bacterial polysaccharide and basic protein, further incubated for 1 hr. at 37°, diluted with water and assayed. In the pyrogenic test the final diluent was 0·9 % NaCl solution.

Bioassays

Tumour-damaging potency was measured in mice bearing 6-day intramuscular implants of Sarcoma 37, using a method which, with minor variations (Shear, Perrault & Adams, 1943; Landy & Shear, 1957) has been routinely employed in this Laboratory for many years. A few micrograms of the bacterial polysaccharide usually produced, within 24 hr. after intraperitoneal injection, haemorrhage and necrosis in the tumours. In the untreated controls the tumours showed no haemorrhage. Affected tumours were those which showed extensive, freshly induced haemorrhage. A minimum of ten mice was used for each dose level. The minimum effective dose (ED50; the dose which produced extensive damage in tumours of half of the mice) was 5 μg.; this value was reproducible within about $\pm$ 50 %.

Pyrogenic activity was measured in rabbits by observing the elevation in rectal temperature under conditions previously described (Landy & Shear, 1957). Lysozyme (16 mg.) was mixed with 20 μg. bacterial polysaccharide, and in the reversal experiment, in addition, with 24 mg. polyglucose sodium sulphate in 1 ml. solution of pyrogen-free water. These aqueous mixtures were incubated for different periods of time at 37°. Thirty minutes before the injection, these concentrated mixtures were diluted with pyrogen-free 0·9 % NaCl solution, and 1 ml. samples were injected into the ear vein of the rabbit. The temperature change was then recorded. Four rabbits were used for each dose level. The minimum pyrogenic dose (MPD) of the bacterial polysaccharide was 0·006 μg. ($\pm$ 50 %); this produced at least 1° F. fever.

The enzymic potency of lysozyme was determined by measuring the decrease of optical density during the lysis of a suspension of the ultraviolet-treated *Micrococcus lysodeicticus* (Skarnes & Watson, 1955). The lysis was complete in 30 min. The assay provided a method with an error of about 5 %.

Chemical methods

The concentration of bacterial polysaccharide was determined by the anthrone procedure, comparing the absorbency at 620 mμ with that of solutions of known concentration.

Lysozyme or ribonuclease concentrations, in interaction complexes with the bacterial polysaccharide, were estimated after adding salt to bring the mixtures to 0·9 % NaCl concentration (which was sufficient to dissolve the precipitates) and then measuring the absorbency of the clear solutions at 280 mμ and calculating the protein concentration from calibration data. The absorbency due to the bacterial polysaccharide was below the average experimental error (about 5 %), and was not used as a correction factor.

Dialysis experiments were carried out in du Pont 400 'gel' cellophan against running distilled water for 64 hr.

Ultracentrifuge experiments

All analytical centrifugation was performed with the Model E Spinco ultracentrifuge using schlieren optics. In the ultracentrifuge experiments the bacterial polysaccharide concentration was 1 % in 0·154 M-sodium chloride at 25°. Sedimentation constants are expressed in Svedberg units $S = S^{\circ}_{20w} \times 10^{-13}$ cm./sec. after correction to water at 20° and using the value of 0·6095 for partial specific volume of the bacterial polysaccharide. The moving platform cell and method of Yphantis & Waugh (1956*a*, *b*) was used in the partition cell analysis experiments.

In the partition cell analysis the centrifugation was at top speed (59,780 rev./min.), and the acceleration and deceleration of the rotor was carefully recorded. After centrifugation the supernatant (upper half), subnatant (lower half of the solution which remained below the partition), rinse of the lower half of the cell, and also an uncentrifuged control solution, were all bioassayed for tumour-damaging activity. Serial dilutions were carried out until the tumour-damaging activity showed a linear decrease with dilution, and the ED 50 was determined. The amount of the original tumour-damaging activity present in the particular portion of the cell (upper half, lower half, lower rinse) was then estimated from such data. The pyrogenic activity of these solutions was then similarly determined. In the uncentrifuged control solution the tumour-damaging activity and the pyrogenic activity was present in the expected ratio (ED 50 (tumour) 5 μg./mouse, MPD (fever) 0·006 μg./rabbit). From bioassays of the supernatant the sedimentation constant was calculated for both the tumour-damaging and for the fever-producing material. The partition cell analysis essentially followed the method suggested by Yphantis & Waugh (1956*b*) and the calculations took into account the integrated angular velocity during acceleration and deceleration as well as during top speed centrifugation (cf. equations 1–4, Yphantis & Waugh, 1956*a*). The calculated value for the sedimentation constant of the biologically active material was found to vary $\pm 0{\cdot}5S$ if ± 100 % error was assumed in the bioassays.

RESULTS

Biological and chemical evidence for interaction

The bacterial polysaccharide at 10–15 μg./mouse caused severe damage in the tumours of most of the mice (Table 1). The same amount of bacterial polysaccharide affected only a small percentage of the tumours after incubation for 30 min. with ribonuclease, polymyxin B, or protamine. Similar incubation with lysozyme, however, led to an opposite result: the tumour-damaging effect of the bacterial polysaccharide increased. The data presented in Table 1 are representative of many such experiments, and also illustrate the reproducibility of the tumour assay.

When polyglucose sulphate was added to the solution containing the bacterial polysaccharide and the polymyxin or RNase, and the incubation was continued for another $\frac{1}{2}$ hr., the decrease in tumour-damaging activity was reversed, and the bacterial polysaccharide essentially recovered its original tumour-damaging activity.

In the case of the solution with lysozyme, the increase in tumour-damaging activity was reversed after further incubation with polyglucose sulphate, and the activity decreased below that of the control.

Table 1. *Tumour-damaging effect of* Serratia marcescens *polysaccharide and the effect of incubation with cationic macromolecules and polyglucose sulphate*

Bacterial polysaccharide before incubation			After incubation with: Cationic macromolecule			After incubation with: Cationic macromolecule and then with polyglucose sulphate		
	Damaged tumours			Damaged tumours			Damaged tumours	
mg.	no.	%	mg.	no.	%	mg.	no.	%
0·015	17/20	85	1 ribonuclease	2/19	10	0·75	11/20	55
0·015	17/20	85	1 ribonuclease	4/19	21	0·5	19/20	95
0·015	37/40	92	1 ribonuclease	12/39	31	0·5	20/20	100
0·015	17/20	85	0·5 ribonuclease	6/20	30	0·5	19/20	95
0·010	17/20	85	0·25 ribonuclease	2/15	13	0·5	15/15	100
0·015	17/19	89	0·4 polymyxin-B	1/16	6	0·5	15/20	75
0·005	7/15	47	2 lysozyme	15/15	100	1	2/10	20

Lysozyme abolished the pyrogenicity of ten times the amount of the minimum pyrogenic dose of the bacterial polysaccharide, while no such effect appeared after incubation with the other cationic macromolecules. However, the concentration of the bacterial polysaccharide had to be sufficiently high (20 μg./ml.) and the incubation had to proceed for a sufficient length of time (1–2 weeks) to obtain consistent and large decrease in pyrogenicity. Bacterial polysaccharide (20 μg.) and commercial lysozyme (16 mg.) were incubated together in 1 ml. of water at 37° for different lengths of time; Fig. 1 gives the average fever curves obtained with various dilutions of this mixture. The control curves were obtained with solutions of the bacterial polysaccharide incubated at 37° without lysozyme.

The presence of sodium polyglycose sulphate abolished lysozyme's ability to reduce the fever-producing activity of the bacterial polysaccharide. When lysozyme (16 mg.) and sodium polyglucose sulphate (24 mg.) were admixed in this ratio (which was known to inhibit completely the enzyme activity; Mora & Young, 1959), and this mixture was then incubated with the bacterial polysaccharide (20 μg.) in 1 ml. water, the fever-producing activity of the bacterial polysaccharide was retained even after incubation for 1 or 2 weeks at 37° (Fig. 2). Apparently the polyglucose sulphate complexed with the lysozyme, as indicated by the appearance of white turbidity, and the action of the lysozyme on the bacterial polysaccharide was inhibited.

Lysozyme decreased the tumour-damaging effect of the bacterial polysaccharide, but to a smaller extent: to about one-half of the original bacterial polysaccharide activity after 2 weeks' incubation at 37°. The bacterial polysaccharide itself did not lose activity after similar incubation.

Thus, the effect of lysozyme on the bacterial polysaccharide was different from the other cationic macromolecules, and indicated that two processes might be

involved: first, an immediate effect, probably due to macromolecular interaction, and secondly, a slower, probably enzymic reaction. The following experiments were carried out to clarify the nature of the lysozyme—bacterial polysaccharide interaction.

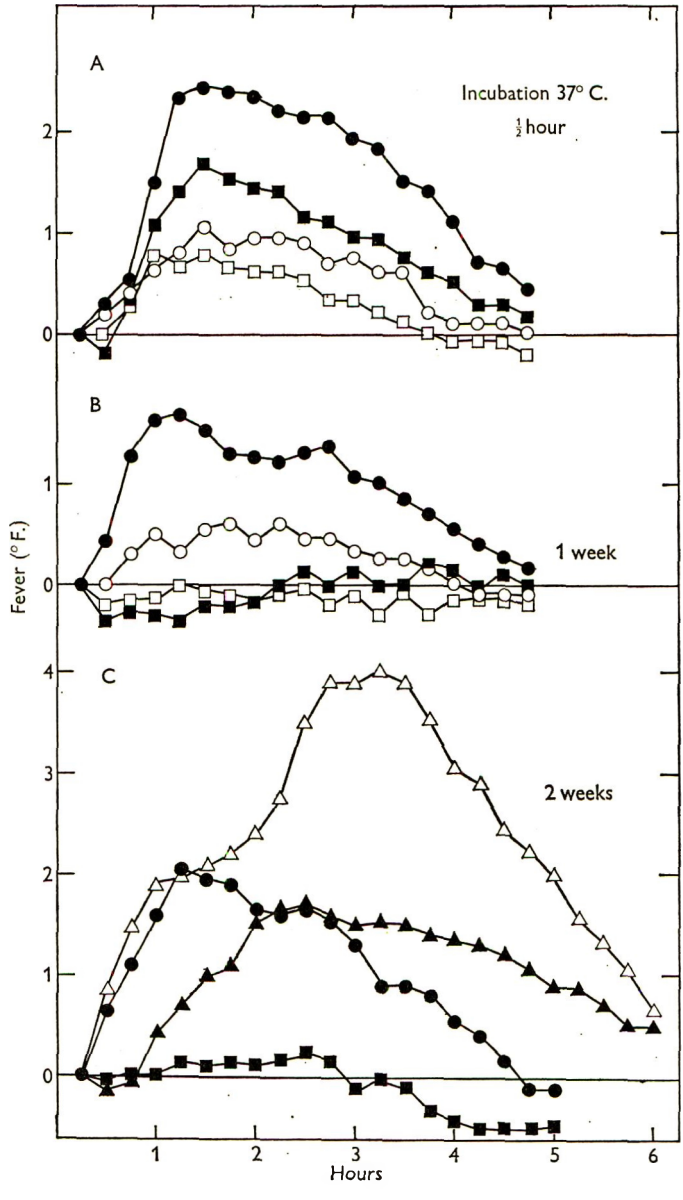

Fig. 1. Pyrogenicity in rabbits of the bacterial polysaccharide after different periods of incubation (37°) with lysozyme. Each point represents average fever from four rabbits after injection. A. Incubation ½ hr., ○ = 0·01 μg. polysaccharide/rabbit, □ = 0·01 μg. polysaccharide +8 μg. lysozyme, ● = 0·1 μg. polysaccharide, ■ = 0·1 μg. polysaccharide +80 μg. lysozyme. B. The same after incubation for 1 week. C. After 2 weeks incubation, △ = 1 μg. polysaccharide, ▲ = 1 μg. polysaccharide +800 μg. lysozyme; ● and ■ as in A.

The bacterial polysaccharide (10 mg.) was titrated at 0° in aqueous solution with 'basic lysozyme'. Figure 3 shows the changes in pH and in optical density. Neutralization occurred after 1·5–2 times as much lysozyme as polysaccharide was added, and also in this same range the optical density (turbidity) increased at a rapid rate. After 28 mg. lysozyme had been added (see arrow in Fig. 3) an aliquot of the suspension was set aside for further studies (see below). The titration was

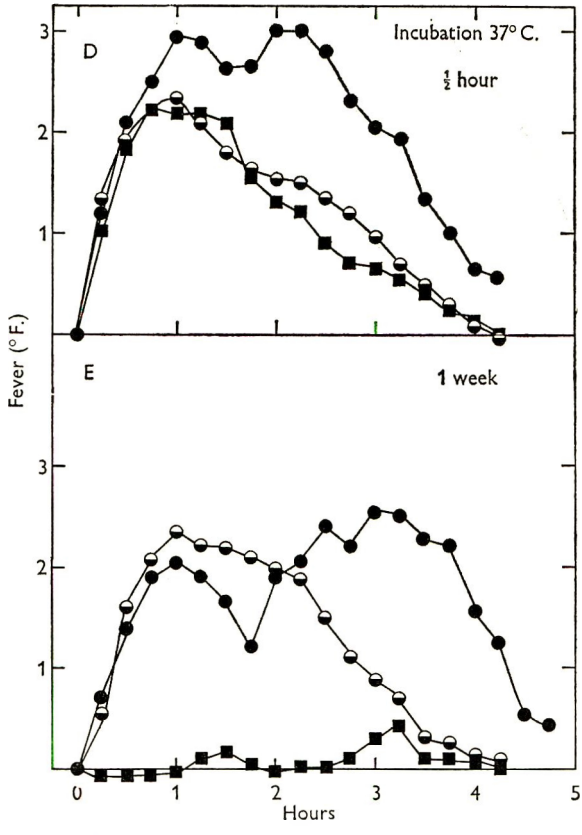

Fig. 2. Inhibition by polyglucose sulphate of lysozyme's ability to decrease pyrogenicity. D. Incubation ½ hr., 37°, ● = 0·1 μg. bacterial polysaccharide, ■ = 0·1 μg. polysaccharide +80 μg. lysozyme, ◒ = 90 μg. lysozyme +120 μg. polyglucose sulphate sodium salt + 0·1 μg. polysaccharide. E. The same after 1 week incubation. Notice that the effect of lysozyme (■) was inhibited by polyglucose sulphate (◒). Results were similar after 2 weeks incubation.

continued on the remainder but there was no indication of further neutralization or precipitate formation (dashed line, Fig. 3). When sodium chloride was added to the final turbid suspension containing the excess lysozyme (establishing 0·1 N-NaCl conc.) the precipitate rapidly dissolved (D = 0·154) indicating dissociation of the complex. Similar immediate clearing occurred after adding a few drops of N-NaOH (D = 0·076, pH = 12·7).

One part of the aqueous suspension titrated to the point indicated by the arrow in Fig. 3 was centrifuged to collect the insoluble precipitate. To another part of the

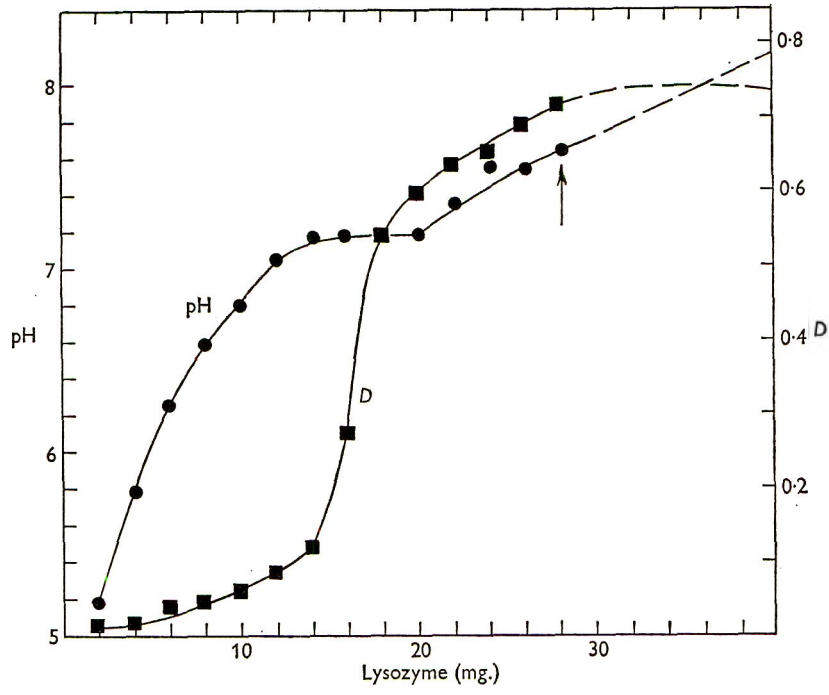

Fig. 3. Titration of bacterial polysaccharide (10 mg.) with 'basic lysozyme' in aqueous solution at 0°; changes in pH, and in optical density (*D*) at 640 mμ.

suspension solid sodium chloride was added to establish 0·154 M-concentration before centrifugation, thereby bringing about a dissociation by the salt similar to that which would be expected upon injection into body fluids. The supernatant fluids and the sediments from both the aqueous and from the saline solution were then analysed chemically for bacterial polysaccharide and for lysozyme concentration, and also

Table 2. *Interaction of bacterial polysaccharide with basic lysozyme in aqueous and in saline solutions; separation of material and of biologic activity into soluble and sedimenting fractions*

10 mg. bacterial polysaccharide titrated with 28 mg. basic lysozyme in aqueous solution

pH | 7·6

H_2O | Saline

32,000 *g* | 30 min. | 32,000 *g* | 30 min.

	Supernatant		Sediment		Supernatant		Sediment	
	Chemical	Bio-assay*	Chemical	Bio-assay	Chemical	Bio-assay	Chemical	Bio-assay
	% recovery							
Bacterial polysacch.	31	50	58	50	78	100	22	0
Lysozyme	51	24	57	24	84	60	16	0

* Bioassay of bacterial polysaccharide by tumour-necrotic potency. Determinations were carried out in serial dilutions and on sufficient tumour-bearing animals to make the estimate valid ±10 %.

Bioassay of lysozyme by enzymic potency, see p. 83.

tested biologically for tumour-damaging potency and for lysozyme activity on *M. lysodeicticus* (Table 2).

The sediment obtained from the preparation in water contained about half of the bacterial polysaccharide with unimpaired tumour-damaging potency. Half of the lysozyme was also in this sediment, but the enzymic activity decreased to about one-quarter of the total. In the supernantant fluid the bacterial polysaccharide had high potency, while lysozyme activity was only one-half of what would have been expected on the basis of the chemical concentration, indicating that a soluble complex was present, but without decrease of the tumour-damaging activity of the bacterial polysaccharide in such a complex.

In saline solution, 22 % of the bacterial polysaccharide was present in the sediment without having demonstrable tumour-damaging activity, but still all the starting tumour necrotic potency was accounted for in the remaining 78 % concentration in the supernatant fluid. Lysozyme enzymic activity was somewhat decreased in the supernatant fluid, while there was no activity in the precipitate.

Dialysis experiments were carried out on aqueous and saline solutions of the bacterial polysaccharide interaction product with excess lysozyme, under conditions in which all the free lysozyme was demonstrated to be eliminated in a control experiment. Table 3 gives the final ratios in the supernatant fluids after dialysis against water and saline solution. Similar data are included on the dialysis of the interaction products of the bacterial polysaccharide and ribonuclease. In both aqueous and saline media the ratio of interacted (non-dialysable) bacterial polysaccharide to lysozyme or to ribonuclease was about one to two.

Table 3. *Dialysis of interaction products of the bacterial polysaccharide with basic lysozyme and with RNase in water and in saline solution*

	Interaction in water		Interaction in 0·9 % N_2Cl	
	Ratio in dialysate* of bacterial polysaccharide to:			
	Lysozyme	Ribonuclease	Lysozyme	Ribonuclease
Dialysed: against water	$\frac{1}{1·9}$	$\frac{1}{1·8}$	$\frac{1}{1·4}$	$\frac{1}{1·9}$
against saline	$\frac{1}{1·9}$	$\frac{1}{2·6}$	$\frac{1}{1·9}$	$\frac{1}{2·2}$

* The portion remaining after dialysis.

Partition cell analysis

Ultracentrifuge sedimentation of the bacterial polysaccharide shows a major component with 3·4*S* and several faster sedimenting components (6·9, 10·5 and 11·5*S*) of much higher molecular weights, with the second summit being at 10·5*S* (Fig. 4A).

Three partition cell analyses were carried out. In the first experiment the bacterial polysaccharide was centrifuged until all the higher molecular weight components passed into the lower half of the cell, but some of the slowest sedimenting material was retained in the upper half (Fig. 4B). In the second experiment the solution was centrifuged for a shorter time, which resulted in retaining also some of the faster sedimenting components in the upper half of the cell (Fig. 4C).

In the third experiment an interaction product of bacterial polysaccharide and lysozyme was centrifuged.

When the bacterial polysaccharide (10 mg.) interacted in aqueous solution (1 ml.) with basic lysozyme (28 mg.), as in the titration experiment above, a precipitate formed, which largely dissolved when the sodium chloride concentration was brought to 0·154 M. The small amount of residual insoluble material was separated by low speed centrifugation (2150 *g*) and the supernatant was kept for 16 hr. at 4°. After this the supernatant fluid had only two slow-moving components in the analytical cell, with 1·92*S* and 3·86*S*, and no faster sedimenting components (Fig. 4D).

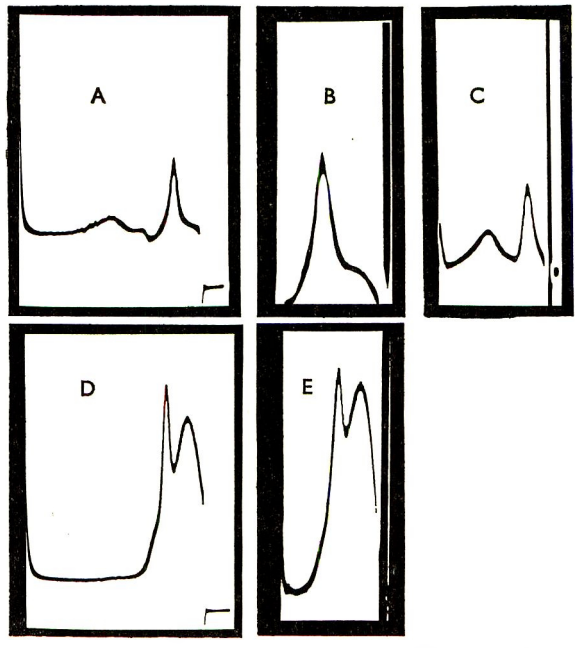

Fig. 4. Sedimentation of bacterial polysaccharide and of the complex with lysozyme in the ultracentrifuge. In all experiments bacterial polysaccharide conc. was 1 % in 0·154 M-NaCl; temp. 25°; centrifugation at top speed (59,780 rev. min.). Sedimentation from right to left. A. Bacterial polysaccharide in analytical cell after 30 min. B. Same in the moving partition cell. Photograph taken during deceleration at 2000 rev. min. after the moving partition re-occupied its median position; the lower half of the cell is dark because the light is blocked out by the supports of the partition. Total effective sedimentation time corrected to top speeds 66 min. C. The same. Total effective sedimentations time 20·70 min. D. Bacterial polysaccharide and lysozyme (orig. conc. 2·8 %) after 30 min. at top speed. E. The same in the moving partition cell during deceleration at 2000 rev. min. Total effective sedimentation time 41·27 min.

In the third partition cell experiment the supernatant from the low-speed centrifugation of the bacterial polysaccharide + basic lysozyme mixture was sedimented to the extent shown in Fig. 4E.

In each partition experiment solutions from the upper and lower half of the cells, careful saline rinses of the lower halves of the cells and also uncentrifuged controls were refrigerated, and the next day were assayed for tumour-damaging and for fever-producing activity. Results are summarized in Table 4.

In the first partition experiment (Fig. 4B) at a dilution which in the uncentrifuged control would have given seventy times the ED 50, the supernatant had no detectable tumour-damaging activity; and also had no pyrogenic activity at a dilution which would have had 1200 times the MPD. All of the tumour-damaging activity and a large amount of the fever-producing activity were recovered, however, from the lower part of the cell. The appearance of the schlieren pattern indicated that a considerable amount of the slower sedimenting component (3·4*S*) was still present in the supernatant. Chemical determination was not possible because of the low level of the bacterial polysaccharide.

Table 4. *Fractions of the total biological activities recovered from compartments of the partition cell*

These values were determined by serial dilution and finding the approximate ED 50 values for the tumour-damaging activity and the MPD values for pyrogenicity and by comparing these to the controls.

Expt. (Fig.)	1 (4B)		2 (4C)		3* (4E)	
Activity	Tumour	Fever	Tumour	Fever	Tumour	Fever
Supernatant fluid	$< \frac{1}{70}$†	$< \frac{1}{1200}$†	$\frac{1}{7}$	$\frac{1}{7}$	$\frac{1}{20}$	$\frac{1}{20}$
Subnatant fluid	$\frac{6}{7}$	Pyrogenic†	$\frac{5}{7}$	$\frac{5}{7}$	$\frac{10}{20}$	$\frac{10}{20}$
Rinse	$\frac{1}{7}$	Pyrogenic†	$\frac{1}{7}$	†	$\frac{4}{20}$	†
Plug removed before centrifuging	—	—	—	—	$\frac{0{\cdot}5}{20}$	Approx. $\frac{1}{2}$†

* Bioassays carried out 1–6 days after the ultracentrifugation.

† Sufficient experiments were not carried out to find the ED 50 or the MPD value, since the available concentration was insufficient.

These results indicated that the active components sedimented rapidly and that the slow sedimenting component (3·4*S*) was not pyrogenic, and that it was also not tumour damaging, when the assays were carried out at the maximum available concentration.

In the second partition experiment where some of the more rapidly sedimenting material remained in the supernatant fluid (Fig. 4C), the supernatant fluid had about one-seventh of the original tumour damaging and also of the pyrogenic activity, while the residual activities were found in the lower half of the cell. On the basis of the bioassays of the supernatant fluid, sedimentation constant calculations by the method of Yphantis & Waugh (1956*a*, *b*) gave a figure of $15{\cdot}7 \pm 0{\cdot}5S$ for both the tumour-damaging and fever-producing activity.

In the third partition experiment (Fig. 4E), in which the bacterial polysaccharide–lysozyme solution was sedimented, bioassays showed that the fraction of the original tumour-damaging and pyrogenic material which remained in the top half of the cell was 1/20, giving a calculated sedimentation constant of $8{\cdot}7 \pm 0{\cdot}3S$ for both of

these activities. In the insoluble material, which was removed before the partition cell analysis, there was only one-fortieth of the tumour-damaging potency, while the pyrogenic activity was high.

DISCUSSION

The tumour-damaging activity of the anionic bacterial polysaccharide was decreased when it was mixed in aqueous solution with typical cationic macromolecules, such as ribonuclease or polymyxin B. Furthermore, this reduction was annulled when an anionic macromolecule (polyglucose sulphate), with stronger dissociating groups and higher negative charge density than the bacterial polysaccharide, was added. These phenomena indicated direct complexing, and uncoupling, of charged macromolecules. In the first step the cationic macromolecules presumably blocked the sites, or components, in the bacterial polysaccharide responsible for its tumour-necrotizing potency. In the second, the stronger anionic polyglucose sulphate formed complexes with the cationic macromolecule, and thus liberated the tumour-damaging site of the bacterial polysaccharide. This mechanism is similar to the one we proposed on the reversible inhibition of enzymes (Mora & Young, 1959), and on the blocking of toxicity (Mora, Young & Shear, 1959).

Lysozyme, however, did not decrease the tumour-damaging activity of the bacterial polysaccharide at first; on the contrary, the tumour-damaging activity was somewhat enhanced when lysozyme and the bacterial polysaccharide were incubated for short periods. Apparently the complexing in this instance was with different components, or sites, of the bacterial polysaccharide, from those involved in the case of the other cationic macromolecules. There was no doubt of immediate complex formation: titration and precipitation curves (Fig. 3) indicated neutralization at a weight ratio of 1·5–2 lysozyme to bacterial polysaccharide, and the lysozyme was present in about the same ratio in non-dialysable complex both in water and in saline (Table 3). Electrostatic complexing with lysozyme must have left the tumour-damaging component or site free to exert its biological action. Addition of polyglucose sulphate to the complex of lysozyme and the bacterial polysaccharide decreased the tumour-damaging effect below that of the bacterial polysaccharide control. The fever-producing component was concentrated in the insoluble interaction product, recovered by low-speed centrifugation (Table 4, expt. 3).

Lysozyme was different from other cationic macromolecules in another respect, it reduced pyrogenicity of the bacterial polysaccharide but only through a slow process, taking 1–2 weeks to show a marked effect. Pyrogenic activity of the bacterial polysaccharide at ten times the minimum pyrogenic dose was completely abolished, and a considerable reduction of fever activity occurred even at 160 times the minimum pyrogenic dose (cf. Fig. 1C). It was necessary to have a relatively high bacterial polysaccharide concentration during incubation. These two features (a slow process, and high substrate concentration increasing the rate of the process) suggest that the reduction of pyrogenic activity occurred through an enzymic process, probably through slow breakdown of certain portions of the bacterial polysaccharide. This enzymic type of activity of lysozyme was blocked by polyglucose sulphate, as expected on the basis of our previous work on lysozyme inhibition (Mora & Young, 1959).

The above findings suggested that different sites or species of the bacterial polysaccharides were responsible for the tumour-damaging and for the fever-producing activity.

Two approaches were used to investigate further the heterogenicity of the bacterial polysaccharide. One was partial precipitation with lysozyme and the study of distribution of the tumour-damaging activity between the supernatant and the sediment upon centrifugation; the other was partition cell analysis of both tumour-damaging and pyrogenic activities.

The precipitation and centrifugation method indicated that about 22% of the bacterial polysaccharide, which sedimented in saline solution, was devoid of, or at least, had very low, tumour-damaging activity; it also confirmed the fact that both soluble and insoluble complexes are formed with lysozyme in aqueous solution (cf. Table 2).

The results of the partition cell analysis of the bacterial polysaccharide showed that both the pyrogenic and the tumour-damaging activities were associated with components which had a high sedimentation rate ($> 10S$), and which probably were large macromolecular complexes. The major component of the bacterial polysaccharide ($3{\cdot}4S$) was not pyrogenic in the maximum dose tested ($1200 \times$ MPD). This component also had lower tumour-damaging activity than the original ($< 1/70$). The limitations of the available quantities for tumour-damaging assay and the limited sensitivity of this assay does not allow further conclusion from this experiment.

The faster sedimenting components did not show up in ultra-centrifugation after the treatment with lysozyme (Fig. 4D). Apparently, these were the components which were preferentially precipitated, and also probably these were the ones digested eventually by the lysozyme. The supernatant fluid of the interaction product showed two slow sedimenting components (Fig. 4, D and E). One of these might be a breakdown product, the other an association complex of the slow sedimenting component with lysozyme.

The ultracentrifuge experiments did not give information on the actual size of the active components. It is possible that the sites which were responsible for pyrogenic or tumour-damaging activity were of much smaller size than the sedimentation constant indicates, and they were only incorporated rather firmly in a larger macromolecular entity which, when broken down by hydrolysis, resulted in complete loss of the biological activities.

It should be kept in mind that the enzymic type of digestion of the high molecular weight fever-producing components was not completely selective, since after 2 weeks' incubation there was also some decrease of tumour necrotic activity, to about one-half of that of the original polysaccharide. Also, our data do not justify the assumption of completely independent physico-chemical entities, one type being purely pyrogenic, the other tumour-damaging. The separation and the different behaviour of the functions was demonstrated, of course, only to the limits of the sensitivity of our biological assays.

The above reported experiments, however, indicated: that two of the biological activities (the pyrogenic and the tumour-damaging activity) of the bacterial polysaccharide complex from *Serratia marcescens* probably are caused by different physico-chemical entities; and that they behave differently in macromolecular

interaction and separation of them is a possibility in an active form when macromolecular size is retained. However fractionation on a preparative scale should be carried out to prove this last point.

The original observations on pyrogenicity were carried out at the suggestion of, and in collaboration with, Dr F. Rosen. Dr W. Carroll and Dr H. Kahler helped with the ultracentrifuge experiments. Mr Ellis Sheets provided experimental assistance. We would like to thank Dr M. J. Shear for his interest throughout this work.

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Spore Formation and 'Dimorphism' in the Mycobacteria

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SUMMARY

In studying 8 strains of *Mycobacterium tuberculosis* and 7 strains of atypical mycobacteria all 15 were found to produce, in addition to the typical acid-fast cells, non acid-fast ones, which gradually developed intracellular spore-like bodies; later free-lying spores were seen in the same cultures. This process occurred in heavily inoculated Löwenstein–Jensen medium cultures, which were at least 8 weeks old and were frequently aerated during incubation. With the atypical mycobacteria it occurred more readily in cultures in Kirschner fluid medium than on solid media. When the cultures containing spores were inoculated on nutrient agar plates, endospore-forming, rapidly growing organisms were obtained, which were not acid-fast. These organisms when obtained from independent cultures of the same strain appeared to be identical in bacillary and colonial morphology at their first isolation on nutrient agar, but the organisms from different strains showed variation in these characters. Thus mycobacteria appear able to grow in two different forms: (*a*) form 1, which is acid-fast and multiplies by fission only; (*b*) form 2, which is not acid-fast, produces endospores regularly and can be maintained in pure culture on nutrient agar. A series of phases of development of form 2 cells in the cultures of form 1 organisms in serial smear examination of Löwenstein–Jensen medium cultures is described. It is suggested that mycobacteria might be considered as dimorphic organisms in the same sense as some of the human pathogenic fungi are known to be dimorphic. Evidence is submitted that form 2 organisms are not contaminants.

INTRODUCTION

During experiments on the bacillary morphology of Mycobacteria (Csillag, 1960, 1961) and *Nocardia asteroides*, smears containing structures which resembled bacterial endospores or some sorts of fungal spores were occasionally seen. Such structures were found in the cultures of 17 of 24 strains of *Mycobacterium tuberculosis*, 20 of 42 strains of atypical mycobacteria (8 of group I, Runyon, 1959; 1 of group II; 7 of group III; 4 of group IV) and all of 4 strains of *N. asteroides*. When cultures containing these 'spores' were inoculated on nutrient agar plates, pure growths of spore-bearing bacilli or coccobacilli were obtained within 2 days, none of which were acid-fast. At first these organisms were considered as contaminants, but the possibility that they were derived from the acid-fast mycobacteria and nocardia was suggested by the following observations: (1) There were differences in the morphology of the spore-bearing, non acid-fast forms obtained from the different species of mycobacteria and nocardia. Thus, strains of *M. tuberculosis* always yielded Gram-

negative organisms, whereas the atypical mycobacteria and nocardia strains yielded bacilli which were Gram-variable or Gram-positive. (2) The spore-bearing organisms obtained from different cultures of the same strains appeared to be identical. (3) The spore-bearing forms were isolated repeatedly in spite of precautions taken to avoid contamination. Systematic experiments were therefore carried out to establish whether these organisms were derived from, and represented a phase in, the life cycle of the acid-fast mycobacteria. The results are reported here. No systematic investigations have been done on the strains of *N. asteroides*. For purposes of convenience, the acid-fast mycobacteria are termed form 1 and the spore-bearing organisms, which are not acid-fast, form 2.

METHODS

Mycobacterium tuberculosis

Organisms used. Seven strains of I 968, I 971, I 977, I 1053, I 1122, I 1133, I 1155 were recently isolated from sputa of seven newly diagnosed and untreated British patients with pulmonary tuberculosis. The isolates were sensitive to isoniazid, streptomycin and *p*-aminosalicylic acid, and were fully virulent in the guinea pig shortly before the *in vitro* experiments were started. The isolation of these strains and the sensitivity tests were made by methods described elsewhere (Tuberculosis Chemotherapy Centre, Madras, 1959). One laboratory strain, H37Rv. Atypical mycobacteria: Group I (Runyon, 1959; photochromogens): 4 strains (260, 266, 353, 1438). Group III (Runyon, 1959; Battey-type): 3 strains (223, 248, 585). All these seven strains were obtained from Dr E. H. Runyon (Veterans Administration Hospital, Salt Lake City, Utah, U.S.A.). They were all obtained from sputa, gastric contents or resected lung material of patients with pulmonary disease. All strains of *M. tuberculosis* and atypical mycobacteria were maintained on Löwenstein–Jensen medium at 37°, and were subcultivated at intervals of a few months.

Media. (1) The Löwenstein–Jensen medium used was without potato starch (Jensen, 1955). (2) 7H-10 oleic acid-albumin agar plates (Cohn, Middlebrook & Russell, 1959). (3) Kirschner medium (*Mackie & McCartney's Handbook*, 1960) and Kirschner-base glycerol medium (containing 10% (v/v) in place of 2% (v/v) glycerol) dispensed in 3 ml. amounts in ½-oz. screw-capped bottles. (4) Nutrient agar prepared by addition of 1·4% (w/v) agar to meat-extract + peptone broth (Oxoid No. 2, Oxo Ltd., London). All solid media were incubated at 37° for 4 days and all fluid media for 5–6 days before use, as a test of their sterility.

Cultivation. All cultures were incubated at 37°. Plates of 7H-10 medium and nutrient agar were sealed in polythene bags during incubation. All bacteriological manipulations were carried out in an inoculation cabinet.

Aeration of cultures. Provision of an additional air supply to the form 1 cultures growing on Löwenstein–Jensen medium was carried out as follows. The medium was normally dispensed in 3 ml. amounts in ½-oz. screw-capped bottles, and incubated with the caps firmly on. Aeration was carried out by flaming the outside of the cap, loosening and flaming it again, removing the cap for a few seconds, flaming the neck of the bottle and replacing the cap. Slopes of Löwenstein–Jensen medium were also prepared in 'aeration tubes' which consisted of a test tube fitted with a rubber

bung through which was passed a 3 cm. diam. glass tube plugged with cotton wool. The tube was attached to 3 mm. length of rubber tubing closed with a clip. The clip was opened for 30 min. to allow the entry of air. During each batch of aeration a nutrient agar plate and a 5% horse blood agar plate were exposed near the hands of the worker in the inoculation cabinet in an attempt to cultivate airborne organisms.

Staining methods. Smears were allowed to dry under an ultraviolet lamp for 8–10 min., and fixed for 1 min. in methanol. Jensen's method of Gram staining (*Mackie & McCartney*, 1960) was modified by decolorizing rapidly with acetone and counterstaining with dilute (1/15) carbol fuchsin. Control smears of *Staphylococcus pyogenes* and *Escherichia coli* were usually stained on the same slide. In the Ziehl–Neelsen staining (*Mackie & McCartney's Handbook*, 1960) smears were decolorized with acid ethanol (NaCl 20 g.; distilled water, 500 ml.; ethanol, 99·3% (v/v), 1500 ml.; HCl conc., 20 ml.) for not less than 5 min. and counterstained with Loeffler's methylene blue for 5–10 min. The Loeffler's methylene blue was at least 5 weeks old. Staining for spores was done with warm 1% (w/v) malachite green in 1% (w/v) phenol in water for 5 min., rinsing in tap water for 3 min., and counterstaining in 1% (w/v) aqueous safranin for 30 sec.

RESULTS

Production of form 2 mycobacteria

Origin of form 2 from form 1 organisms. The following experiment was carried out to obtain form 2 organisms from cultures of form 1 mycobacteria. A total of 15 strains (8, *Mycobacterium tuberculosis*; 4, group I atypical; 3, group III atypical) were plated out on 7H-10 medium plates and, after incubation for 17 days, single colonies were plated out again. With each strain, 6 slopes of Löwenstein–Jensen medium were each heavily inoculated from a separate well-isolated colony grown on the second plate for 17 days. An equal number (90) of Löwenstein–Jensen medium slopes from the same batch of medium were 'inoculated' in a similar manner, but with a sterile loop. These control slopes were subsequently subjected to exactly the same procedures as their counterpart slopes which had been inoculated with living organisms. The slopes were inoculated in a random order.

Groups of slopes containing one slope inoculated with a colony from each strain and an equal number of control slopes inoculated with a sterile loop were subjected to different procedures which are set out diagrammatically in Fig. 1. The slopes of groups A 1 and B 1 were in aeration tubes, while all the remaining slopes were in screw-capped bottles. The procedures for groups A and A 1 were otherwise identical, and similarly for groups B and B 1. All cultures were incubated for 14–24 weeks. In groups A, A 1, B, B 1 and C the cultures were aerated for portions of the incubation period, with the aim of investigating the relationship between aeration and the occurrence of form 2 organisms. Cultures of group D were not aerated. At 14 weeks cultures of group B 1 were subcultured on to fresh Löwenstein–Jensen medium slopes (group S) and, after incubation for 8 weeks, the growth from group S was again subcultured into tubes of Kirschner and Kirschner-base glycerol medium (groups K and Kg). Further control slopes or tubes inoculated with a sterile loop were also included in groups S, K and Kg.

At the times indicated in Fig. 1, samples of the initial inoculum and of the growth in the cultures were inoculated in two nutrient agar plates and examined in smears stained by the Ziehl–Neelsen methods. The nutrient agar plates were incubated for 5 days and colonies were examined and smears were made from them daily. After the end of the experiment each Löwenstein–Jensen medium culture in group C was re-incubated for a further 3 months and was aerated twice weekly. At the end of this period smears were stained from the growths on these slopes.

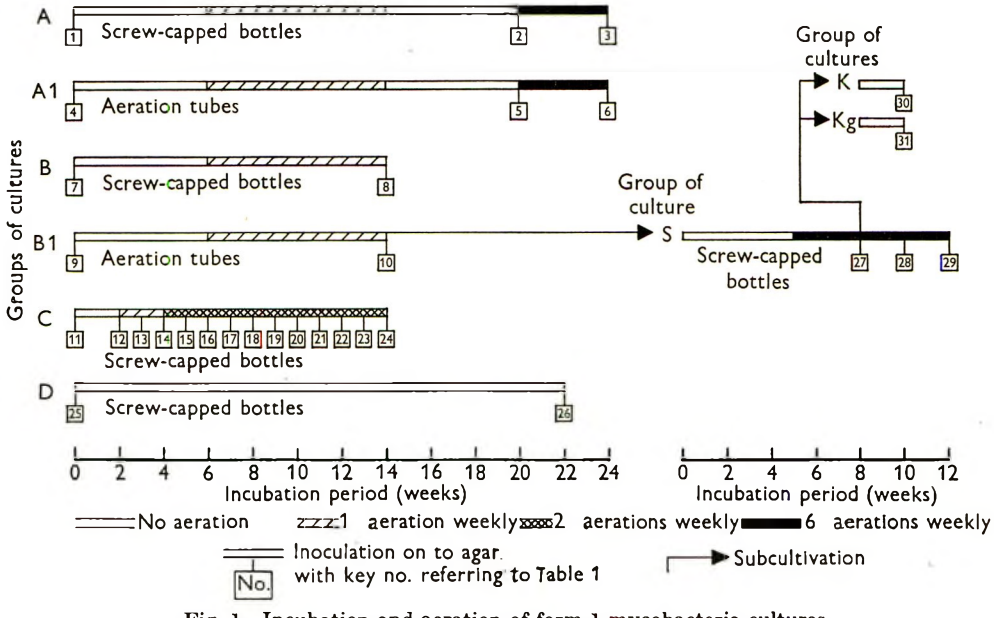

Fig. 1. Incubation and aeration of form 1 mycobacteria cultures.

The results of the cultures on nutrient agar plates inoculated at the intervals indicated in Fig. 1, from the growths on Löwenstein–Jensen and Kirschner media, are set out in Table 1. All samples of the colonies on 7H-10 medium plates used for inoculating the Löwenstein–Jensen medium slopes failed to yield growth when inoculated on to nutrient agar plates. All 15 strains yielded form 2 colonies on nutrient agar plates on 3 to 10 occasions of sampling. Form 2 colonies were obtained only from form 1 cultures on Löwenstein–Jensen medium which had been incubated for 8 weeks or more. An isolation from a culture was not always followed by further isolations from the same culture, possibly because the samples were usually taken from a small area on the slope and were not representative of the entire growth. At each isolation a heavy growth of at least 100 form 2 colonies was obtained on incubation of the nutrient agar plates for 1–2 days. None of the control slopes, which had been initially inoculated with a sterile loop yielded any growth on the nutrient agar plates. None of the cultures of group D, which were not aerated, yielded form 2 colonies, when they were sampled at 22 weeks. However, form 2 colonies were obtained on each occasion of sampling at 8–14 weeks from 3 to 8 cultures in group C, which were being aerated twice weekly.

Of the 88 pairs of nutrient and blood agar plates exposed for cultures of airborne

Table 1. *Isolation of form 2 organisms from form 1 mycobacteria*

Group of cultures	Period of incubation (weeks)	Key no. from Fig. 2	*M. tuberculosis*								Atypical mycobacteria						
											Group I				Group III		
			H37 Rv	I 968	I 971	I 977	I 1053	I 1122	I 1133	I 1155	260	266	353	1438	223	248	585
A	0	1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	20	2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	24	3	+	−	−	−	−	−	+	+	−	−	−	−	−	−	−
A1	0	4	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	20	5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	24	6	−	+	+	−	−	−	+	+	−	−	−	−	−	−	−
B	0	7	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	14	8	−	+	+	+	−	+	+	−	−	−	−	−	−	−	+
B1	0	9	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	14	10	+	+	+	+	−	+	+	−	−	−	−	−	−	−	+
C	0	11	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	2	12	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	3	13	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	4	14	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	5	15	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	6	16	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	7	17	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	8	18	+	−	−	−	−	−	−	−	−	−	−	+	−	+	−
	9	19	+	−	−	−	−	+	+	−	+	−	+	+	+	+	−
	10	20	−	−	−	−	−	+	−	−	+	−	+	+	−	−	−
	11	21	−	+	−	−	+	−	−	+	−	−	−	+	+	+	−
	12	22	−	−	−	+	−	+	−	−	+	−	−	+	−	−	−
	13	23	+	−	−	−	−	+	−	−	−	+	+	+	−	−	−
	14	24	−	−	+	−	+	−	−	−	+	−	+	+	−	−	+
D	0	25	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	22	26	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S	8	27	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	10	28	−	−	−	−	+	−	−	−	−	−	−	+	+	−	−
	12	29	+	+	−	−	−	−	−	−	−	−	+	+	+	−	−
K	2	30	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+
KG	2	31	−	−	−	−	−	−	−	−	+	+	+	+	+	−	+

− indicates no growth of samples on nutrient agar plates.
+ indicates growth of form 2 colonies on nutrient agar plates.

contaminants during the aeration of the cultures, only two plates yielded growth on subsequent incubation, and the colonies on both consisted of Gram-positive cocci.

After incubation for 2 days, plates bearing form 2 colonies of the first isolate from each strain were formolized for comparison with the other isolates. The bacillary morphologies of the form 2 organisms were compared in smears, stained by the Ziehl–Neelsen methods, when endospores first become apparent, that is, after the nutrient agar plates had been incubated for 1–5 days. Both the bacillary and colonial morphologies of the form 2 organisms, isolated from the same form 1 strain on different occasions from the same culture and from different cultures appeared identical. The similarity of the bacillary morphologies of five pairs of form 2

organisms, each member of the pair being obtained from a different group, and the variation from strain to strain are illustrated in Pl. 1, figs. 2–6.

The effect of aeration. Aeration of the Löwenstein–Jensen medium cultures appeared to be necessary for the development of the form 2 organisms in the form 1 cultures, as seen with the cultures of group D, which were not aerated and did not yield form 2 colonies. It also appears probable that the interruption of aeration resulted in the form 2 organisms losing their ability to grow on nutrient agar. In groups B and B 1 at 14 weeks, following an 8-week period of aeration form 2 colonies were obtained from 13 of the 30 cultures. However, cultures in groups A and A 1 were aerated in the same way until the 14th week; they were not aerated for 6 weeks, when sampling failed to yield any form 2 colonies, but after a further period of intensive aeration 7 of the 30 cultures yielded these colonies. Subculturing followed by a period of growth without aeration also appeared to prevent the isolation of form 2 colonies. Colonies were obtained from 7 of the 15 cultures in group B 1 at 14 weeks, when they were subcultivated as group S. After growth without aeration for 5 weeks and with aeration for a further 3 weeks, none of the cultures of group S yielded form 2 colonies.

The effect of different media. The effect of different media on the development of form 2 mycobacteria appeared to differ according to whether the form 1 strains were *Mycobacterium tuberculosis* or atypical mycobacteria. Form 2 colonies were obtained from sampling of 7 of the 8 strains of *M. tuberculosis*, but from only 1 of the 7 strains of atypical mycobacteria in the Lowenstein–Jensen cultures of groups A, A 1, B and B 1. Again, the subcultures in Kirschner-base glycerol medium (group Kg) yielded form 2 colonies from none of the 8 strains of *M. tuberculosis*, but from 6 of the 7 strains of the atypical mycobacteria.

Morphology of form 2 organisms in form 1 cultures

Phases of development. The phases of development of form 2 organisms are described as they appeared in the cultures of group C (Table 1), since in this group smears were made at weekly intervals. Samples of the initial inoculum and of the growth in the cultures were stained by the Ziehl–Neelsen method. The microscopic appearances are illustrated diagrammatically in Fig. 2, since the early phases were difficult to see and even more difficult to photograph. Some of the phases appeared together in the smears and it is therefore difficult to be certain that the order of development is exactly as described. Considerable variation occurred from culture to culture in the time intervals between the occurrence of the phases, and only the average time is reported.

Form 2 cells first appeared between the fourth and sixth week of incubation as just visible non acid-fast dots lying free among the form 1 bacilli (Fig. 2, phase 1). At about 6 weeks short rods with one pointed end and a polar granule at the other, appeared, giving the impression of a germination tube (Fig. 2, phase 2). At about 8 weeks, slender rods with pointed ends were seen, and these were either stained evenly or contained a single deeper stained granule located centrally, or two granules located at the poles (Fig. 2, phase 3). A minority of these cells were arranged parallel to each other. Between 9 and 14 weeks the intracellular granules often became larger than the rest of the cell, distorting the cell wall and giving the impression of an early stage of intracellular spore-formation. At 14 weeks a pro-

portion of the cultures yielded smears which contained either one or both of the following forms: (*a*) evenly and deeply stained rods with squared ends, often lying in chains or parallel (Fig. 2, phase 4*a*); (*b*) free-lying oval spores, either isolated or in clumps (Fig. 2, phase 4*b*). An example of phase 4*b* is shown in Pl. 1, fig. 1. All of the cultures in group C (Table 1) at the end of the additional 3-month period of incubation and aeration yielded smears containing organisms in phases 4*a* and *b*.

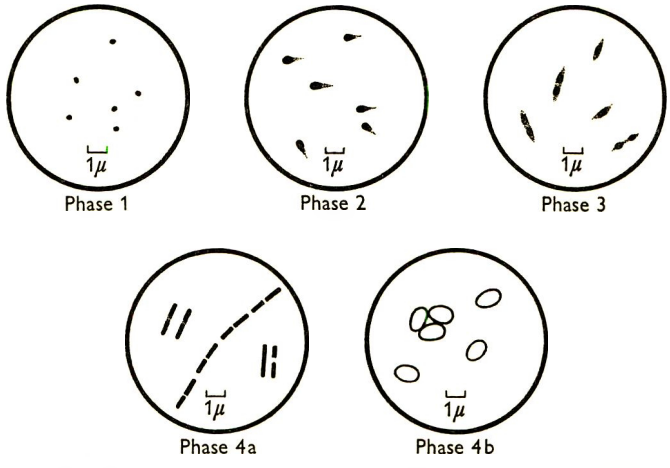

Fig. 2. Diagrammatic illustrations of development of form 2 organisms in form 1 cultures.

Form 2 organisms were stained in their early phases with difficulty, and did not stain with an aqueous solution of methylene blue or with Loeffler's methylene blue when the latter was less than 5 weeks old. The vegetative forms were never acid-fast. The central area of the free spores was usually unstained, but occasionally weakly acid-fast; the walls were stained blue. The spores stained green with safranin-malachite green. The number of the form 2 organisms in proportion to the number of form 1 organisms was highly variable. In phases 1 and 2, form 2 organisms were present only sparsely and could easily be overlooked. When in phase 3, the ratio of their number to the number of form 1 organisms was not less than 1:100.

Changes in the culture medium. The Löwenstein–Jensen medium did not alter until the form 2 organisms were in phases 1 and 2; when they were about to change from phase 3 to phases 4*a* and 4*b* the medium became yellowish, particularly at the edges of the slopes. When form 2 organisms reached phase 4, the growth often developed a brownish pigmentation or liquefied the medium, giving the impression that it was contaminated.

Ability to grow on nutrient agar. Form 2 organisms in phases 1, 2 and early in phase 3 did not grow on nutrient agar. In phase 3, when the granules became large enough to distort the cell wall, and in phase 4*a* and 4*b*, colonies were invariably obtained on nutrient agar plates. Form 2 organisms in phases 1, 2 and early 3 disappeared from smears and lost their ability to grow on nutrient agar when aeration of the Löwenstein–Jensen medium cultures was interrupted for a long period, as described above. Once form 2 organisms reached phase 4, they were not

influenced by interrupting the aeration, as shown by a further experiment in which cultures contained form 2 organisms in phase 4*b* and were incubated without aeration for 12 months. The spores remained unchanged and yielded colonies on nutrient agar.

Growth on Löwenstein–Jensen medium. When form 1 cultures containing form 2 organisms in phases 1–3 were subcultured to fresh Löwenstein–Jensen medium, normal growth of form 1 organisms appeared and the growth did not contain form 2 organisms. However, when form 1 cultures on Löwenstein–Jensen medium, containing form 2 organisms in phases 4, were subcultured on the same medium, the medium of the subculture turned yellow or brown and was often softened. Form 1 organisms never grew on it and at most, scanty colonies of form 2 were obtained on it.

Properties of form 2 organisms isolated on nutrient agar

Bacillary morphology. The bacillary morphology of the form 2 organisms is described from smears prepared from the colonies of the first isolate on nutrient agar. The appearance in smears stained by the Ziehl–Neelsen method are described when endospores first became apparent (1–5 days). Gram staining is described in smears taken at 2 days, irrespective of sporulation. Staining with safranin-malachite green was done when the cultures contained numerous free spores.

The bacillary morphology of individual strains is not described in detail, because further experience suggests that it is profoundly affected by the nature of the medium, the period of incubation, the age and previous history of the inoculum, and the temperature of incubation. Nevertheless, it is important to emphasize that the bacillary morphology of the different isolates from the same form 1 strain always appeared identical at the first isolation on nutrient agar. The form 2 organisms obtained from 12 strains were rods (Pl. 1, figs. 2, 3, 5, 6), which varied from strain to strain in their size (range $0{\cdot}5 \times 3{\cdot}0\ \mu$–$1{\cdot}0 \times 2{\cdot}5\mu$), in the shape of their ends, in the arrangement of cells, in the location of their endospores and in the extent to which the spores distorted the cell walls. None of the rods was acid-fast. The rods from *M. tuberculosis* were Gram-negative, those from the atypical mycobacteria were Gram-variable or Gram-positive. After longer periods of incubation (3 days–2 weeks) small, Gram-positive granules appeared within some of the rods from all strains. The free spores were oval (size range $0{\cdot}7 \times 1{\cdot}0\ \mu$–$1{\cdot}2 \times 1{\cdot}5\ \mu$), their cell walls were blue in the Ziehl–Neelsen smears and Gram-positive, the central area was usually unstained, but occasionally weakly acid-fast and Gram-negative. They were uniformly green when stained with safranin-malachite green. The form 2 organisms obtained from the remaining three strains (H37Rv, I 977, I 1133) were cocci of very uneven size and shape, often arranged in chains (Pl. 1, fig. 4), which were not acid-fast and were Gram-negative. The large, oval forms seen in Pl. 1, fig. 4, were not as typical of spores as those seen in the rod-shaped form 2 organisms, but they were still considered as spores since they stained green with safranin-malachite green. These spores stained uniformly Gram-negative. Although these coccoid forms were obtained from all of the 3 strains at all isolations, it has been observed that strain H37Rv yielded rod-shaped form 2 organisms, with typical endospores after prolonged incubation and aeration on Löwenstein–Jensen medium. This finding suggests that the coccoid form may be an intermediate stage followed by a final rod-shaped stage similar to the remaining strains of *M. tuberculosis*.

Viability. The form 2 organisms which were composed of cocci all died out after 1–2 subcultivations on nutrient agar. With the remaining strains, subcultures of colonies which did not contain free spores had a similar poor viability. When free spores were present, subcultures on nutrient agar could often be maintained in a viable state at room temperature for 2 months, but during regular subcultures at intervals of 2 months for a year most have died out.

Colonial morphology. The morphology of the colonies of the form 2 organisms is described from the first isolate from each strain obtained on nutrient agar plates after incubation for 2 days. The colonies were examined at $\times 10$ magnification with a plate microscope. As with bacillary morphology, no attempt has been made to describe the detailed colonial morphology of each strain since the appearances were markedly variable on further subcultures. Each strain, however, yielded the same colonial type when first isolated from the Löwenstein–Jensen medium slopes. The colonies obtained from the *Mycobacterium tuberculosis* strains (Pl. 2, figs. 1, 2) were usually small (0·1–0·9 mm. diam.), but larger colonies (2·0–3·0 mm. diam.) were obtained from three strains. The colonies were usually discrete, but one strain produced a confluent thin film on the surface of the medium; they were circular, or irregular, effuse or raised, smooth or finely granular; some strains had colonies with one central umbo, or two umbos or were umbilicate; the edges were usually entire though the large colonies had crenated or slightly fimbriate edges; all colonies were grey and opaque, most were friable, but the larger colonies were butyrous; one strain had colonies firmly adherent to the medium. The colonies obtained from the atypical mycobacteria were larger (3·0–8·0 mm. diam.); they were circular or irregular, smooth, glistening, butyrous either with a uniform structure and entire edges (Pl. 2, figs. 3, 4) or a beaten-copper, worm-cast surface with rhizoid edges (Pl. 2, fig. 5).

DISCUSSION

Evidence that the form 2 organisms were derived from the form 1 organisms and were not contaminants can be derived from the following observations. (1) There was variation in the bacillary and colonial morphologies of form 2 organisms from strain to strain and from species to species, yet these characters were closely similar in multiple isolations from the same form 1 strain. It will be appreciated that the form 1 cultures in groups A, A 1, B, B 1 (in this context B 1 includes its subcultures S, K and Kg) and C, were each derived from a separate colony on 7H-10 medium plates (Fig. 1). The similarity of the bacillary morphologies of five pairs of form 2 organisms, each member of the pair being obtained from a different group and the variation from strain to strain are illustrated in Pl. 1, figs. 2–6. Some of the strains of *Mycobacterium tuberculosis* and atypical mycobacteria were again examined for the production of form 2 organisms in further experiments. The form 2 isolates had colonial and bacillary morphologies identical to those described here. (2) The conditions under which form 2 organisms were obtained, such as length of incubation and type of medium, were different with strains of *M. tuberculosis* and of the atypical mycobacteria. If the form 2 organisms arose as airborne or medium contaminants then a random pattern of these characters would be expected and would not be related to the species of the form 1 strains; however, the observations reported provide strong evidence against this view, particularly since the inoculation and aeration of the cultures was done in a random order. (3) The controls incorporated

in the experiments also provided evidence that the form 2 organisms did not originate from the air or the culture medium. The failure to obtain form 2 organisms from the media 'inoculated' with a sterile loop and aerated during the course of the experiment is against these organisms arising from the air or the medium. The failure to obtain any colonies resembling the form 2 organisms on the plates exposed in the inoculation cabinet during aerations suggest that they did not result from aerial contamination. The failure to obtain form 2 colonies from those slopes which were not aerated (series D, Fig. 1) suggest that they were not medium contaminants. (4) Evidence that contamination arising during aeration of the Löwenstein–Jensen medium cultures was not responsible for the isolation of form 2 organisms is provided furthermore by comparing the frequency with which form 2 colonies were obtained from cultures aerated either by removal of their screw-caps or by allowing air to enter through a cotton-wool plug. Form 2 colonies were obtained from 9 of 30 cultures in groups A and B, which were aerated by removal of their caps and in 11 of 30 cultures in groups A1 and B1, which were set up in aeration tubes (Fig. 1, Table 1). (5) Although most of the form 2 organisms resemble the genus *Bacillus* when grown on nutrient agar, all of the isolates from *M. tuberculosis* strains were definitely Gram-negative and it would be remarkable to obtain such a high proportion of Gram-negative *Bacillus* species. Further experiments, to be described elsewhere, show that the form 2 strains have a complex morphology and life-cycle resembling but not identical with certain species of the families Actinomycetaceae and Streptomycetaceae (Waksman & Henrici, 1943).

The existence of form 2 organisms in the form 1 cultures used as the inoculum is improbable. The Löwenstein–Jensen medium slopes were inoculated with form 1 cultures which had been purified by single colony selection on two occasions. If a form 2 organism had survived this purification it should have been visible in the smears or should have grown in the cultures on nutrient agar which were made from the inocula in these experiments.

As considered above, it is reasonable to conclude that form 2 organisms were derived from the form 1 cells in certain stages of their life cycle. The exact stage at which the transition between these two forms occurs is uncertain. The serial examinations of Löwenstein–Jensen medium cultures (group C) suggest that in the early phases of their development the form 2 organisms are not able to grow on nutrient agar. In the late phases of their development when spores were present either in an advanced stage in the cells or were lying free, positive isolates were obtained from the spores. These structures were considered as spores since they resemble the spores of different micro-organisms (Bacillaceae, Streptomycetales) in their morphological and staining characteristics and in their capability to survive for longer periods than the vegetative cells. Heat resistance of spores was not investigated, since it is known that spores of Streptomycetales are reproductive bodies rather than resistant bodies and are destroyed by heat at 60–65° (Waksman, 1950). Spore formation (conidia) in organisms closely related to mycobacteria has been known for a long time. The order Actinomycetales (Waksman & Henrici, 1943) contains five genera (*Mycobacterium*, *Actinomyces*, *Nocardia*, *Streptomyces*, *Micromonospora*) of which two, *Streptomyces* and *Micromonospora* have been known for long to form a certain type of spores (conidia), while spores have recently been described in a third genus, *Nocardia*, by Gordon & Mihm (1958). The close relationship between the

genera *Mycobacterium*, *Actinomyces* and *Nocardia* has been commented on by many authors (Jensen, 1931; Skinner, Emmons & Tsuchiya, 1947; Gordon & Mihm, 1957; Cummins & Harris, 1958). Spore formation of Mycobacteria was described and illustrated by Xalabarder (1954) and by Brieger & Glauert (1956). The latter authors observed round bodies in the filaments of an avian strain of *Mycobacterium tuberculosis* by electron microscopy; these bodies were identified as spores and had a structure similar to the spores of *Bacillus cereus* and *B. megatherium*. The existence in acid-fast mycobacteria cultures of forms that are not acid-fast, is extensive, and has been reviewed by Bassermann (1953) and Koelbel (1957). Rosenthal & Heagan (1955) described by using bright field, phase and electron microscopy certain cells in the culture of *M. tuberculosis* strain BCG which correspond in bacillary morphology and staining properties to the description of form 2 organisms reported here. They considered that the bacilli which correspond most closely to the early phase 3 described here reverted to normal acid-fast bacilli. In Lack & Tanner's (1953) paper illustration of organisms in mycobacteria cultures closely resembling the phases 3 and 4*b* of the present paper are shown in Pl. 3, fig. 10, and Pl. 1, fig. 3. The authors found that these forms were not acid-fast (Dr C. H. Lack, personal communication) except for the middle of the spore-like structures in Pl. 1, fig. 3, which they considered as abnormal nuclei.

Although form 2 organisms differ from form 1 organisms in a variety of important characters such as bacillary morphology, staining characters, colonial morphology, speed of growth and nutritional requirement, they seem to be not two different organisms but two different forms of the same organism. In mycology some species are known to exist in two entirely different forms (dimorphism); this phenomenon is considered by Langeron & Vanbreuseghem (1952) as a special manifestation of polymorphism. In the pathogenic dimorphic fungi, the morphology of the parasitic form is usually simple but the saprophytic form is complex. For example, *Histoplasma capsulatum* always grows in the host as a budding yeast, but *in vitro* it grows either as a yeast, producing smooth, glistening colonies, or reverts to a hyphal type of growth with cottony, mould colonies in which chlamidospores occur regularly. It is reasonable to suggest that the forms 1 and 2 of the mycobacteria described here are examples of such dimorphism. There appears to be no previous report of proved dimorphism in the mycobacteria. Since the form 2 organisms when in phase 4 and when growing on nutrient agar are similar to strains of the genus *Bacillus*, it would seem probable that form 1 cultures containing them would usually have been discarded as contaminated. The results of attempts to convert form 2 to form 1 mycobacteria and a fuller description of the characteristics of form 2 organisms will be described at a later date.

I wish to express sincere thanks to Dr D. A. Mitchison for his interest in this work and for his severe criticism which nevertheless proved extremely valuable. Our stimulating discussions contributed much to the soundness of the experiments. Finally, I am grateful to him for reading this paper. I am also grateful to Mr K. Robinson and Miss Janet Lloyd for their conscientious technical assistance.

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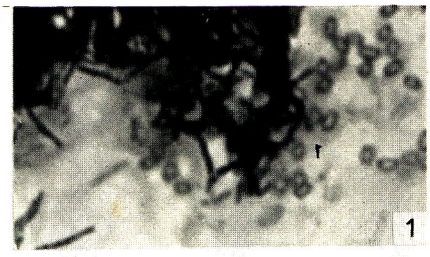

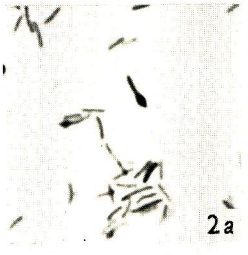

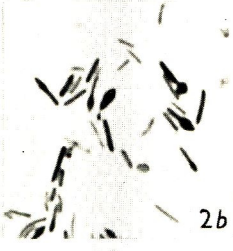

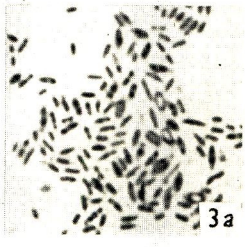

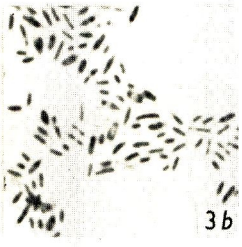

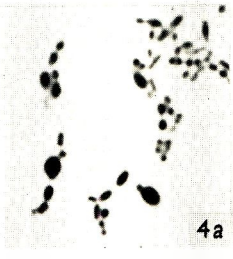

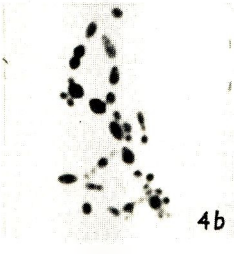

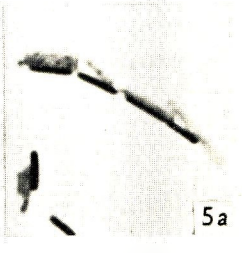

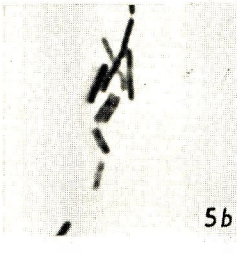

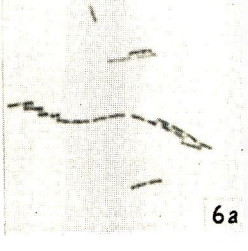

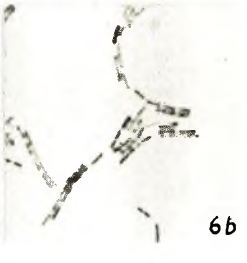

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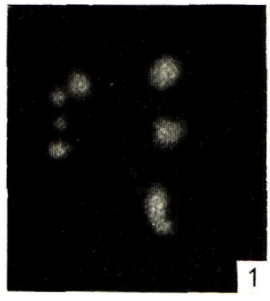
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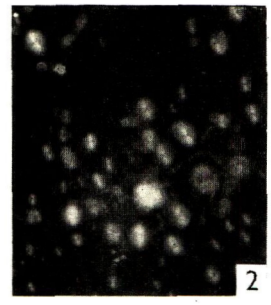
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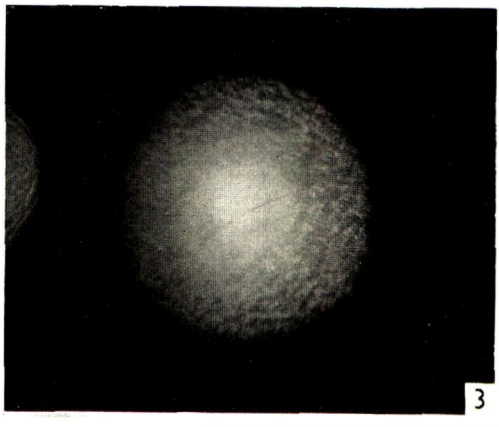
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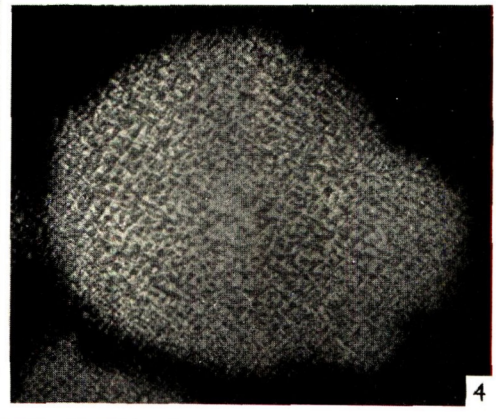
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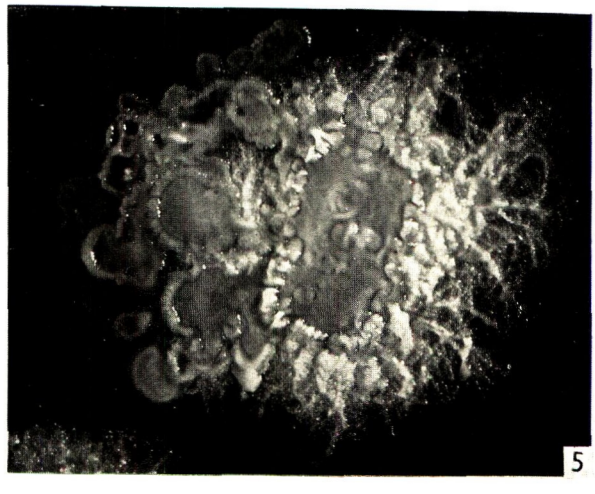
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EXPLANATION OF PLATES

Plate 1

Fig. 1. *Mycobacterium tuberculosis*: 1–6 weeks' incubation with frequent aeration, on Löwenstein–Jensen medium. Form 1 cells and free spores are shown. Ziehl–Neelsen stain. ×2000.

Figs. 2–6. Form 2 organisms isolated on nutrient agar plates, incubated for 1–5 days. Pairs of photographs of the same strain; the 2 members of the pairs having been obtained in separate groups (Fig. 1). Ziehl–Neelsen stain. ×1500.

Fig. 2*a*. *M. tuberculosis*, strain I971, group B.

Fig. 2*b*. *M. tuberculosis*, strain I971, group B1.

Fig. 3*a*. *M. tuberculosis*, strain I1155, group A.

Fig. 3*b*. *M. tuberculosis*, strain I1155, group A1.

Fig. 4*a*. *M. tuberculosis*, strain I1133, group A.

Fig. 4*b*. *M. tuberculosis*, strain I1133, group A1.

Fig. 5*a*. Group I, atypical, strain 260, group C.

Fig. 5*b*. Group I, atypical, strain 260, group Kg.

Fig. 6*a*. Group I, atypical, strain 1438, group C.

Fig. 6*b*. Group I, atypical, strain 1438, group C.

Plate 2

Figs. 1–5. Colonies of form 2 organisms at their first isolation on nutrient agar plates, after 2 days' incubation. ×10.

Fig. 1. *M. tuberculosis*, strain I968.

Fig. 2. *M. tuberculosis*, strain I971.

Fig. 3. Group I, atypical, strain 260.

Fig. 4. Group I, atypical, strain 1438.

Fig. 5. Group III, atypical, strain 223.

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Further Observations on Changes in the Phage-Typing Pattern of Phage Type 80/81 Staphylococci

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SUMMARY

Two distinct temperate phages (594a, 594n) lysogenizing phage type 80/81 staphylococci and producing gains in sensitivity to typing phages 52 and 52A were found in lysogenic clones of a strain of staphylococcus of phage type 52/52A/80/81. When either of these phages infected type 80/81 cocci a new phage (594b) appeared in the lysates. This phage was unable to replicate in type 80/81 cocci but could grow in them when they had been lysogenized with phage 594a or 594n. The source of phage 594b was found to be recombination of phage 594a or 594n with a prophage present in cocci of phage type 80/81. This prophage was completely defective and could be demonstrated only by recombination. The gains in phage sensitivity that follow lysogenization of type 80/81 strains with the so-called 'converting' phages can be most satisfactorily explained on the basis of prophage substitution.

INTRODUCTION

Asheshov & Rippon (1959) and Rountree (1959) described changes in the phage-typing pattern of staphylococci of phage type 80/81 occurring after lysogenization with certain temperate phages, which were called 'converting' phages for want of a better name. The change of particular interest was that by which the lysogenized cocci became sensitive to the typing phages 52 and 52A so that their typing pattern became 52/52A/80/81. The mechanism of this change remained obscure. By replica plating Rountree demonstrated the presence in old broth cultures of the propagating strain of phage 80 (P.S. 80) of mutants sensitive to phages 52 and 52A and she suggested that the converting phages might select such pre-existent mutants, which might be more readily lysogenized than the wild type. On the other hand, Asheshov & Rippon found no mutants sensitive to phages 52 and 52A in their type 80 strain. Furthermore, when they had converted this strain by lysogenization and then 'cured' it of the converting phage by ultraviolet irradiation, the cured strain remained sensitive to phages 52 and 52A. In addition both the original strain and the cured strain gave identical lytic and lysogenic responses to a converting phage. These observations indicated that neither lysogenic conversion nor selection of pre-existing mutants could explain the phenomenon. Prophage substitution

remained a possibility, but all attempts to demonstrate lysogenicity in cocci of phage type 80/81 were unsuccessful. It has now been found, however, that type 80/81 staphylococci contain a completely defective prophage and that the change in the typing pattern after lysogenization with one of the converting phages is due to substitution of this prophage by the converting phage.

METHODS

Phage type 80/81 staphylococci. Seven staphylococcal strains typing as 80/81 and isolated in a number of different localities were chosen for study. A strain was considered to be phage type 80/81 if it was lysed only by these two phages amongst the 21 phages comprising the basic set of typing phages, both when typing was carried out with the phages at the routine test dilution (RTD) and with the phages at 1000 times greater concentration. The seven type 80/81 strains comprised the following: *P.S. 80*, the propagating strain for phage 80, isolated in Sydney in 1953. A streptomycin-resistant mutant of P.S. 80 was used in some experiments. It will be referred to as P.S. 80^{sr}; *P.S. 81*, the propagating strain for phage 81, isolated in Canada in 1956 (Bynoe, Elder & Comtois, 1956); *U9*, isolated in Philadelphia, Pa., U.S.A. and received from Dr J. Baldwin, Columbus, Ohio, in 1956; *8428*, isolated in Oamaru, New Zealand in 1954; *1406/1*, obtained from Miss K. Key of the Commonwealth Serum Laboratories, Melbourne, in 1957; *590*, isolated in Sydney in 1958; *6346*, isolated in England in 1958.

Phage type 52/52A/80/81 staphylococci. Four strains of this phage type were used. They were as follows: *strain 594*, isolated from the same patient as strain 590 and presumed to have been converted from strain 590 *in vivo*. Lysogenic clones of this strain were used as the source of two converting phages and are described in detail below; *strain 6346 DL*, derived from strain 6346 which was lysogenized with a converting phage, 7287[1], and subsequently cured (Asheshov & Rippon, 1959). This strain is believed to be completely non-lysogenic; *strain 80* la, a mutant of P.S. 80 isolated by Rountree by replica plating from an old broth culture of P.S. 80 which differed from the parent culture in being sensitive to the two typing phages 52 and 52A (Rountree, 1959); *strain 1406/2*, presumed to have been derived from 1406/1 (type 80/81) during mouse passage and differing from the parent culture in being type 52/52A/80/81.

Preparation of phage stocks. Phage stocks were prepared either by ultraviolet (u.v.) induction of singly lysogenic strains or by propagation on a non-lysogenic strain in order to avoid contamination with phages carried by lysogenic strains. The method of u.v. induction was essentially that of Gorrill & Gray (1956). The cocci were grown with aeration in glucose veal broth for 2 hr., centrifuged and resuspended in phosphate buffer pH 7·0. They were irradiated with a Westinghouse 'Sterilamp' for 50 sec. at a distance of 38 cm., the suspension being agitated in a Petri dish during exposure. The irradiated cocci were inoculated into glucose veal broth at 5×10^7 cocci/ml. and incubated in a water bath at 37°. After 60 min. they were transferred to the bench where clearing of the culture took place within 2–5 hr. of irradiation. Such stocks had titres of the order of $1–5 \times 10^9$ infective particles/ml. In other cases phage stocks were prepared by propagating the phage on the non-lysogenic strain, 6346 DL, by the method of Swanstrom & Adams (1951).

Counting phages. Agar plates were flooded with a young broth culture of the

appropriate indicator strain of staphylococcus, the excess broth pipetted off and the plates allowed to dry before the phage dilutions in 0·01 or 0·02 ml. volumes were deposited on their surface. Three or four replicates were plated from each dilution and were not spread over the surface of the plate.

Lysogenization. This was carried out on agar as previously described (Rountree, 1959). All phages used for lysogenization were made by u.v. induction, filtered through Gradocol membranes of 0·8 μ A.P.D. and tested for sterility before use. Some strains were lysogenized in broth, a quantity of phage theoretically sufficient to infect all cocci being added to young aerated cultures.

The lysogenized strains are described in the usual way, e.g. P.S. 80 (594a) or, more simply P.S. 80(*a*) is P.S. 80 lysogenized by the phage derived from strain 594A.

RESULTS

Phages isolated from strain 594

Staphylococcus strain 594, of phage type 52/52A/80/81, showed numerous plaques when grown on a phage-typing plate shortly after its isolation, and the original broth culture showed evidence of spontaneous clearing after a day at room temperature. It was plated on agar on two occasions and a number of entire single colonies picked after incubation. These clones, lettered A to R, were all of phage type 52/52A/80/81. All of the clones were lysogenic but three different phages were identified in the various clones. The behaviour of these three phages and of cocci lysogenized by them were valuable in demonstrating the mechanism of the conversion of type 80/81 strains to type 52/52A/80/81.

Phage 594a and 594n were converting phages present in the prophage state in clones 594A and 594N, respectively. Both belonged to serological group A and were inducible by u.v. radiation and by superinfection with the other phage; there was, however, no cross-immunity between them.

The third phage, 594b, was found in a single lysogenic clone, 594B. It also belonged to serological group A and was inducible by u.v. radiation and by super infection with phage 594a or 594n. Cocci of P.S. 80 were immune to it but such cocci became sensitive to this phage after they had been lysogenized by either phage 594a or 594n and could then be used as an indicator for the phage. Clone 594G, doubly lysogenic for 594a and 594n could also be used as an indicator for phage 594b but was less sensitive than 594A or 594N. For the sake of brevity these three phages will be referred to subsequently as phage *a*, *n*, and *b*.

Table 1 summarizes the reactions of clones isolated from strain 594. Several representatives of each clonal type were identified. The *n* prophage in clone F was partly defective since no *n* phage was produced spontaneously and only a small amount when the clone was induced by u.v. radiation. The host range of the three phages was determined against a set of test strains of various phage types. Only minor differences were detected. The main difference between the three phages therefore lay in their failure to show cross-immunity and in the failure of phage *b* to lyse strains of phage type 80/81.

Origin of phage b

The origin of phage *b* and of clone 594B was examined. The working hypothesis that strain 594 was derived from a type 80/81 strain, 590, that had been infected

in vivo with the converting phages, *a* or *n*, did not explain the occurrence of phage *b* and clone 594B, since type 80/81 cocci (either P.S. 80 or 590) were immune to the phage. It seemed possible that phage *b* represented a mutant of phages *a* or *n* sufficiently virulent to overcome the immunity of strains lysogenic for phages *a* or *n*, and which, at the same time, had mutated in such a way that it was no longer able to lyse cocci of P.S. 80. However, high-titre phage preparations made by u.v. induction of strains 594A and P.S. 80(*a*) and strains 594N and P.S. 80(*n*) showed no trace of phage *b* when tested on their homologous strains. That phage *b* arose as a mutant of phage *a* or *n* seemed unlikely. Another possibility was that phage *b* represented a hybrid phage resulting from recombination between phage *a* and phage *n*. Once again, however, high-titre preparations of phage *a* propagated on strain 594N and of phage *n* propagated on strain 594A failed to show the presence of phage *b*. When, however, either phage *a* or *n* was propagated on P.S. 80 or strain 590, phage *b* was regularly recovered in the lysates. It seemed, therefore, that phage *b* might have its origin in a hitherto undetected prophage in P.S. 80 or else be a recombinant of such a hypothetical prophage with the phage *a* or *n*.

Table 1. *Reactions of clones isolated from staphylococcus strain 594*

Clone no.	Sensitivity to phages			Lysogenicity for				Prophage state of clone		
	a	*b*	*n*	PS 80	PS 80(*a*)	PS 80(*n*)	594G			
594A	−	+	+	+	−	+	−	*a*	.	.
594N	+	+	−	+	+	−	−	.	.	*n*
594B	+	−	+	−	+	+	+	.	*b*	.
594C	−	−	+	+	+	+	+	*a*	*b*	.
594F	−	+	−	+	−*	+	−	*a*	.	n_d*
594G	−	+	−	+	+	+	−	*a*	.	*n*
594K	−	−	−	+	+	+	+	*a*	*b*	*n*

* = defective prophage, trace only on u.v. induction.

Lysogenicity of type 80/81 strains

Previous attempts to demonstrate lysogenicity in type 80/81 strains had been unsuccessful (Asheshov & Rippon, 1959; Rountree, 1959) but the appearance of phage *b* during the growth of phage *a* and *n* in these strains indicated that a further search should be made. When supernatant fluids of 2–4 hr. broth cultures of P.S. 80 or 590 were filtered and plated on 594A or 594N no plaques were formed. When, however, the supernatant fluids were tested before filtration, minute fuzzy plaques developed which were difficult to count even with a hand lens. When broth cultures were centrifuged for increasing lengths of time, the number of plaques appearing was found to be directly proportional to the number of unsedimented cocci of P.S. 80 in the inoculum. These results suggested that the appearance of plaques was intimately connected with the number of cocci of P.S. 80 able to develop on the surface of the plate. Furthermore, when the unfiltered supernatant fluid of a streptomycin-sensitive clone of P.S. 80 was plated on agar containing 1000 μg. streptomycin/ml. inoculated with the streptomycin-resistant strains P.S. $80^{sr}(a)$ or P.S. $80^{sr}(n)$, a situation in which the unsedimented P.S. 80 cocci were killed, no plaques were seen. When the supernatant of P.S. 80^{sr} was plated under these same conditions, phage plaques were visible, so that failure to see them in the platings from the strepto-

mycin-sensitive strains was not due to the phage itself being streptomycin-sensitive. Propagation of the phage which produced the fuzzy plaques yielded preparations identical in serological reactions and host range to phage *b*.

It was concluded that no free phage corresponding to phage *b* was produced by P.S. 80 cocci and that interaction between the converting phages and the type 80/81 cocci was necessary for its production. This interaction would occur on plates if a few type 80/81 cocci were deposited on a lawn of lysogenic cocci that spontaneously released some phage during incubation and thus infected the type 80/81 cocci. The most satisfactory explanation of these findings was that P.S. 80 cocci contained a completely defective prophage that could be detected only when it formed a recombinant with phage *a* or phage *n*. This prophage will be termed 80′. We have referred to phage *b* as a single entity regardless of whether it represents a recombinant between phage *a* and 80′ or phage *n* and 80′. We have not yet detected any difference between these two recombinants and for the purpose of this paper no distinction will be made between them. A more detailed study of them is in progress.

Prophage substitution during conversion

If a defective prophage exists in type 80/81 cocci, it is clear that the changes in typing pattern that result from lysogenization might be explicable on the basis of prophage substitution. Type 80/81 cocci are immune to the recombinant phage, *b*, and also show 'blocking' of the typing phages 52 and 52A. After lysogenization with a converting phage, such as phage *a* or *n*, the cocci are sensitive to phages *b*, 52 and 52A. Thus the 80′ prophage may determine the immunity to all three phages and its removal by lysogenization may lead to increased phage sensitivity of the cocci. Further evidence for the loss of defective prophage by substitution with a converting phage is derived from the fact mentioned above, that propagation of phage *a* in P.S. 80(*n*) and vice versa, failed to reveal the presence of any of the recombinant phage, *b*.

It follows that the spontaneous mutants of type 80/81 strains which are sensitive to phage 52 and 52A may have gained this phage sensitivity by spontaneous loss of the 80′ prophage. Two such mutants were available, strains 80 1a and 1406/2. Both strains were sensitive to phage *b*. Neither strain yielded a recombinant phage when infected with phage *n*, and neither showed the minute fuzzy plaques when their supernatant fluids were plated on strain 594N. Similarly strain 6346 DL, which had been lysogenized with a converting phage and subsequently cured, was found to be fully sensitive to phage *b* and to be incapable of giving rise to it by recombination. It was concluded that all three strains had lost their defective prophage, the first two spontaneously, the latter by prophage substitution, and that this defective prophage determined the typing pattern of the type 80/81 strains and gave rise to the recombinant phage.

Origin of clone 594B

The origin of clone 594B, the singly lysogenic colony, containing only the *b* prophage, remained to be explained. It had been isolated from the original broth culture of strain 594 in which the recombinant phage was being produced. Since type 80/81 cocci are resistant to phage *b* they cannot be lysogenized by this phage. However, a spontaneous mutant which had lost its 80′ prophage would be sensitive

and could be lysogenized. Alternatively, the doubly lysogenic colonies containing prophage *a* and *b* or *n* and *b* might be unstable and lose their *a* or *n* prophage.

The reconstruction of clones of various prophage states was attempted. Cocci of strain 590 were infected in broth with phage *a* or phage *n* and, after clearing, allowed to become turbid again. The culture was diluted and plated so as to obtain 100–200 colonies/plate after incubation. These master plates were replicated on to a plate flooded with strain P.S. 80 as an indicator of phage *a* or *n*, and on to either strain 594A or 594N, depending on which phage had been used for infection, as an indicator of phage *b*. By this means, colonies containing prophages *a* alone, *n* alone, *a* and *b*, and *n* and *b* were identified and isolated. Some of the colonies containing *a* and *b* or *n* and *b* were unstable when subcultured in broth and showed plaques when flooded on to agar plates, thus behaving like 'suicide' strains. Some single colonies picked from subcultures of these strains contained only the *b* prophage. Similarly, although doubly lysogenic colonies were readily obtained when clones 594A or 594N were treated with phage *b*, such cultures were sometimes unstable on continued subculture and cocci containing the *b* prophage alone could be isolated from them. The singly lysogenic clone 594B could therefore have arisen either by lysogenization of a spontaneous mutant or else by double lysogenization, first with a converting phage and second with the recombinant phage, and the subsequent loss of the 'converting' prophage.

Lysogenicity of other type 80 strains

All of the seven type 80/81 strains examined have yielded the recombinant phage after infection with phage *n*. Table 2 shows the count of phage *n* and phage *b* at the end of one-step growth experiments of phage *n* in five strains isolated from widely different places. All five strains were examined for the presence of free phage but none could be found and it was concluded that all contained the defective prophage. All changed their typing pattern to 52/52A/80/81 after lysogenization with phage *n*.

In addition, 85 strains which had been sent to the Staphylococcus Reference Laboratory in London for routine phage typing and which were phage type 80/81 when tested with the typing phages at the routine test dilution (RTD) were examined for carriage of the defective phage in the following way. The supernatant fluids of 24 hr. broth cultures of the strains were spotted on to plates flooded separately with strains 594A, 594N, 594B, and the non-lysogenic strain 6346 DL. The appearance of small fuzzy plaques on the first two strains and the absence of any reaction of the latter two strains was considered to be fairly good evidence of the carriage of the defective prophage 80′. Of the 85 strains 68 gave the expected result; 10 of the 68 were tested with the typing phages at 1000 RTD and all 10 were type 80/81. The supernatant fluids of the remaining 17 strains showed strong lytic reactions on all four indicator strains. On typing these strains at 1000 RTD all 17 were found to be type 52/52A/80/81, i.e. not typical 'phage type 80/81' strains. These results indicated that the carriage of the defective prophage 80′ was characteristic of all typical type 80/81 strains.

Frequency of recombination

One-step growth experiments were run to determine the rate at which phage *a* or phage *n* recombined with phage 80′ to form the recombinant phage *b*. A log-phase broth culture of P.S. 80 was spun down, washed once in phage adsorption medium

Table 2. *Production of phage 594b by staphylococcal strains of type 80 infected with phage 594n; counts made at the end of one step growth curves*

Strain no.	Phage 594n (particles/ml.)	Phage 594b (particles/ml.)
PS 80	$1{\cdot}7 \times 10^9$	$5{\cdot}9 \times 10^6$
PS 81	$3{\cdot}9 \times 10^9$	$4{\cdot}2 \times 10^6$
U9	2×10^9	7×10^6
8428	$2{\cdot}6 \times 10^9$	$1{\cdot}1 \times 10^7$
1406/1	$6{\cdot}4 \times 10^9$	$2{\cdot}7 \times 10^6$

(PAM; Hershey & Chase, 1952) and finally resuspended in PAM to a concentration of $1–2 \times 10^8$ viable units/ml. Sufficient phage *a* or *n* was added to infect about 40–50 % of the cocci in 15 min. at 37°. At the end of this time the adsorption mixture was centrifuged, washed once in PAM and finally resuspended in nutrient broth warmed to 37°, the time of resuspension being taken as 0 hr. Tenfold dilutions were made in warm nutrient broth to a point where 0·1 ml. contained about 1×10^2 infected cocci/ml. The dilution tubes were held at 37° throughout the experiment and at intervals samples were removed from the appropriate dilutions and titrated for phage *a* (or *n*) and for the recombinant phage. Titrations for the recombinant phage were made on agar containing 1000 μg. streptomycin/ml. The streptomycin-resistant indicator strain $80^{sr}(a)$ was used to titrate the recombinant phage where phage *a* was the infecting phage and strain $80^{sr}(n)$ where phage *n* was the infecting phage. In the presence of streptomycin, uninfected cocci of P.S. 80 which are deposited on the plate are unable to grow and no recombination can take place on the plate. Infected cocci in which recombination has taken place at the time of plating will probably produce a plaque, provided that at least one recombinant particle is already mature or matures within 2–3 min. of plating; cyanide-lysis experiments had shown that streptomycin at the concentration used required this length of time to exert its inhibitory effect. As a control on the streptomycin effect, titrations for phage *a* (or *n*) were made on nutrient agar and on streptomycin agar plates both flooded with strain 80^{sr}.

The results of a typical experiment are shown in Fig. 1. In this experiment phage *a* which was used to infect P.S. 80 had a latent period of about 35 min. and an average burst size of 20 particles of phage *a*/infected coccus. The actual burst size may be smaller since, staphylococci having a tendency to clump, the actual number of infected cocci during the latent period may be higher than the number recorded. Plaques of phage *a* on streptomycin agar began to appear at 5 min. and increased logarithmically up to 35 min., when the count equalled that on nutrient agar. This was interpreted to mean that mature particles of phage *a* appeared at 5 min. when some 3·4 % of infected cocci contained at least one mature phage particle. There was a delay in the appearance of mature recombinant particles until 20 min. At this time about 50 % of infected cocci contained mature *a* particles while only 0·0002 % contained a recombinant particle. The proportion of infected cocci containing recombinant particles increased rapidly until at 35 min. the proportion was about 0·07 %.

It was not clear from the results of a number of experiments whether or not the cocci that released the recombinant phage released more than one recombinant

particle. In most experiments the count of phage *b* continued to increase during the rise period to a final concentration approximately 2–3 times the count at the beginning of the rise period. This might represent a true burst size for the recombinant or it might be the result of late-lysing cocci contributing to the count of recombinant particles. When the infected cocci were lysed with cyanide during the latent period, curves such as that for phage *n* shown in Fig. 2 were obtained. Replication of phage *n* was complete at 50 min. but the release of the phage was not complete until 60 min. During this last 10 min. period the amount of phage *b* increased from $7{\cdot}1 \times 10^5$/ml. to $9{\cdot}5 \times 10^5$/ml. These results do not exclude the possibility of the replication of the recombinant in individual cocci. They do, however, suggest that the initial step of recombination occurs later in the latent period than the initial step of replication of the infecting phage which is in keeping with current knowledge of the recombination process in coli phages (Hershey, 1958).

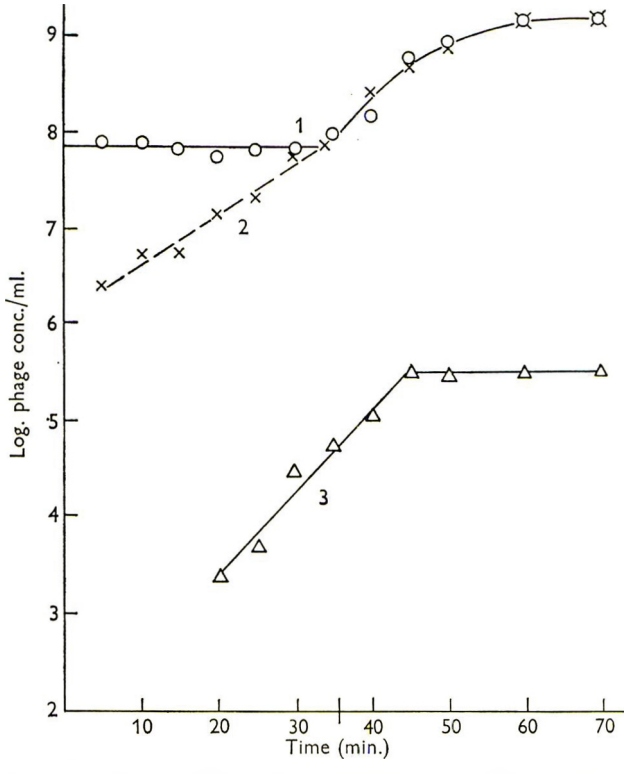

Fig. 1. One step growth curve of phage *a* in staphylococcus PS 80. Titrations for phage *a* were made on strain 80^{sr} on plain agar (curve 1) and on streptomycin agar (1000 μg./ml.) (curve 2). Titrations for the recombinant phage *b* were made on strain $80^{sr}(a)$ on streptomycin agar (curve 3).

The rate of recombination was calculated as the ratio of the number of infected cocci releasing the recombinant to the total number of infected cocci. However, without knowing whether the increase in the number of recombinant particles during the rise period represented an actual burst size or simply the contribution of late-lysing cocci it is impossible to know how many infected cocci actually released

at least one recombinant particle. If it be assumed that the recombinant replicates in those cocci that produce it and that the increase represents a true burst, the ratio would be best calculated on the number of cocci releasing any recombinant phage at the end of the latent period. If, on the other hand, the increase in the recombinant during the rise period be due entirely to the contribution of late lysing cocci, and no replication of the recombinant takes place in any coccus that releases it, then the ratio should be calculated on the total amount of the recombinant released at the end of the rise period.

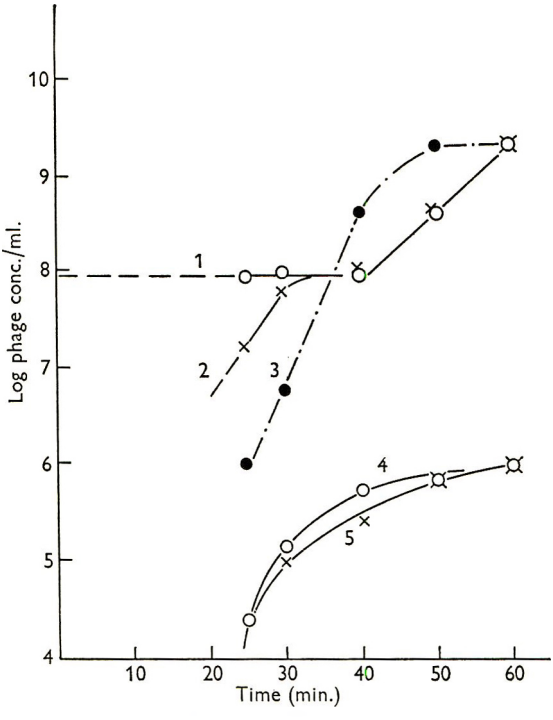

Fig. 2. Cyanide lysis experiment of staphylococcus PS 80 infected with phage *n*. Infected cocci were diluted into broth containing 0·02 M sodium cyanide after 25, 30, 40 and 50 min. These dilutions were held at 4° for 30 min. then at 37° for 60 min. before titrating for phage *n* on strain 80^{sr} and for the recombinant phage *b* on $80^{sr}(n)$. Curve 1 = phage *n* titrated on plain agar. Curve 2 = phage *n* titrated on agar containing 10 μg. streptomycin/ml. Curve 3 = phage *n* released by cyanide-lysed cocci at the time indicated. Curve 4 = phage *b* titrated on streptomycin (10 μg./ml.) agar. Curve 5 = phage *b* from cocci prematurely lysed by cyanide at the times indicated.

The figures obtained differed in different experiments but there was little difference regardless of whether phage *a* or phage *n* was used as the infecting phage. When calculations were based on the count of the recombinant at the end of the latent period (assuming a small burst size) the figures varied between 1/100 and 1/1000 with an average of about 1/600. When calculations were based on the final count of the recombinant (assuming no replication of the recombinant) the figures varied between 1/30 and 1/250 with an average of about 1/150.

DISCUSSION

The experimental results described in this paper are compatible with the hypothesis that the 'conversion' of type 80/81 staphylococci to type 52/52A/80/81 after lysogenization is due to prophage substitution. A similar example of prophage substitution in staphylococci was described by Gorrill (1957) who showed that a prophage carried by the propagating strain of typing phage 76 was displaced when the strain was lysogenized with phage 76. There are several similarities between the system studied by Gorrill and that investigated in the present paper. These include the instability of some of the doubly-lysogenic colonies and the gain in phage sensitivity following lysogenization. The main difference between the present system and that of Gorrill is the complete defectiveness of the 80′ prophage. The prophage state in the type 80/81 cocci resembles that described by Cohen (1959) for the prophage of *Escherichia coli* B, which is completely defective and recognizable only by its ability to enter into recombination with phage P2.

The present results throw some light on to the relationship of the prophages in type 80/81 cocci. The presence of the 80′ prophage causes a 'blocking' of the sensitivity of the cocci to infection with phages 52 and 52A. It may be postulated that this blocking is due to a steric interference by the 80′ prophage which makes loci for phage 52 and 52A inaccessible to these phages. When the 80′ prophage is displaced by the converting phages or by spontaneous loss, this steric interference disappears. The recombinant phage *b* can lysogenize converted type 80/81 cocci from which the 80′ prophage has disappeared and this does not cause blocking of phage 52 and 52A. In other words, interference with phages 52 and 52A is a characteristic of 80′ prophage but not of its recombinant. If, as Jacob & Wollman's work (1959) implies, attachment of the prophage to the bacterial chromosome may involve overlapping by the prophage of some genetic loci, then it may be postulated that the prophages *a*, *n* and *b* overlap fewer sites than does the 80′ prophage.

The phages *a*, *n* and *b* are closely related. Work in progress shows that infection with any of them will induce the others when they are present as prophages. It was considered that phage *a* might be a 'dismune' (Bertani, 1958) mutant of phage *n* or vice versa. However, no evidence for such a mutation has been found. It must therefore be concluded that the original type 80/81 strain in the patient from whom the 594 clones were isolated was infected with two converting phages in the respiratory tract.

Cocci of type 80/81 infected with a converting phage in broth culture behave as suicide cultures. They survive the attack of the phage by becoming lysogenized with it; this, however, renders them sensitive to the recombinant phage which has been produced in a proportion of the infected cocci and on plating they will show plaques due to this phage. If such broth cultures are continued they may show a second clearing. The cocci surviving this second attack will be doubly lysogenic. Strains Bundaberg, 308, 313 and 315 which had been previously used as sources of converting phages (Rountree, 1959) were examined and found to be resistant to phages *a* and *b* but sensitive to phage *n* and it was concluded they were doubly lysogenic. On the other hand, strain 9684, which was the source of a converting phage identical with that of Bundaberg, had shown free phage on its original plate

and then been picked four times from single colonies; it contained only prophage *a* and had evidently lost the recombinant phage.

The type 80/81 strains used in this study were selected as being representative of isolations made in widely separated parts of the world. The fact that all behaved similarly on lysogenization supports the view that they are closely related, if not identical. This similarity is not, however, proof of a single common origin of all these strains.

Some interesting problems are posed by the appearance of the recombinant phage following infection with the converting phages. So far as we are aware, there is no information on phage crosses and recombination in staphylococcal phages, since experimental systems with suitable genetic markers are at present lacking. On analogy, however, with the coli phages, recombination in the *a* or *n* phages would imply that the 80′ prophage is able to enter the mating pool of the infecting phages. The late maturation of the recombinant particles compared with the infecting particles suggests either that this entry occurs at a relatively late stage in the latent period or that, entry having occurred, there is a random chance of the 80′ prophage forming a recombinant. Further, there is no definite evidence for or against further replication of the recombinant. Entry into the mating pool also implies that, after infection with the converting phage, there is an alteration in the physical state of the 80′ prophage leading to its detachment from its site on the bacterial chromosome. Whether this happens in every type 80/81 coccus infected with a converting phage is unknown but it certainly occurs in all cocci giving a lysogenic response to infection, since all of these lose their 80′ prophage.

The term 'converting' phage has been used to describe the phages concerned in this phenomenon. The term is inaccurate when applied to phages *a* or *n*, since the change in the typing pattern of strains of type 80/81 which results from lysogenization with either of these phages is not due to the presence of the phage *per se* but simply to the loss of the defective phage 80′. All the evidence suggests, however, that phage 80′ is a converting phage since its presence in the coccus is essential for maintaining the typing pattern of the strain. Its loss, either spontaneously or by prophage substitution, caused an immediate change in the typing pattern. A closer analysis is required of the characteristics of individual recombinants following infection with different phages in order to find out what variations, if any, there are in the genetic contributions made to the recombinants by the infecting phages.

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Pseudomonas maltophilia, an Alcaligenes-like Species

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SUMMARY

Pseudomonas maltophilia is frequently encountered in specimens submitted to the clinical laboratory for bacteriological examination. This report describes morphological, physiological and serological attributes of this species. Photomicrographs show the presence of polar multitrichous flagella in stained preparations. These pseudomonads do not produce acid from glucose but readily produce acidity from maltose oxidation. A historical review of the epithet *Alcaligenes bookeri* is presented.

INTRODUCTION

'Bacterium bookeri' NCTC 6572 was isolated in 1943 by J. L. Edwards (Public Health Department, County Bacteriology Laboratory, Stafford, England) from a specimen of pleural fluid and was reported as being non-motile and probably a skin contaminant. It was later found (Hugh, 1953) to have multitrichous flagella. Hugh (1953) recorded the morphological and physiological characteristics of six strains that are now classified as *Pseudomonas maltophilia* including NCTC strain 6572, in a study of Alcaligenes. It became apparent that *P. maltophilia* had been unwittingly misidentified as *Alcaligenes faecalis* by Ulrich & Needham (1953); see their strains 249 and 282. An additional 28 strains of *P. maltophilia* have since been isolated from the following sources: river water, well water, raw milk, frozen fish, rabbit and human faeces, blood, pericardial fluid, ascitic fluid, pleural fluid, spinal fluid, oropharyngeal swabs, and contaminated tissue culture. Twenty-six of the above strains have now been restudied and the detailed results are recorded here. An abstract (Hugh & Ryschenkow, 1960) has already been published. The present report serves to amend the previously published description; the name dates from May, 1960 and should be cited as *Pseudomonas maltophilia* Hugh & Ryschenkow, 1960.

METHODS

Morphology. Leifson's (1960) technique was employed to stain flagella.

Physiology. The effects of the bacteria on carbohydrates were determined by the OF (oxidative-fermentative) principle of Hugh & Leifson (1953) and Leifson (1958). The medium used had the following composition: Difco Casitone (pancreatic digest of casein), 5 g.; agar, 3 g.; bromthymol blue (2%, w/v, aqueous solution), 4 ml.; carbohydrate, 10 g.; distilled water, 1000 ml.; final pH 7·1.

Seitz-filtered 10% (w/v) carbohydrate solution was added aseptically to the cool autoclaved (15 min. at 121°) melted basal medium. The medium was then dispensed

aseptically into 13 × 100 mm. sterile tubes to a depth of 50 mm. Ethanol medium was prepared by adding 3 ml. Seitz-filtered absolute ethanol to 97 ml. of the above melted OF basal medium. The ethanol medium was dispensed into sterile 13 × 100 mm. tubes to a depth of 50 mm. and immediately cooled. Ability to grow in an acid medium was studied in yeast extract broth adjusted to pH 4·5 with HCl (Rhodes, 1959) as described by Shimwell, Carr & Rhodes (1960).

Modified Kovacs's reagent (Gadebusch & Gabriel, 1956) was used to detect indole in 1 % (w/v) tryptone broth. Simmons's citrate, Christensen's urea, Møller's (1954, 1955) KCN broth, Kligler's iron agar for hydrogen sulphide detection, Kohn's (1953) charcoal gelatin, Carlquist's (1956) test for lysine decarboxylase activity, phenylalanine deaminase activity (Ewing, Davis & Reaves, 1957), the cytochrome oxidase slope test of Ewing & Johnson (1960), and the 2-ketogluconate test of Moore & Pickett (1960) were used. Physiological tests were incubated at 37°; time of incubation and criteria for the tests being positive are given in the references cited and are described by Ewing (1960).

Serology

Preparation of antigen for production of O antisera. Brain heart infusion broth cultures, incubated at 37° for 24 hr., were placed in flowing steam (100°) for 2 hr. and preserved by adding 0·3 ml. of commercial 40 % (w/v) formaldehyde to 100 ml. of the broth.

Preparation of antigen for production of H antisera. Actively motile organisms, picked from spreading growth in semi-solid agar Gard (1938) plate incubated at room temperature, were inoculated into brain heart infusion broth. After incubation at 22° for 18–24 hr., the broth was diluted with an equal volume of saline containing 0·6 % (v/v) formalin.

Schedule of immunization. Rabbits were given four intravenous injections of H or O antigen at intervals of 4 days. The volume administered was increased each time as follows: 0·5, 1·0, 2·0 and 4·0 ml. The animals were bled on the sixth day after the last injection. Normal serum for control was obtained from each rabbit before immunization. The sera were preserved by the addition of an equal volume of glycerol and stored at 4°.

Preparation of antigens for O and H agglutination. Antigen for O slide-agglutination tests was prepared from an infusion agar slope incubated at 37° for 24 hr. The growth was emulsified in 0·5 ml. saline to form a dense homogeneous suspension. The suspensions were placed in a boiling water bath for 15 min., cooled and tested. Antigen for H tube agglutinations was prepared in the same manner as antigen used for the production of H antisera.

Serological technique. The somatic agglutination of an organism was determined by slide agglutination with a 1/10 dilution of the O antisera. The technique described by Edwards & Ewing (1955) for the somatic agglutination of salmonellas was followed. A twelve tube serial dilution of H antiserum was used to determine flagellar agglutination of the organisms. Tubes were examined for flocculent agglutination after incubation at 45° for 1 hr. followed by 18 hr. at 10°.

RESULTS

Morphology

The organism in peptone broth was a Gram-negative rod about $0{\cdot}5 \times 1{\cdot}5\ \mu$. Capsule and spore formation was not demonstrable. All the strains were motile and showed a tuft of polar flagella. Plate 1 illustrates the polar multitrichous morphology of *Pseudomonas maltophilia*. The flagella appear to be similar in wavelength and amplitude to the flagella of a typical strain of *P. aeruginosa*.

Physiology

This strictly aerobic organism produced a dense turbidity in peptone broth in 18–24 hr. The strains studied did not produce a distinctive pigment in ordinary media. Smooth glistening growth with an entire margin readily appeared on nutrient agar and blood agar in 24 hr. at 37°. All strains produced grey to white colonies on Leifson's deoxycholate agar. A few strains required 48 hr. to grow out on this medium. Sheep erythrocytes in infusion agar base around well-isolated surface colonies of all 26 strains were not haemolysed. The tube haemolysis test with sheep erythrocytes, as performed in the study of cholera vibrios (Pollitzer, 1959), was negative for all strains. None of the strains grew in peptone broth at pH 4·5.

The following physiological reactions were negative for all 26 strains: acidity from arabinose, glucose, galactose, lactose, mannitol, rhamnose, sucrose, xylose; the indole, methyl red, and Voges-Proskauer tests; urea hydrolysis, nitrate conversion to nitrogen gas, hydrogen sulphide production, phenylalanine deaminase, and 2-keto-gluconate production.

The following physiological reactions were positive for all 26 strains: acidity from maltose (acidity was produced in the open tube, but not in the closed tube), motility, gelatin, catalase and lysine decarboxylase. The gelatin test generally became positive before the fourth day. Acidity from maltose generally became evident on the first or second day. Freshly isolated strains of *Pseudomonas maltophilia* inoculated into the basal medium and basal medium containing glucose or other carbohydrates which were not metabolized, produced a strong alkaline reaction at the surface of the medium after 24–48 hr. of incubation. Some old strains which have been kept alive in the laboratory for 8–10 years by periodical transfer in semi-solid medium now produce a very weak acid reaction after prolonged incubation in glucose medium. Some strains produced a positive reaction, while others produced a negative reaction in the tests listed in Table 1.

Antigenic structure

O antigens. Fifteen distinct somatic serotypes were encountered among the 26 strains of *Pseudomonas maltophilia* (Table 2). Organisms of one somatic serotype did not appear to share major antigens with organisms of other serotypes, since cross-reactions between the various O groups were not pronounced. Slow and weak minimal agglutination reactions, which occurred infrequently, were interpreted to be due to minor antigens and were recorded as negative (−) in Table 2. The serum with which the antigen first reacted established the O group to which the organism was assigned. These positive (+) reactions were prompt and strong.

Table 1. *Physiological variability found in* 26 Pseudomonas maltophilia *strains*

	Number of strains		Time required for the strains to become positive at 37°
	Positive	Negative	
Acidity from fructose (aerobic)	3	23	Positive on the 1st day, after 2nd day positives began to become alkaline
Acidity from mannose (aerobic)	23	3	Generally positive on the 1st or 2nd day
Citrate utilization	6	20	Generally positive on 2nd day
KCN resistance	23	3	Positive on 1st or 2nd day
Nitrate reduction to nitrite	11	15	Test performed after 48 hr. incubation
Cytochrome oxidase slope test	16	10	Test performed after 24 hr. incubation
Acidity from ethanol (aerobic)	1	25	4 days

Living suspensions of *Pseudomonas maltophilia* and suspensions treated with ethanol and heat were also used in the O slide-agglutination test. Of the three different O antigen preparations tested, the boiled suspensions were most suitable. The ethanol treated suspensions and the living suspensions frequently produced a slow and weak agglutination, or no agglutination, with homologous antisera. The information recorded in Table 2 was obtained with boiled suspensions of *P. maltophilia*. Undiluted glycerol-preserved normal control serum did not agglutinate the corresponding organism used for immunization.

Table 2. *O agglutination of* Pseudomonas maltophilia *with antisera prepared against the corresponding organism*

Antigen	Antiserum														
	560	810–2	294	555	609	601	557	447	558	653–4	788–3	556	109–4	363–4	873–3
560, 430, 559, 661–1	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
810–2, 104–2, 245–3	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
294	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
555	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
609, 229, 194–1, 457–1	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
601, 611	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
557, 751–4	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–
447	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–
558, 483–2	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–
653–4	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
788–3	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–
556	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
109–4	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
363–4	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
873–3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+

\+ = strong agglutination reaction.
– = no agglutination or weak and slow agglutination.

The 15 *Pseudomonas maltophilia* O antisera did not agglutinate O antigens prepared from 2 strains of each of the following 8 organisms: *Aeromonas hydrophila*, *Alcaligenes faecalis* (peritrichous), *Bordetella bronchiseptica*, *Herellia vaginicola*,

Lophomonas alcaligenes, Pseudomonas aeruginosa, Mima polymorpha, Pseudomonas diminuta. The 16 O antigens of the above organisms were prepared in the same manner as were the *P. maltophilia* O antigens.

There was no evidence of an O antigen common to the following three groups of *Pseudomonas maltophilia*: 12 strains of oropharyngeal origin, 5 strains which apparently were the cause of natural infections, and 2 strains of faecal origin.

Two of the O antigen groups were examined by absorption to confirm that the O antigens within each were serologically similar. The homologous somatic antibodies in antiserum produced by immunization with a boiled suspension of strain 560 were removed by absorption with a live suspension of strain 430 or 559 (see Table 3). The homologous somatic antibodies in antiserum produced by immunization with a boiled suspension of strain 810–2 were removed by absorption with a live suspension of strain 104–2 or 245–3 (see Table 4).

Table 3. *Agglutination following absorption of homologous O antibodies to* Pseudomonas maltophilia *strain 560 with heterologous organisms*

Living suspension (strain number)	Unabsorbed O antiserum to strain 560	O antiserum to strain 560 absorbed with strain	
		430	559
560	+	−	−
430	+	−	−
559	+	−	−
661–1	+	−	−

Table 4. *Agglutination following absorption of homologous O antibodies to* Pseudomonas maltophilia *strain 810-2 with heterologous organisms*

Living suspension (strain number)	Unabsorbed O antiserum to strain 810–2	O antiserum to strain 810–2 absorbed with strain	
		104–2	245–3
810–2	+	−	−
104–2	+	−	−
245–3	+	−	−

H antigens. The reciprocal of the homologous titres of each of the six H antisera are tabulated in Table 5. All 26 strains of *Pseudomonas maltophilia* were agglutinated by one or more of the six H antisera. The H antiserum prepared with *P. maltophilia* strain 560 agglutinated 23 of the 26 strains (88%). Eighteen of the strains (69%) appear to have a very similar flagellar antigenic structure. There appear to be at least 4 distinct flagellar antigens.

Glycerol-preserved normal serum in dilutions of 1/20, 1/40 and 1/80 did not agglutinate the corresponding organisms used for immunization.

A fine granular agglutination reaction occurred in some of the H agglutination tests in antiserum dilutions of 1/80 or less. This was best seen with a $\times 10$ hand lens. This phenomenon was interpreted to be due to O antibodies in the H antiserum reacting with the cell bodies of the formalinized flagellar antigen. Such reactions are recorded as negative (−) in Table 5.

Table 5. *Flagellar agglutination of* Pseudomonas maltophilia *strains with antisera prepared against the corresponding organism*

Suspension (strain no.)	Antisera against strains					
	555	560	810–2	104–2	294	557
	Reciprocal of dilutions					
555	**20,480**	10,240	2,560	—	—	—
560	20,480	**10,240**	2,560	—	—	—
447, 556*	5,120 to 20,480	2,560 to 10,240	1,280 to 5,120	—	—	—
810–2, 245–3	10,240	5,120	**20,480**	20,480	—	—
104–2	—	—	10,240	**40,960**	—	—
294	—	—	—	—	**5,120**	—
611	20,480	10,240	2,560	—	5,120	—
229	20,480	2,560	—	—	—	—
430	—	10,240	—	—	—	—
557	—	—	—	—	—	**10,240**

— = less than 80.

* These organisms and the following were agglutinated over the stated range: 558, 559, 601, 609, 109–4, 194–1, 363–4, 457–1, 483–2, 653–4, 661–1, 751–4, 788–3 and 873–3.

DISCUSSION

The 26 strains of *Pseudomonas maltophilia* examined were morphologically and biochemically much alike, but serologically highly diverse. The following characteristics generally serve to identify the species:

Flagellation	Polar multitrichous
Acid from glucose (aerobic)	No acid
Acid from maltose (aerobic)	Acid
Acid from maltose (anaerobic)	No acid
Acid from mannose (aerobic)	Acid
Citrate utilization	Negative
Potassium cyanide resistance	Positive
Nitrate reduced to nitrogen gas	No gas
Charcoal gelatin	Hydrolysed
Lysine decarboxylase (Carlquist)	Positive
2-Ketogluconate production	Negative

Although no haemolysis was seen around well-isolated colonies, blood agar medium around colonies in the heavily inoculated areas showed a greenish discoloration. This discoloration was interpreted to be the result of excessive accumulation of alkaline metabolic by-products.

The oxidase test for this species of *Pseudomonas* is often positive, delayed and generally difficult to interpret. The positive reactions, obtained with some strains of *Pseudomonas maltophilia*, are generally not as intense as those seen with cholera vibrios and *Aeromonas*. The value of the oxidase test as the sole criterion for the recognition of species of *Pseudomonas* has marked limitations since some species do not produce a positive test and *Vibrio comma* and aeromonads produce a positive test. On the basis of these observations it does not appear judicious to incorporate a positive oxidase test as part of the description of the genus *Pseudomonas*.

The physiological variability found among the 26 strains of *Pseudomonas maltophilia* studied (see Table 1) is interpreted to be variation within the concept of species. No attempt was made to designate physiological varieties of the species in view of the limited number of strains studied and the apparent absence of a practical reason for such division.

Serological reactions did not serve the authors as a useful tool to screen unknown organisms in search of *Pseudomonas maltophilia*. Cursory studies on a strain of *P. maltophilia* might result in confusion with *Alcaligenes faecalis*, *Bordetella bronchiseptica* or *Lophomonas alcaligenes*, since all are motile and produce an alkaline reaction in glucose peptone broth. *A. faecalis* and *B. bronchiseptica* have peritrichous flagella. *L. alcaligenes* has lophotrichous flagella and does not attack carbohydrate, hence it differs from *P. maltophilia* which has polar multitrichous flagella and oxidizes maltose and mannose. Clinical bacteriologists frequently fail to differentiate the above organisms because the identification is erroneously thought to be based on a single character or at most only a few characters, and often these are not necessarily the most important for recognition of the taxon. Before the identity of an aerobic, asporogenous Gram-negative, glucose-nonfermenting rod should be regarded as established, the complete morphological and physiological reaction pattern should be determined and compared with the complete pattern of the species in the genus. If the reaction patterns have not been suitably worked out the unknown organism must be compared with recognized authentic type or neotype cultures.

The authors recognize that other species of *Pseudomonas* might be closely related *P. maltophilia*. However, it is difficult to state how other species are related or how they may be precisely differentiated at this stage of our understanding. Ninety-four differently named species of *Pseudomonas* deposited in the American Type Culture Collection were studied; not one of the strains possessed the characteristics of *P. maltophilia*.

Pseudomonas maltophilia 810–2 is designed the type strain. Strains 560, 611, 661–1 and 873–3 may also be useful in future comparative studies. These five strains have been placed in various repositories and have the following corresponding accession numbers:

RH number	Canadian National Research Council number	American Type Culture Collection number
560	—	13636
611	727	13843
661–1	728	—
810–2	729	13637
873–3	730	—

The species name *Alcaligenes bookeri* has been inadvertently assigned to strains included in the above described taxon; hence it would appear appropriate to review the history of this name. Booker (1887) isolated an organism from the faeces of children with diarrhoea. He labelled the organism Bacillus A and described it as actively motile; milk coagulated, alkaline and peptonized; gelatine liquefied. No mention was made of the Gram reaction, carbohydrate reaction or flagellation. Booker (1890) stated that his Bacillus A might have been *Proteus vulgaris*. Ford

(1903) proposed the name 'Bacillus bookeri' for an organism he isolated from the intestine of a child and considered to be like Booker's Bacillus A. He described the organism as: rod shaped; no spores, no effect on glucose, lactose or sucrose; aerobic; yellow or yellow-brown growth on agar slope; litmus milk alkaline and reduced; nitrate to nitrite reduction negative; gelatin, casein and coagulated serum liquefied; indole not produced. There is no statement of Gram reaction or flagellar morphology in Ford's report. It does not seem to us that Ford's 'Bacillus bookeri' was adequately enough described to enable one to recognize the species today with certainty. Levine & Soppeland (1926) proposed the name 'Bacterium bookeri'. Weldin (1927) and Kutscher (1937) did not describe the flagellar morphology of their strains of *Alcaligenes bookeri*. Subsequent literature reviews, such as *Bergey's Manual* (1923, 1957) also used the name *A. bookeri*. *A. bookeri* is described as an organism with the general properties of alcaligenes and specifically with peritrichous flagella and ability to liquefy gelatin; however, the source of this description has not been made evident. It is generally agreed that the type species *Alcaligenes faecalis* is a peritrichous organism. It follows that any other flagellated species included in the genus must also be peritrichous. In view of these comments we consider that *A. bookeri* is a species *incertae sedis* and therefore the epithet *bookeri* should not be applied to *Pseudomonas maltophilia*. In addition, *P. maltophilia* is a polar flagellated organism; hence it is not appropriate to place the taxon in the genus *Alcaligenes*. A review of the literature has not as yet been productive in establishing an earlier epithet for the taxon described in this report.

The specific epithet and noun *maltophilia* is derived from the old Anglo-Saxon word *malt* and the Greek work *philia*. It has the literal word meaning 'malt friend' or 'friend of malt'. The etymology of the specific epithet *P. maltophilia* makes it clear that it is a hybrid epithet. This is a subsidiary point and does not affect the acceptance of the name.

The utilization by bacteria of disaccharides without utilization of either of its constituent monosaccharides has been observed repeatedly. Pelczar & Doetsch (1949) described a *Neisseria* sp. which utilized maltose with acid production while glucose was not utilized. The authors can confirm the existence of such an unnamed *Neisseria* sp. in the oropharyngeal region. *Pseudomonas maltophilia* is yet another example of an organism with this type of physiology.

Liu (1961) concluded that identification of pseudomonads is difficult because of the lack of a biochemical reaction pattern for each species and that identification of species is dependent upon unstable pigment production and unstable pathogenicity. The observations presented in this study of *Pseudomonas maltophilia* do not offer support for these conclusions. The following redefinition of the genus *Pseudomonas* has been found useful: Gram-negative straight or curved rods, polar-flagellated when motile; strict aerobes which do not produce acid from glucose under anaerobic conditions in a glucose peptone medium, glucose may be oxidized to acid end products in the presence of oxygen; indole, methyl red and Voges-Proskauer tests negative; pigments when present are usually water soluble.

During the later stages of preparation of this manuscript in 1961 Dr J. Tannenberg (Genessee Laboratory, Batavia, New York, U.S.A.) informed us that *Pseudomonas maltophilia* strain 1144 was isolated in pure culture at a necropsy from granulomatous lesions in the lung and heart musculature. Bacterial colonies appeared to be present

within small granulomata at the boundary between necrosis and granulomatous cells. Strain 1144 was agglutinated by O antiserum 560. Strains 560, 430, 559 and 661–1 were isolated from spinal fluid, human oviduct, chest fluid and buccal cavity of a normal adult, respectively. Dr J. Tannenberg will amplify these observations and prepare a report later.

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EXPLANATION OF PLATE

Fig. 1. *Pseudomonas maltophilia* strain 611 showing a rod with a polar tuft of seven short flagella; ×2000.

Fig. 2. *P. maltophilia* strain 611 showing a rod with a polar tuft of five flagella; ×2000.

Fig. 3. *P. maltophilia* strain 611 showing a rod with a polar tuft of four flagella; ×2000.

Fig. 4. *P. maltophilia* strain 611 undergoing binary fission with one of the two daughters showing a polar tuft of three flagella; ×2000.

Fig. 5. *P. maltophilia* strain 611 showing a rod with a polar tuft of three flagella; ×2000.

Fig. 6. *P. maltophilia* strain 611 showing a rod with a polar tuft of two flagella; ×2000.

Fig. 7. *P. maltophila* strain 611 undergoing binary fission with both daughters showing a polar tuft of two flagella at the distal ends; ×2000.

Fig. 8. *P. maltophilia* strain 611 showing a rod with a single long polar flagellum; ×2000.

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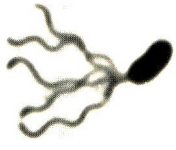

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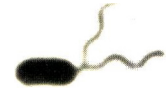

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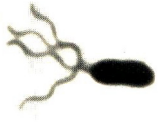

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Taxonomic Relationships of *Xanthomonas uredovorus*

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SUMMARY

The cultural and physiological characteristics of some *Xanthomonas* spp. that are typical of the genus were compared with two atypical species, *Xanthomonas uredovorus* and *X. stewartii*. Electron microscopic studies of *X. uredovorus*, prepared by two methods, show that this organism possesses peritrichous flagella. This fact, together with evidence of the fermentative metabolism of carbohydrates, should exclude this bacterium from the genus *Xanthomonas*. The interpretation of electron micrographs is discussed, and the systematic position of *X. uredovorus* and *X. stewartii*.

INTRODUCTION

From the systematic examination of numerous xanthomonads from culture collections and of fresh isolates from diseased plant material, and from a comparison of these with published descriptions of the genus *Xanthomonas* (Burkholder & Starr, 1948; Dowson, 1957; Dye, 1959) it became obvious that the definition of the genus required clarification. At least two species, *X. uredovorus*, a parasite of the uredia of cereal rusts, and *X. stewartii* the cause of Stewart's disease of sweet corn, were thought to have a number of characteristics which should exclude them from the genus. This study was undertaken in order to compare some physiological characteristics of a selection of seven *Xanthomonas* spp. with *X. stewartii* and *X. uredovorus*, and thus elucidate some fundamental differences between them. An electron microscopic study of *X. uredovorus* was carried out to provide unequivocal evidence of the mode of flagellation. In the original publication (Pon *et al.* 1954) *X. uredovorus* was described as possessing a single polar flagellum, and by this criterion, together with pigmentation, the bacterium was classified in the genus *Xanthomonas*.

METHODS

Source of cultures. The following cultures were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB, Harpenden, Hertfordshire): *Xanthomonas stewartii* NCPPB 67, NCPPB 449, *X. uredovorus* NCPPB 391, *X. phaseoli* NCPPB 557, *X. campestris* NCPPB 279, *X. vasculorum* NCPPB 795 (from Madagascar), NCPPB 796 (from Mauritius), *X. vesicatoria* NCPPB 701, *X. nigromaculans* f.sp. *zinniae* NCPPB 799, *X. malvacearum* NCPPB 634. Three other cultures of *X. uredovorus* were received from Professor M. P. Starr (University of California); these strains, XU 102, XU 103 and XU 104 have been deposited in the

National Collection of Plant Pathogenic Bacteria and given the accession numbers 800, 801 and 802. Three cultures of *X. malvacearum* from C. Logan (Empire Cotton Growing Corporation, Uganda) and two freshly isolated strains of *X. pruni* from Professor H. H. Thornberry (University of Illinois), were included in the group of strains used for systematic examination.

Staining reactions. Weigert's modification of Gram's method was used. The presence of sudanophilic inclusions in heat-fixed smears of the test bacteria was determined by the method of Burdon (1946), but without clarification of the smears stained with Sudan Black B (G. Gurr Ltd.) by dipping in xylene. After staining with the Sudan stain for 10–15 min. the preparation was washed under a gentle stream of tap water. Smears for Sudan staining were prepared after incubation for 3 and 6 days on glucose peptone agar of the following composition: glucose, 20·0 g.; K_2HPO_4, 0·5 g.; $MgSO_4.7H_2O$, 0·25 g.; peptone (Oxo Ltd.) 5·0 g.; agar (Davis Gelatine Ltd.), 20·0 g.; distilled water 1 l. ; pH about 7·2.

Motility. This was determined periodically in a semi-solid medium (3·0 g. agar/l.), using hanging drop preparations.

Oxidation v. *fermentation of carbohydrates.* The medium of Hugh & Leifson (1953) was modified for use with the weakly oxidative plant pathogenic bacteria, as follows: peptone (Oxoid), 1·0 g.; $NH_4H_2PO_4$, 1·0 g.; $MgSO_4.7H_2O$, 0·2 g.; KCl, 0·2 g.; agar, 3·0 g.; bromthymol blue 0·03 g.; distilled water 1 l.; pH about 7·2. Five ml. of a 10 % (w/v) solution of glucose was added to 45 ml. quantities of the molten agar base which was dispensed in sterile plugged test tubes (5 × 0·4 inch) to a depth of about 1·5 in. Sterile liquid paraffin (B.P. grade) was used as a seal, following stab inoculation of the test bacteria from agar cultures. Salicin (10·0 g./l) was sterilized with the medium. Inoculated tubes were examined for a period of 14 days.

Lipase. The method of Sierra (1957) was used; the cultures were discarded after incubation for 6 days.

Hydrolysis of soluble starch, gelatin and casein. The following agar medium was dispensed in 45 ml. quantities in 2 oz. bottles: peptone (Oxoid), 5·0 g.; yeast extract (Difco), 3·0 g.; agar, 20·0 g.; distilled water, 1 l.; adjusted to pH 7·2. Five ml. quantities of 4·0 % (w/v) gelatin (British Drug Houses Ltd.), 4·0 % (w/v) casein (Judex Ltd., light white soluble), or 2·0 % (w/v) soluble starch (British Drug Houses Ltd., Analar) were added to the molten agar base from which three plates were poured. Three or four organisms were inoculated to each plate, into the centre of 0·5 cm. diameter cavities made with a surface sterilized cork borer. After incubation for 6 days starch plates were flooded with Gram's iodine solution and gelatin plates with acid mercuric chloride solution (Frazier, 1926). Zones of hydrolysis were recorded.

Aesculin hydrolysis. The liquid and solid media of Sneath (1960) were used and observed for blackening and loss of fluorescence for a period of 14 days.

Production of H_2S *and indole.* Peptone water supplemented with casein hydrolysate (British Drug Houses Ltd., Laboratory reagent) 1·0 g./l., and L-cysteine hydrochloride, 0·1 g./l. was dispensed in 5 ml. quantities in ½ oz. screw-capped bottles. Lead acetate papers were held over the medium by the screw cap which was kept loose. At 6 days final observations were made for H_2S production, and indole was tested for by addition of ether and Ehrlich's rosindole reagent (Mackie & McCartney, 1960, p. 609). For H_2S production consistent results were only obtained

by using a relatively massive inoculum, i.e. one loopful of growth on glucose peptone agar. From small inocula some *Xanthomonas* spp. gave inconsistent results.

Urease. The method of Christensen (1946) was used, with incubation for 14 days.

Oxidase. Kovacs's method (1956) was used.

Catalase. Loopfuls of agar growth were emulsified in 10 vol. – H_2O_2 on a slide and examined microscopically for evolution of oxygen.

Voges–Proskauer reaction. This was made at 6 days in standard glucose phosphate medium, using Barritt's modification (Mackie & McCartney, 1960).

Salt tolerance. The following medium was dispensed in 10 ml. quantities in 1 oz. screw-cap bottles, and inoculated with two drops of a light suspension of the test organism in distilled water: peptone (Oxoid), 5·0 g.; sucrose (British Drug Houses Ltd., Analar), 5·0 g.; K_2HPO_4, 0·5 g.; $MgSO_4$, 0·25 g., sodium chloride (Analar), 20·0, 30·0, 40·0 or 50·0 g.; distilled water 1 l.; adjusted to pH 7·2. The culture medium was examined for turbidity during incubation for 14 days.

Nitrite formation from nitrate and nitrite destruction. A medium of the following composition was used: peptone (Oxoid), 10·0 g.; K_2HPO_4, 5·0 g.; yeast extract (Difco), 1·0 g.; KNO_3, 1·0 g. or $NaNO_2$, 0·1 g.; agar, 3·0 g.; distilled water, 1 l.; adjusted to pH 7·0; 10 ml. medium per 1 oz. screw-capped bottles. At 5 days tests for nitrite formation or nitrite destruction were made by standard procedures.

Growth temperatures. Two drops of a light suspension of organisms in distilled water were added to glucose peptone agar slopes maintained in a water bath at 40° or 37° for 2 days. Otherwise all cultures were incubated at 28° for the period of test.

Electron microscopy. One culture of *Xanthomonas uredovorus*, strain XU 102, was examined extensively under the electron microscope by one of us (W.H.). Cultures for electron microscopy were grown on nutrient agar slopes and incubated at 20° for 3 days or at 25° for 24 hr. The resultant growth was suspended in distilled water and washed three times by centrifugation. The washed suspensions were then stored overnight at +5°, a procedure which causes partial lysis and consequently causes the cell contents to shrink more readily upon drying. Two methods of preparation, metal-shadowing and phosphotungstic acid 'staining', were used. For the former, the washed suspensions were mounted on formvar films and shadowed with gold-palladium at an angle of 15°. For the phosphotungstic acid preparations (Brenner & Horne, 1959) an equal volume of 2% (w/v) aqueous phosphotungstic acid solution (adjusted to pH 7·4 with KOH) was added to washed suspensions and the resultant mixture mounted on carbon films. The specimens were examined in a Siemens Elmiskop I using the single condensed system with a 200 μ condenser aperture and a 50 μ objective aperture. Electron micrographs were taken at initial magnifications of ×8000 and ×10,000 on Ilford N 50 plates.

RESULTS

All the bacteria examined were unequivocally Gram-negative in young (16 hr.) cultures and did not form prominent polar or central, refractile, sudanophilic inclusions on glucose peptone agar after incubation for 3 or 6 days. This is in accordance with previous observations (Hayward, 1960). With the known exception of *Xanthomonas albilineans*, bacteria of the genus *Xanthomonas* do not form sudano-

philic inclusions in culture on a medium containing utilizable carbohydrate, as do many pseudomonads (Morris & Roberts, 1959).

There were differences between the cultural characteristics of the *Xanthomonas* spp. on the one hand and the cultures of *X. stewartii* and *X. uredovorus* on the other. The latter did not produce the raised, convex, shiny, slimy, mucoid colonies which are typical of the majority of *Xanthomonas* spp. grown on an agar medium containing glucose or sucrose. The exception in this study was the Madagascar strain of *X. vasculorum* which was non-mucoid, in common with several other strains of this species. Elrod & Braun (1947) noted the increase in degree of mucoidness with serial transfer on media rich in carbohydrate, an increase we have noted. This increase in mucoidness was observed in some strains which were non-mucoid on first isolation. All the bacteria examined were yellow in the mass on agar.

The cultures examined were catalase positive. The Kovacs oxidase test did not give unequivocal results on different media and after different periods of incubation: on Difco nutrient agar cultures at 2 days all the *Xanthomonas* spp. were positive, i.e. they gave a strong colour reaction in less than 30 sec., while the cultures of *X. uredovorus* and *X. stewartii* were negative at 30 sec. However, on a medium

Table 1. *Characters of some* Xanthomonas *spp.*, Xanthomonas stewartii *and* Xanthomonas uredovorus

Characteristic	*Xanthomonas* spp. (7 species, 12 strains)	*X. stewartii* (2 strains) NCPPB 449 NCPPB 67	*X. uredovorus* (4 strains)
Motility	+	−	+
Metabolism of glucose	Oxidative (3–6 days)	Fermentative, anaerogenic (24–48 hr.)	Fermentative, anaerogenic (12–24 hr.)
Metabolism of salicin	−	−	Fermentative, anaerogenic
Hydrolysis of 'Tween 80' (lipase)	+	−	+
Soluble starch hydrolysis	+($\frac{9}{12}$)	−	−
Gelatin hydrolysis	+	−	+
Casein hydrolysis	+	−	Weak +
Aesculin hydrolysis	+	−	+
H_2S from cysteine	+	−	−
Nitrite from nitrate	−	−	+
Nitrite destruction	−	−	−
Tolerance of sodium chloride	2–3 %	5 %	5 %
Urease	−	−	−
Voges–Proskauer reaction	−	−(449), +(67)	+
Indole production	−	−	+
Growth at 37°	+	+	+
Growth at 40°	−	−	−

− = Negative reaction, + = positive reaction.

Soluble starch hydrolysis: for *Xanthomonas* spp. zone diameter 36·0–56·0 mm. The two cultures of *X. pruni* did not hydrolyse starch, the Madagascar strain of *X. vasculorum* did not produce a clearly defined zone of hydrolysis but showed a trace of activity.

Casein hydrolysis: for *Xanthomonas* spp. zone diameter 17·0–46·0 mm. The cultures of *X. uredovorus* did not produce a clearly defined zone of hydrolysis but a decrease in the opacity of the medium in a zone 10·0 mm. diameter.

Gelatin hydrolysis: for *Xanthomonas* spp. zone diameter 20·0–46·0 mm., for the cultures of *X. uredovorus* *c.* 15·0 to *c.* 23·0 mm.

containing 2 % (w/v) glucose or glycerol the Kovacs reaction given by some of the *Xanthomonas* spp. was retarded and often indistinguishable from the reaction given by *X. uredovorus* and *X. stewartii*. On a rich tomato juice agar medium the cultures of *X. uredovorus* and *X. stewartii* gave a positive oxidase reaction.

In the modified Hugh & Leifson (1953) technique *Xanthomonas* spp. produced an indicator change in the top 1 cm. of medium after incubation for 3–6 days, whereas the cultures of *X. uredovorus* produced an indicator change throughout the depth of the medium in 12–24 hr. and *X. stewartii* in 24–48 hr. Other characters are given in Table 1.

Electron microscopy of Xanthomonas uredovorus *strain XU 102.*

Shadow-cast specimens showed that the organisms usually possess one or two lateral flagella (Pl. 1, figs. 1, 2, 3); occasionally more flagella were seen (Pl. 1, figs. 4, 5 and 6). No organisms with polar flagella were seen. The phosphotungstic acid preparations were most interesting as these illustrate the difficulties encountered in the interpretation of flagella-preparations. Plate 2, fig. 7, is easy to interpret and clearly shows the lateral insertion of the flagellum. However, were this organism to be rotated on its own axis through 90° the flagellum could appear to be polar in origin, particularly in shadow-cast specimens. Plate 3, fig. 8, illustrates the true path of the flagellum and the origin of the flagellum in an organism in exactly this position.

Plate 4, fig. 9, shows a shadow-cast specimen in which the true path of the flagellum can be traced. Partial lysis and the consequent amount of shrinkage of cell contents allows the true (lateral) position of origin of the flagellum to be clearly defined.

DISCUSSION

The electron micrographs indicate that the original authors (Pon *et al.* 1954) were in error in their interpretation of their electron micrographs. Critical examination of their published micrograph (Pon *et al.* 1954) reveals the possible source of this error. The complete organism shown in the micrograph is full of cell material, it is metal-shadowed and there is a considerable amount of debris near the pole. The true path of the flagellum is probably obliterated by the metal shadowing (cf. Pl. 3, fig. 8; Pl. 4, fig. 9). In addition the Pon *et al.* micrograph includes part of another cell in which the origin of the flagellum is undoubtedly lateral. Attention has already been drawn to the difficulties of interpretation of flagella stains when using the optical microscope (Hodgkiss, 1961). It is apparent from the present results that electron-micrographs of bacterial flagella require equally careful interpretation. As an illustration, an overexposed negative or an overexposed and/or overdeveloped print of Pl. 3, fig. 8 could show a 'polar' flagellum.

The occurrence of mixed polar and peritrichous forms in pure cultures of organisms as described by Leifson & Hugh (1953), Sneath (1956) and Sreenivasan & Venkataraman (1956) merits discussion. Careful electron microscopic studies at Torry Research Station of various organisms have so far failed to yield similar results. It would appear that the interpretation of results requires special care in optical-microscopic examinations of flagella stains when detail such as that seen in Pls. 2, 3, 4 cannot possibly be resolved.

Together with the evidence provided by electron microscopy, the results of the biochemical tests show that *Xanthomonas uredovorus* has no place in the Pseudo-

monadaceae, but should be included in the family Enterobacteriaceae. *X. uredovorus* is not a plant parasite, but a parasite of the fructifications of a fungal pathogen and the bacterial genus *Erwinia* is so defined in the 7th edition (1957) of *Bergey's Manual* to include only plant pathogens. However, *X. uredovorus* is closely related to the anaerogenic *Erwinia* spp., to which genus we propose that this bacterium be transferred.

Misgivings about the classification of *Xanthomonas uredovorus* were expressed by Lovrekovich & Klement (1960) in a study of the tolerance of triphenyltetrazolium chloride by organisms of several genera of bacterial plant pathogens. In the genus *Xanthomonas*, *X. uredovorus* strains, and also *X. stewartii*, were markedly more tolerant than the other species tested.

Xanthomonas uredovorus is related to *Erwinia lathyri* (Manns & Taubenhaus) Holland as described by Graham (1958), who summarized the principal characters of this bacterium as follows: coliform (i.e. fermentative) metabolism of carbohydrates, without gas formation, peritrichous flagellation, positive gelatin liquefaction, Voges–Proskauer and nitrate reduction both usually positive, formation of a yellowish pigment on many agar media. Although discredited as a plant pathogen (Graham, 1958), *E. lathyri* or its close relatives are regularly isolated from moribund plant material. They occur as saprophytes on plants and plant debris and in the soil, which is also characteristic of *Xanthomonas uredovorus* according to Pon *et al.* (1954). *E. lathyri* grows more rapidly on isolation media than xanthomonads, and consequently has often been implicated, mistakenly, as the cause of plant disease. In differentiating the Gram-negative bacteria on plant material the technique of Hugh & Leifson (1953) has proved invaluable for screening a large number of bacteria, and in avoiding the confusion between *E. lathyri* and *Xanthomonas* spp.

The definition of the genus *Xanthomonas* (Dowson, 1957; Dye, 1959) should be modified to include only bacteria with an oxidative metabolism of glucose, and thus exclude organisms such as *X. stewartii* and *X. uredovorus* which are clearly distinct from *Xanthomonas* on other grounds (see Table 1). *X. stewartii*, in addition to having a fermentative metabolism of carbohydrates, is non-motile, has a high salt tolerance (Burkholder & Starr, 1948), does not hydrolyse aesculin, potato starch or gelatin, and does not produce hydrogen sulphide in a medium containing cysteine. However, the systematic position of *X. stewartii* is obscure, and without detailed comparative study with other yellow Gram-negative bacteria, for example, of the genus *Flavobacterium*, it is not yet possible to propose a change in the classification of this organism. It has long been recognized as an atypical xanthomonad (Burkholder & Starr, 1948), and may be a degenerate member of the Enterobacteriaceae.

The authors wish to thank Professor H. H. Thornberry, Professor M. P. Starr, Mr C. Logan and Mr R. A. Lelliott (Curator of the National Collection of Plant Pathogenic Bacteria, Harpenden, Hertfordshire) for generously providing the cultures used in this investgation. The electron microscopy described in this paper was carried out as part of the programme of the Department of Scientific and Industrial Research.

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EXPLANATION OF PLATES

All preparations were of cultures of *Xanthomonas uredovorus*, strain XU 102 grown on nutrient agar at 20° for 3 days.

The scale marked on figures = 1·0 μ.

PLATE 1

Fig. 1. Gold palladium shadow at 15°. ×16,000. Neg. no. 454.

Fig. 2. Gold palladium shadow at 15°. ×25,000. Neg. no. 452.

Fig. 3. Gold palladium shadow at 15°. ×16,000. Neg. no. 481.

Fig. 4. Gold palladium shadow at 15°. ×20,000. Neg. no. 458.

Fig. 5. Gold palladium shadow at 15°. ×24,000. Neg. no. 456.

Fig. 6. Phosphotungstic acid preparation. ×32,000. Neg. no. 472.

PLATE 2

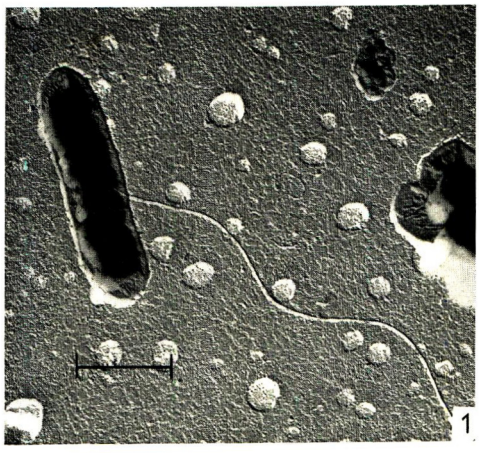
Fig. 7. Phosphotungstic acid preparation. ×32,000. Neg. no. 472.

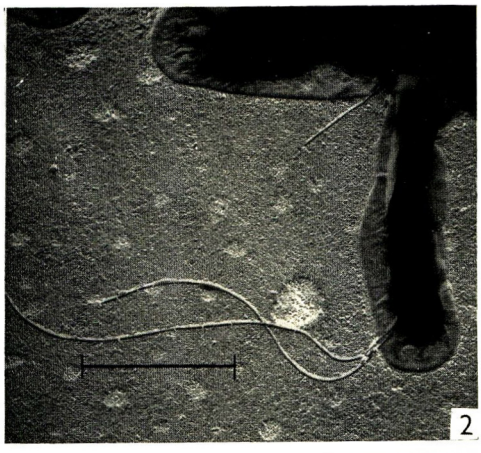
PLATE 3

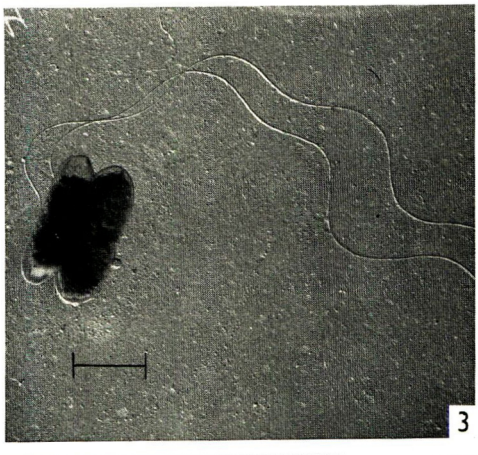
Fig. 8. Phosphotungstic acid preparation. ×32,000. Neg. no. 478.

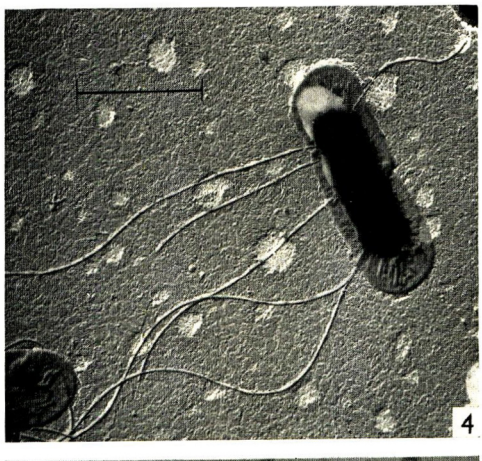
PLATE 4

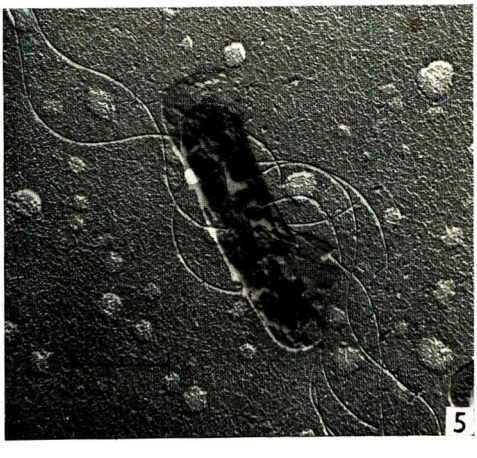
Fig. 9. Gold palladium shadow at 15°. ×32,000. Neg. no. 482.

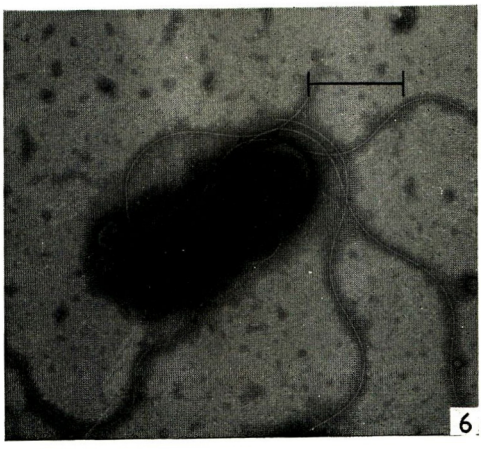












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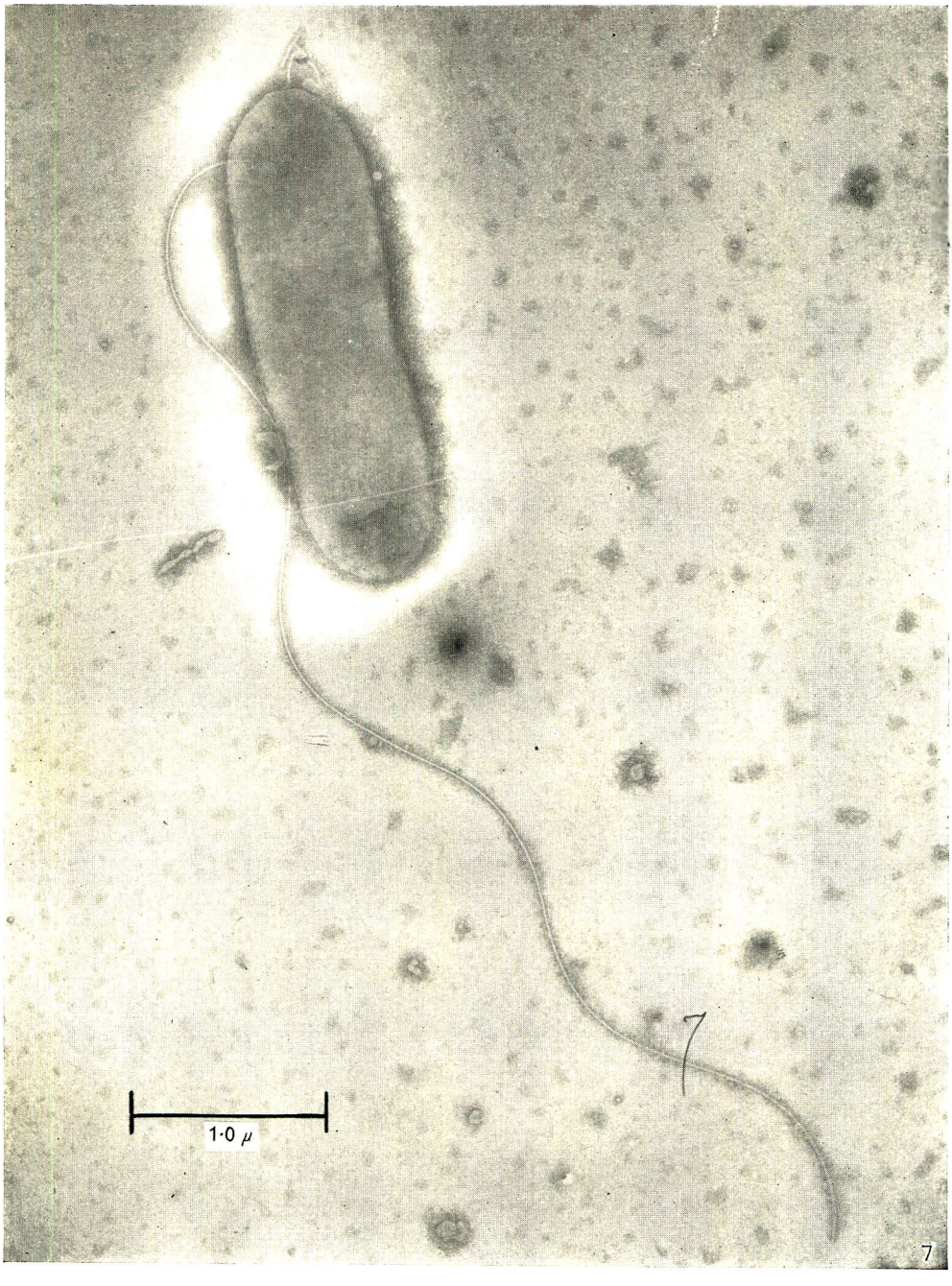
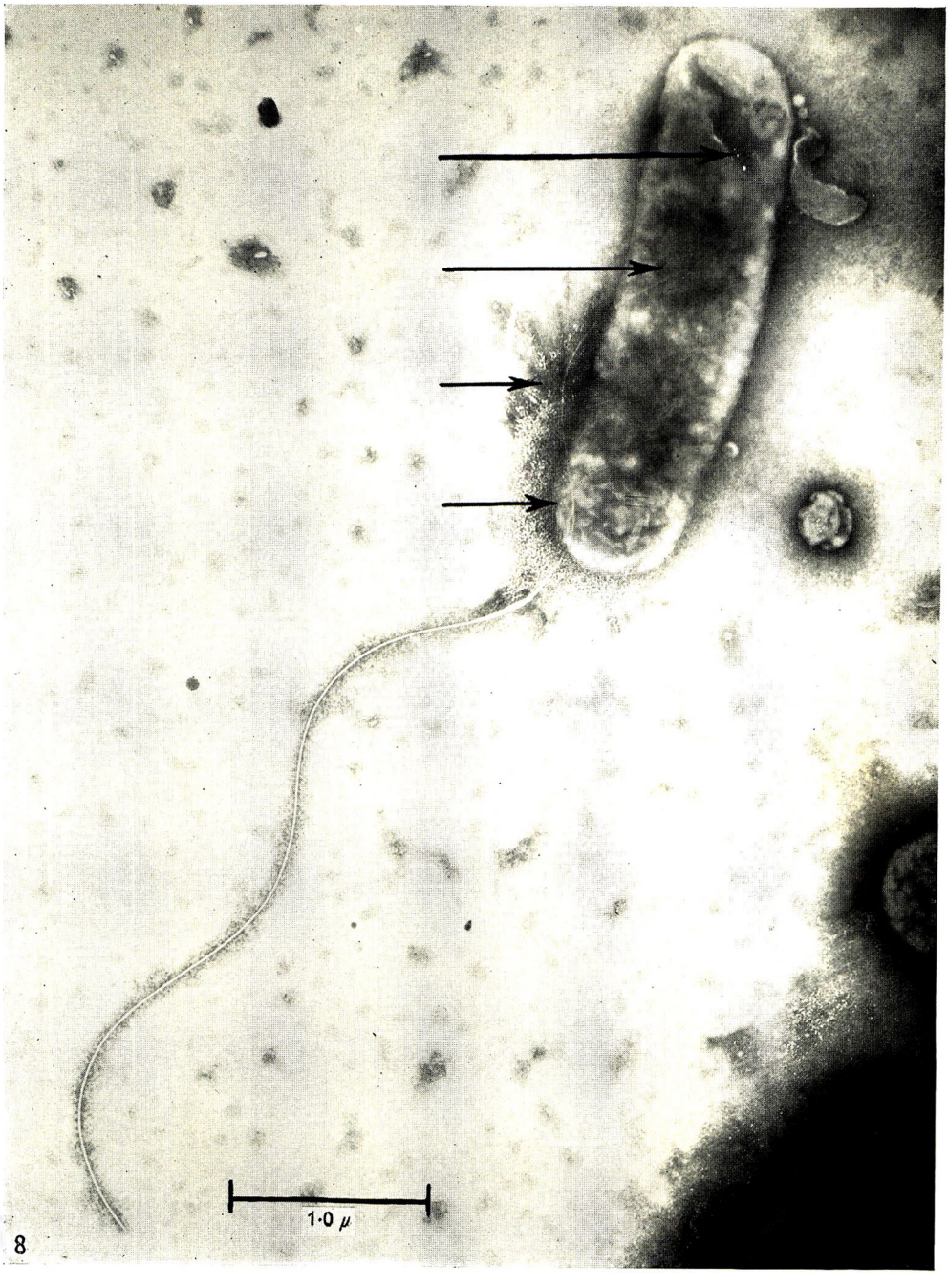


A. C. HAYWARD AND W. HODGKISS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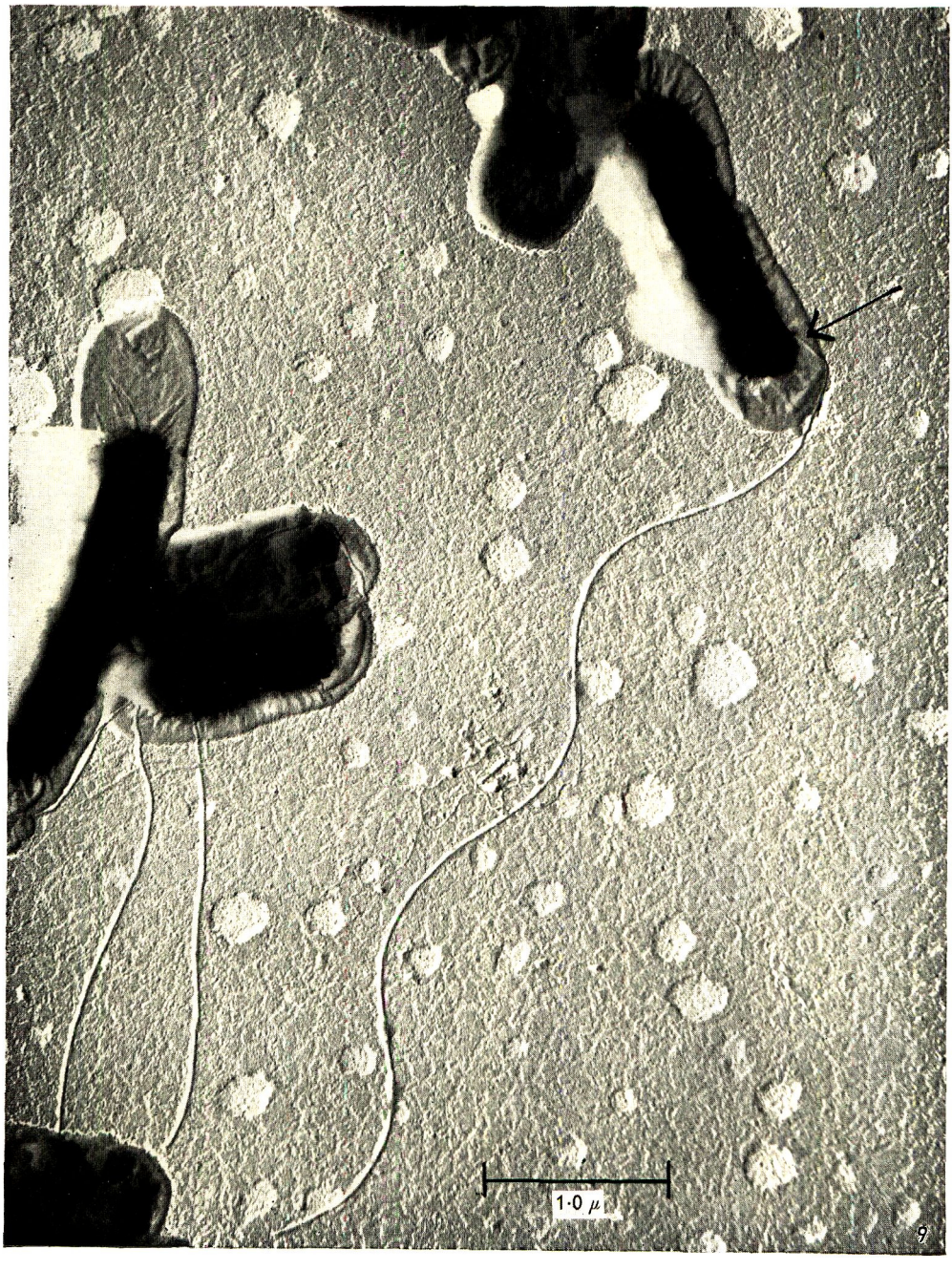
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Metschnikowiella zobellii sp.nov. and *M. krissii* sp.nov., two Yeasts from the Pacific Ocean Pathogenic for *Daphnia magna*

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SUMMARY

Metschnikowiella zobellii sp.nov. and *M. krissii* sp.nov. are described and Latin diagnoses given. Both species form on V8 agar (Wickerham, 1951) club-shaped asci containing a single needle-shaped ascospore and are capable of parasitizing *Daphnia magna* under experimental conditions. *M. zobellii* differs from *M. krissii* by its capacity to ferment glucose and to assimilate galactose, L-sorbose, D-xylose, D-glucosamine, adonitol, D-sorbitol and pyruvate. In infected daphnias the asci and cells of both yeasts resemble Metschnikoff's drawings of *M. bicuspidata*. As cultures of *M. bicuspidata* do not exist and its physiological properties are unknown, the possible identity of either *M. zobellii* or *M. krissii* with *M. bicuspidata* cannot be verified and *M. bicuspidata* (Metschnikoff 1884) Genkel 1913 is therefore considered a *nomen dubium*. Both yeasts were repeatedly isolated from marine substrata on and off the coast of La Jolla, California, U.S.A. Minimum numbers of viable organisms in positive samples varied for *M. zobellii* as follows: sea water, 2–58/100 ml.; fish gut contents (of *Atherinopis affinis littoralis* and *Trachurus symmetricus*), 25–5,730/ml.; surface of Giant Kelp (*Macrocystis pyrifera*), 520–39,200/g. *M. krissii* was isolated only from sea water, 1–57/100 ml.

INTRODUCTION

Metschnikoff (1884) described as *Monospora bicuspidata* a yeast-like organism, parasitic in the body cavity of the fresh-water crustacean *Daphnia magna*. Keilin (1920) summarized Metschnikoff's observations as follows: 'when the cavity of the hosts is entirely invaded by the parasites, these grow in size, become elongated, and form club- or sausage-shaped asci in each of which is developed a single needle-like spore having both ends pointed. When the parasitized host dies, it is filled with ripe spores, and healthy daphnias, which feed on the detritus of their dead and diseased fellows, become infected by ingesting the asci. The latter, when they enter the host's alimentary canal, set free the needle-shaped spores which perforate the gut wall and penetrate in the body cavity, where they germinate laterally, thus starting the new infection'. It was in this material that Metschnikoff made his early observations on the important phenomenon to which he gave the name of phagocytosis.

According to Kudryavtsev (1954), the generic name *Monospora* was changed by Kamensky (1899) to *Metschnikowia* because *Monospora* had been in use, prior to Metschnikoff, for a genus of Algae. Genkel (1913) found out that *Metschnikowia* had also been in previous use and proposed the name *Metschnikowiella*. Keilin (1920),

unaware of this renaming, introduced the name *Monosporella* and described as *Monosporella unicuspidata* a second species, observed by him in the body cavity of larvae of the peratogonid fly *Dasyhelea obscura*. Kudryavtsev (1954) renamed it *Metschnikowiella unicuspidata*.

Monosporella unicuspidata, as described by Keilin (1920), differs from *Monosporella bicuspidata* by the morphology of its spores: pointed at one end and truncated at the other in the former species, pointed at both ends in the latter. According to Keilin (1920) a similar yeast was observed by Bütschli in the coelom of a free-living nematode, *Tylenchus pellicidus*. Mesnil & Caullery (1911) found yeast-like fungi of elongated shape, in a polychaete worm *Potamilla torelli*, which they considered related to Monospora, although they did not succeed in finding spores. Also in a pelagic copepod Acartia they observed what they considered to be a similar yeast. None of these yeasts has been cultivated by their authors or by others. Recently one of us isolated from various marine substrata on and off the coast of La Jolla, California, U.S.A., a number of yeasts, which, based on their fermentative and assimilative properties, belong to two distinct species, both morphologically similar with *Monosporella bicuspidata*. We propose for these two species the names *Metschnikowiella zobellii* and *M. krissii* (in honour of Professor Dr C. E. ZoBell and Professor Dr A. E. Kriss, pioneering marine microbiologists, based at La Jolla, California, U.S.A. and Moscow, U.S.S.R., respectively).

METHODS

Isolation medium. The isolation broth had the following composition (%, w/v): glucose, 2; peptone (Difco), 1; yeast extract (Difco), 0·5; filtered sea water; isolation agar had 2% (w/v) agar added. To discourage bacterial growth the medium was adjusted to pH 4·5 with lactic acid.

Isolations from sea water. Subsurface samples were taken at the end of the pier of the Scripps Institution of Oceanography, University of California, La Jolla, California, U.S.A.; 100 ml. samples were run through 'Millipore' filters (Millipore Filter Corp.) of the following specifications: HA, pore size 0·45 μ; disk diameter 47 mm.; white; plain. Each filter was then placed on top of plated isolation medium.

The plates were incubated at a temperature between 18° and 20°. After 3–5 days yeast colonies appeared on the filters. These were subcultured to the periphery of the plates. The subcultured colonies were distinguished by macroscopic and microscopic morphology. The numbers of each type were recorded and representatives subcultured to slopes of isolation medium for later identification.

Isolations from fish gut contents. Fish of the species *Atherinopis affinis littoralis* Ayres ('Topsmelt') and *Trachurus symmetricus* Ayres ('Pacific Jack Mackerel') were caught with unbaited hooks off the coast of La Jolla, California. The gut contents of each specimen were suspended in twice their volume of filter-sterilized sea-water and 0·2 ml. amounts of the suspensions were spread with a glass rod on the surface of isolation medium plates. The plates were incubated at 18°–20°, the numbers of yeast colonies recorded according to macroscopic and microscopic morphology. Representative colonies of each type were subcultured for later identification.

Isolations from Giant Kelp. Huge heaps of Giant Kelp (*Macrocystis pyrifera*) are washed ashore on the beaches of Southern California. Pieces of stems and leaves

were cut from the subsurface of such heaps, placed in sterile Erlenmeyer flasks and weighed. After the addition of four times the weight of filter-sterilized sea water, the flasks were shaken for 50 min. Serial dilutions of the wash water were then plated on isolation medium and the plates, treated in the same way as the gut-contents plates.

Identification of the yeast isolates. The methods described by Lodder & Kreger-van Rij (1952), Wickerham (1951) and Van Uden & Farinha (1958), were used. Only isolates belonging to the *Metschnikowiella* genus are considered in this paper.

Experimental pathogenicity. Yeast-free pond water was placed in 50 ml. amounts in wide-mouthed jars together with twenty adult embryo-bearing females of *Daphnia magna* from a yeast-free laboratory population. Duplicate jars were inoculated with a loopful of growth of a 10-day sporulating culture of either *Metschnikowiella zobellii* or *M. krissii*; one set was left uninoculated as a control. The jars were incubated at 20° in a water bath and inspected daily for the presence of dead daphnias. The latter were examined microscopically in unstained preparations and used for making cultures.

RESULTS

A total of twenty-nine isolates of *Metschnikowiella zobellii* and six isolates of *M. krissii* were obtained. Their abundance in the various substrata examined can be seen from Table 1. As the possibility of seasonal fluctuations of the *Metschnikowiella* populations has not been excluded, the dates of collection are given.

Table 1. *Minimum numbers of viable cells of* Metschnikowiella *spp. yeasts in marine substrata*

Date of collection (1960)	In 100 ml. seawater		*M. zobellii* in 1 ml. fishgut contents of		*M. zobellii* on the surface of 1 g. Giant Kelp (*Macrocystis pyrifera*)
	M. zobellii	*M. krissii*	*Atherinopis affinis littoralis*	*Trachurus symmetricus*	
12. ii.	8	1	.	.	12,600
19. ii.	28	4	.	.	23,000
21. ii.	2	0	.	.	760
3. iii.	2	0	.	.	5,720
8. iii.	0	14	2,100	0	.
11. iii.	58	0	275	.	.
14. iii.	16	0	.	.	.
15. iii.	0	25	520	0	.
23. iii.	15	30	.	1,950	0
29. iii.	4	0	.	.	.
30. iii.	3	57	.	.	.
1. iv.	.	.	.	5,730	39,200
11. iv.	42	0	0	.	.
9. v.	17	0	85	.	.
10. v.	47	0	930	.	520
11. v.	22	0	.	25	29,600
13. v.	15	0	.	.	0

0 = no yeast present in sample.
. = no sample taken.

Metschnikowiella zobellii sp.nov.

In medio liquido cum dextroso et peptono et extracto levedinis cellulae rotundae et ovoideae, (6–11) × (7–11) μ, singulares, binae aut catenatae; pellicula tenuis formatur. In agaro peptonato cum dextroso et extracto levedinis cultura flavalbida, mollis, subnitida, subreticulata. Pseudomycelium primitivum formatur. Asci in agaro V8 clavati, 18–20 μ; asci in *Daphnia magna* elongato-subclavati, 30–45 μ. Ascosporae acuiformes, bicuspidatae, ad 1 in asco; in agaro V8, 15–18 μ, in *Daphnia magna*, 28–43 μ. Dextrosum fermentatur at non galactosum, saccharum, maltosum, raffinosum, trehalosum et inulinum. Dextrosum, galactosum, L-sorbosum, maltosum, saccharum, cellobiosum, trehalosum, melecitosum, D-xylosum, D-glucosaminum, alcohol aethylicum, glycerinum, adonitolum, D-mannitolum, D-sorbitolum, alpha-methyl-glucosidum, salicinum, arbutinum, acidum pyruvicum et acidum succinicum assimilantur at non nitras kalicus. Necessaria ad fortem crescentiam sunt vitamina externa: biotinum et thiaminum. Temperatura maxima crescentiae 34°–35°.

Morphology. In isolation broth after 48 hr. at 25° cells are round and oval, (6–11) × (7–11) μ, single, in pairs and in small groups. A thin pellicle is formed. The streak culture on isolation agar after 30 days at 25° is yellowish white, soft, dull-glistening and slightly reticulated. A primitive pseudomycelium is formed.

Sporulation. Asci are formed on Henrici's vegetable juice medium (V8 medium, Wickerham, 1951). No sporulation has been observed to occur on isolation agar, malt agar and corn-meal agar. The asci are club-shaped, 18–21 μ long and contain a single, needle-shaped ascospore, pointed at both ends and 15–18 μ long (Fig. 1).

Fermentation, growth in the absence of single vitamins at 25° *and assimilation* (see Table 2).

Maximum temperature still permitting growth: 34°–35°.

Experimental pathogenicity for Daphnia magna. During the 21 days of observation daphnia populations in the control jars showed no signs of disease and produced a numerous offspring. In the jars inoculated with *Metschnikowiella zobellii*, dead daphnias began to appear after 3–5 days and the entire population had died after 10–14 days. The dead specimens were densely filled with yeasts; on subculture the dead daphnias produced only *M. zobellii*, no other yeasts. The morphology of *M. zobellii* in the daphnias showed a striking similarity with Metschnikoff's drawings of *M. bicuspidata*, as reproduced by Keilin (1920) and Kudryavtsev (1954). The asci were elongated or slightly clavate, 30–45 μ long, and contained a single needle-shaped ascospore, 28–43 μ long. The vegetative cells were more slender than the cells on artificial media (Fig. 2).

Type strain. An isolate from sea water has been designated as the type strain of *Metschnikowiella zobellii*. It is maintained in this laboratory with the number 2892. Subcultures have been deposited with the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Netherlands and the Fermentation Division of the Northern Utilization Research and Development Division, Peoria, Illinois, U.S.A.

Metschnikowiella krissii sp.nov.

In medio liquido cum dextroso et peptono et extracto levedinis cellulae rotundae et ovoideae, (4·5–6) × (6–11) μ et longovoideae, (4·5–6) × (11–13) μ, singulares, binae

aut catenatae. In agaro peptonato cum dextroso et extracto levedinis cultura flavalbida, mollis, subnitida, subpunctata. Pseudomycelium primitivum formatur. Asci in agaro V8 clavati, 18–24 μ; asci in *Daphnia magna* elongato-subclavati vel elongato-angulares, 35–40 μ. Ascosporae acuiformes, bicuspidatae, ad 1 in asco; in agaro V8, 15–21 μ; in *Daphnia magna* 32–38 μ. Non fermentat. Dextrosum, maltosum, saccharum, cellobiosum, trehalosum, melecitosum, alcohol aethylicum, glycerinum, D-mannitolum, alpha-methyl-glucosidum, salicinum, arbutinum et acidum succinicum assimilantur, ad non nitras kalicus. Necessaria ad fortem crescentiam sunt vitamina externa: biotinum et thiaminum. Temperatura maxima crescentiae 34–35°.

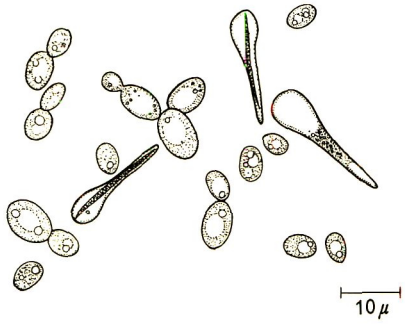

Fig. 1

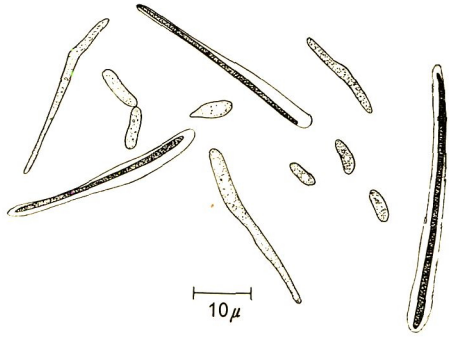

Fig. 2

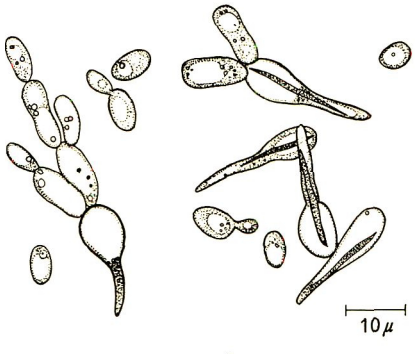

Fig. 3

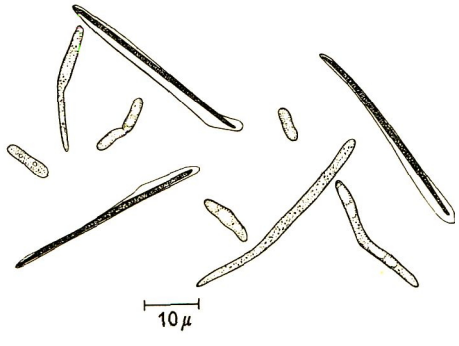

Fig. 4

Fig. 1. *Metschnikowiella zobellii.* Asci and vegetative cells on V8 medium. Camera lucida drawing.

Fig. 2. *Metschnikowiella zobellii.* Asci and vegetative cells in parasitized *Daphnia magna.* Camera lucida drawing.

Fig. 3. *Metschnikowiella krissii.* Asci and vegetative cells on V8 medium. Camera lucida drawing.

Fig. 4. *Metschnikowiella krissii.* Asci and vegetative cells in parasitized *Daphnia magna.* Camera lucida drawing.

Morphology. In isolation broth after 48 hr. at 25° cells are round and oval, (4·5–6) × (6–11) μ, and long oval, (4·5–6) × (11–13) μ, single, in pairs and small groups. A thin pellicle may form after prolonged incubation. The streak culture on

Table 2. *Fermentation, vitamin dependency and assimilation patterns of* Metschnikowiella zobellii

Fermentation +gas	Growth in the absence of single vitamins at 25°	Assimilation			
Glucose −gas	Without biotin ±	Glucose +	Raffinose −	D-Glucosamine +	Salicin +
Galactose	Without pantothenate +	Galactose +	Melecitose +	Ethanol +	Arbutin +
Sucrose	Without inositol +	L-Sorbose +	Inulin −	Glycerol +	Pyruvate +
Maltose	Without niacin +	Maltose +	Soluble starch −	Erythritol −	Lactate −
Lactose	Without folic acid +	Sucrose +	D-Xylose +	Adonitol +	Succinate +
Raffinose	Without *p*-aminobenzoic acid +	Cellobiose +	L-Arabinose −	Dulcitol −	Citrate −
Trehalose	Without pyridoxin +	Trehalose +	D-Arabinose −	D-Mannitol +	Ethylacetoacetate −
Inulin	Without riboflavin +	Lactose −	D-Ribose −	D-Sorbitol +	Inositol −
	Without thiamin ±	Melibiose −	L-Rhamnose −	α-Methyl glucoside +	Nitrate −

+ = good growth, ± = weak growth, − = no growth.

Table 3. *Fermentation, vitamin dependency and assimilation patterns of* Metschnikowiella krissii

Fermentation −gas	Growth in the absence of single vitamins at 25°	Assimilation			
Glucose	Without biotin ±	Glucose +	Raffinose −	D-Glucosamine −	Salicin +
Galactose	Without pantothenate +	Galactose −	Melecitose +	Ethanol +	Arbutin +
Sucrose	Without inositol +	L-Sorbose −	Inulin −	Glycerol +	Pyruvate −
Maltose	Without niacin +	Maltose +	Soluble starch −	Erythritol −	Lactate −
Lactose	Without folic acid +	Sucrose +	D-Xylose −	Adonitol −	Succinate +
Raffinose	Without *p*-aminobenzoic acid +	Cellobiose +	L-Arabinose −	Dulcitol −	Citrate −
Trehalose	Without pyridoxin +	Trehalose +	D-Arabinose −	D-Mannitol +	Ethylacetoacetate −
Inulin	Without riboflavin +	Lactose −	D-Ribose −	D-Sorbitol −	Inositol −
	Without thiamin ±	Melibiose −	L-Rhamnose −	α-Methyl glucoside +	Nitrate −

+ = good growth, ± = weak growth, − = no growth.

isolation agar after 30 days at 25° is yellowish white, soft, dull-glistening and slightly pointed. A primitive pseudomycelium is formed.

Sporulation. Asci are formed abundantly on V8 medium (Wickerham, 1951). Sporulation has not been observed on isolation agar, malt agar and corn meal agar. The asci are club-shaped, 18–24 μ long and contain a single, needle-shaped ascospore, pointed, at both ends and 15–21 μ long (Fig. 3).

Fermentation, growth in the absence of single vitamins at 25° *and assimilation* (see Table 3).

Maximum temperature still permitting growth: 34°–35°.

Experimental pathogenicity for Daphnia magna. *Metschnikowiella krissii* behaved in the same way as *M. zobellii*. Its morphology in *Daphnia magna* can be seen in Fig. 4. Many asci were slightly angular.

Type strain. An isolate from sea water has been designated as the type strain of *M. krissii*. It is maintained in this laboratory with number 2895. Subcultures have been deposited with the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Netherlands, and the Fermentation Division of the Northern Utilization Research and Development Division, Peoria, Illinois, U.S.A.

DISCUSSION

Taxonomy. As *Metschnikowiella bicuspidata* and *M. unicuspidata* have not been cultivated in the laboratory, their physiological properties are unknown and no cultures are available for comparative studies. As yeast species identification is largely based on physiological properties, an eventual re-identification of either species would probably imply an arbitrary decision. Their known properties, as described by Metschnikoff and Keilin, are however unique among the yeasts and characterize the genus *Metschnikowiella*: (1) asci containing a single needle-shaped ascospore; (2) pathogenicity for arthropods. We feel justified therefore to place *M. zobellii* and *M. krissii* in *Metschnikowiella*. An emended description of the genus *Metschnikowiella*, a discussion of its possible phylogenetic relationships and the designation of a type species is better postponed, we feel, till the life cycles of *M. zobellii* and *M. krissii* have been worked out. Both *M. zobellii* and *M. krissii* are morphologically similar to *M. bicuspidata* and both are capable, like *M. bicuspidata*, of parasitizing *Daphnia magna*. As far as Metschnikoff's description of *M. bicuspidata* goes, our two yeasts would have to be identified with Metschnikoff's organism. This, however, is impossible since *M. zobellii* and *M. krissii* belong to distinct species. *M. zobellii* differs from *M. krissii* by its capacity to ferment glucose and to use galactose, L-sorbose, D-xylose, D-glucosamine, adonitol, D-sorbitol, and pyruvate. This situation exemplifies that Metschnikoff's description of *M. bicuspidata* is not sufficiently complete to allow its re-identification. As, furthermore, no cultures of *M. bicuspidata* are available to permit an emended description, we have to consider the name *Metschnikowiella bicuspidata* (Metschnikoff, 1884) Genkel, 1913 as a *nomen dubium*.

Ecology. Metschnikowiella zobellii and *M. krissii* were repeatedly isolated from sea water and *M. zobellii* also from Giant Kelp and fish gut (Table 1). This shows that both species, though capable of infecting and killing *Daphnia magna*, are facultative, rather than obligate parasites. The numbers of viable *M. zobellii* on the surface of Giant Kelp were very high as compared with the numbers found in sea water. In

most heaps of Giant Kelp from which *M. zobellii* was isolated, slight signs (smell, consistency, temperature) of beginning microbial decomposition were noted. Possibly the breakdown of algal polysaccharides by bacterial action provides certain simple carbohydrates, easily used as a carbon source by *M. zobellii*. The inability of *M. krissii* to assimilate galactose and other carbohydrates may possibly explain why this species was not found in the kelp heaps. The numbers of *M. zobellii* were much higher in fish gut than in sea water. This suggests that *M. zobellii* either multiples in fish gut more actively than in sea water or is associated with some of the marine organisms on which the fish feed. Both *M. zobellii* and *M. krissii* are capable of parasitizing *Daphnia magna* under experimental conditions. *Daphnia magna*, however, is a fresh-water crustacean, whereas *M. zobellii* and *M. krissii* seem well adapted to marine environments. Whether these species may, under natural or experimental conditions, parasitize marine crustaceans or other marine animals is an open question.

The isolation work was done during a stay of one author (N. van U.) at the Microbiology Laboratories, Scripps Institution of Oceanography, University of California, La Jolla, California, U.S.A. To Professor Dr C. E. ZoBell and his associates Dr G. E. Jones, Mr H. L. Scotten, Mrs Jean S. ZoBell and Miss Susan Wright warm thanks are expressed for their hospitality and help.

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Isolation, Identification and Growth of some Soil Hyphomycetes and Yeast-Like Fungi which Utilize Aromatic Compounds Related to Lignin

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SUMMARY

Fungi were isolated from soil under several vegetational types by an enrichment technique with vanillin or *p*-hydroxybenzaldehyde as sole source of carbon. Although similar morphologically, the isolates obtained are classified in two separate groups, yeasts and hyphomycetes. A study was made of the growth in pure culture of representative species, namely, *Pullularia pullulans*, *Margarinomyces heteromorpha* and *M. mutabilis* on several aromatic compounds related to lignin.

INTRODUCTION

In previous studies concerning the decomposition of lignin by soil fungi (Henderson & Farmer, 1955) a number of isolates were obtained from soil by means of the dilution-plate method. Apart from incorporating tannic acid in the medium used, in the hope that lignin-decomposing fungi might be indicated by the production of a brown coloration round the colonies, no special selective methods were applied. Subsequent work (Henderson, 1960) showed that the coloration with tannic acid was not linked with an ability to utilize compounds related to lignin and to decompose them; a similar conclusion was reached recently by Ross (1960). In the present investigations a soil enrichment technique with vanillin or *p*-hydroxybenzaldehyde as substrate was used with the aim of isolating lignin-decomposing fungi, and a survey of vegetational types similar to those studied previously carried out. This paper describes the isolation and growth of a number of yeast-like fungi which can utilize compounds related to lignin and which were not encountered in the previous studies.

METHODS

Soil samples were obtained from a heath, Scots Pine forest (actively decomposing litter and humus layers), peat moss, garden and field. With the exception of the Scots Pine litter and humus samples, which were collected by means of sterilized forceps from the appropriate exposed layer, the samples were obtained by scraping a freshly exposed face, from a depth of $6\frac{1}{2}$ in. to $\frac{1}{2}$ in. below the surface, with a sterilized scoop. The samples, three from each area, were immediately transferred to sterile jars and were thoroughly mixed before the removal of samples for the isolation of micro-organisms.

Isolation of fungi. Portions (2·5 g.) of the 'soil' samples were added to 50 ml. sterile water contained in a Waring micro-blender, which was run at top speed for

1 min. Samples (0·5 ml.) of the resulting suspensions were added to 20 ml. lots of Turfitt's medium (Turfitt, 1944) omitting cholesterol and $FeSO_4.7H_2O$ and adding 0·01 % (w/v) vanillin or *p*-hydroxybenzaldehyde as carbon source. After incubation at 27° for 7 days the flasks were shaken by hand for 1 min. and 1 ml. from each culture flask was transferred to a replicate flask which was incubated for a further 7 days. After incubation the flasks were again shaken by hand for one minute and 1 ml. portions of the resulting suspensions were used for the preparation of dilutions at 1/1000, 1/10,000 and 1/100,000. One ml. from each dilution was added to each of two plates of Waksman's agar (Waksman, 1922) modified by the replacement of peptone by 0·25 % (w/v) $(NH_4)_2SO_4$. The plates were incubated for 14 days at 27° and representative organisms were isolated from them during this period. The isolates were maintained in culture on potato glucose agar.

Growth experiments. Suspensions for inoculation were obtained by growing the organisms on 50 ml. modified Czapek mineral salts ($NaNO_3$, 1·5 g.; $MgSO_4.7H_2O$, 0·25 g.; KH_2PO_4, 0·5 g.; KCl, 0·25 g.; glucose, 5 g.; Bacto yeast extract (Difco Laboratories, Detroit, Michigan), 2·5 g.; water, 500 ml.) and incubating at 27° for 2 days. The resulting growth was centrifuged off, washed three times with sterile water and suspended in 10 ml. water. One ml. portions of these suspensions were added to 99 ml. water and single drops, from a 1 ml. pipette, of the resulting suspensions served as inocula.

The basal mineral salts medium for the growth experiments was the same as that used above for the preparation of inocula, but with the glucose and yeast extract omitted. It was sterilized by autoclaving at 120° for 20 min. The required substrates, sterilized by filtration, were added to give final concentrations of 0·01 % (w/v). Ten ml. lots of media were dispensed in 1 oz. vials closed with cotton-wool plugs. After inoculation the bottles were incubated on a shaking machine for 5 days at 27°.

Estimation of growth. In agitated liquid media the fungi grew in a predominantly unicellular form and growth was estimated by counting the number of viable organisms by the technique of Miles & Misra (1938). Occasionally small pellets of mycelium were formed, which led to an abnormally low count. In order to obtain zero time counts, bottles containing 10 ml. water were inoculated in a similar way to the media. One ml. samples were removed aseptically immediately from such bottles and after 1, 2, 3, 4, and 5 days of incubation from the experimental bottles. These samples were used for the preparation of dilutions in the range 1/10 to $1/10^5$, according to the amount of growth. The medium used for making the counts was potato glucose agar which, after pouring, was allowed to solidify and the plates, with the lids slightly raised, were then placed in an incubator at 60° for 2 hr. to dry the surface of the agar. Three drops from each dilution were placed on plates which were then incubated at 27° for 2 days (*Pullularia pullulans*, *Margarinomyces mutabilis*) or 3 days (*M. heteromorpha*) before counting. The volume of the drops added to the plates being known, it was possible to calculate the number of organisms/ml. in the inocula and cultures.

RESULTS

Isolation of fungi by the enrichment technique

The yield of organisms from the different areas varied markedly, there being in some cases a definite enrichment of fungi, while in others there was none. When enrichment did occur it was noticeable that most of the isolates obtained were similar morphologically. They were either mycelial yeasts or Fungi Imperfecti closely resembling these yeasts. These isolates were sent to the Centraalbureau voor Schimmelcultures, Baarn, Netherlands, for identification. A marked growth in the enrichment cultures was observed on incubating the Scots Pine litter sample in the presence of vanillin. Ten representative isolates were obtained in pure culture and were subsequently identified as *Pullularia pullulans* (6); *Trichosporon cutaneum* var. *multisporum* (1); *Phialophora aurantiaca* (1).

Two isolates (no. 8, 10) were not identical with any known species. Although not identical in all respects they were considered by the Baarn workers to belong to one species. The description of these two isolates was given as follows (personal communication):

'Elements, occurring in a young malt extract culture are very different in appearance: small cells round, about 1·2–3 μ in diameter, oval to long-oval cells (2·8–4·5) × (5–10) μ. True mycelium, thinly septate, is produced. After 1 week a sediment is developed and a thin pellicle. The streak culture on malt agar after 1 week at room temperature is creamy and tough, hairy all over the surface in no. 8, in no. 10 hairy in the lower part, wrinkled in the upper part of the streak. In slide cultures on potato-agar outside the coverslip: good growth of true mycelium, the cross-walls of which are thin. Small round spores develop all along the mycelium, often on denticules. Under the coverslip a pseudomycelium develops as well, with oval or elongate blastospores. Asci and ascospores have not been found. These two strains do not ferment sugars. Aerobically glucose, galactose, saccharose, maltose and lactose are assimilated, the latter latently, in the auxanographic test. Ethanol is used as an only source of carbon in the liquid medium according to Wickerham; KNO_3 is not utilized as an only source of nitrogen; external vitamins are required for development in a synthetic medium. There is no growth at 37°. Arbutin is split after 1 week at 25°. The "starch" reaction after 3 weeks of growth in a medium of low pH in shaking cultures was negative. Comparison of the assimilation of the two strains of thirty carbon sources in Wickerham's liquid medium resulted in differences only with sorbitol and erythritol.'

The heath soil sample also gave a definite enrichment when incubated in the presence of vanillin; six representative isolates were identified as *Margarinomyces heteromorpha* (2) and *M. mutabilis* (4). There was no enrichment from Scots Pine humus, while from peat moss a few colonies of *Fusarium* sp. were obtained when the samples were incubated with vanillin. Both vanillin and *p*-hydroxybenzaldehyde were used as substrates for the garden- and field-soil enrichments. No enrichment was obtained on either substrate from the field soil, but the garden soil gave an enrichment of fungi, one of which was identified as *Geotrichum candidum* and the description of the other (isolate no. 18), is as follows:

'On malt extract after 2 days at 25° a white mouldy pellicle is formed. Microscopic examination shows elongate and cylindrical arthrospores, measuring

(3–4) × (5–13) μ, and also true mycelium 3·5–5 μ in diameter. A young potato agar slide culture reveals true mycelium 2·5–4·5 μ, and young "buds", arising on small projections, situated laterally on the mycelium, sometimes also on the joints of the arthrospores. Sometimes these "buds" are formed inside an empty cell of the mycelium. They contain an oildrop. There is no sign of development of ascospores. The organism is not capable of alcoholic sugar fermentation. Glucose and galactose are utilized as sources of carbon; sucrose, maltose, lactose and raffinose are not assimilated, neither is ethanol. KNO_3 is not used as a sole source of nitrogen; arbutin is not split. The organism needs external vitamins for growth in a synthetic medium. It will not develop on malt agar at 37°.'

Growth experiments

The following isolates were used for growth experiments: *Pullularia pullulans* from Scots Pine litter; *Margarinomyces heteromorpha* and *M. mutabilis*, both from heath soil. Glucose was included as a substrate in all growth experiments, for

Table 1. *Growth of* Pullularia pullulans, Margarinomyces heteromorpha *and* M. mutabilis *on aromatic substrates*

Aromatic compounds or glucose were added to the basal growth medium ($NaNO_3$, 1·5 g.; $MgSO_4.7H_2O$, 0·25 g.; KH_2PO_4, 0·5 g.; KCl, 0·25 g.; water, 500 ml.) to give final concentrations of 0·01 % (w/v) experiments 1 to 4 or 0·01 M experiments 5 to 6. Ten ml. lots of media were used. One drop of suspension used as inocula gave zero hour counts (Miles & Misra, 1938; no. viable particles $\times 10^{-4}$/ml.): *P. pullulans*, 1·4–2·9; *M. heteromorpha*, 3·5–3·9; *M. mutabilis*, 14·3–17·3). Growth was for 5 days at 27° on a shaking machine.

Expt. no.	Substrate	Number of viable particles ($\times 10^{-5}$)/ml. *P. pullulans*	*M. heteromorpha*	*M. mutabilis*
1	Control	0·82	57·0	3·1
	Glucose	13·0	390·0	95·0
	p-Hydroxybenzaldehyde	6·4	230·0	53·0
2	Control	1·6	28·0	6·3
	Glucose	—	—	77·0
	Ferulic acid	25·0	120·0	77·0
3	Control	2·4	28·0	9·1
	Glucose	14·0	—	41·0*
	Syringaldehyde	10·0	99·0	29·0*
4	Control	2·8	41·0	7·6
	Glucose	27·0	170·0	160·0
	Vanillin	11·0	81·0	69·0
5	Control	3·7	40·0	9·6
	Glucose	28·0	110·0	57·0*
	Benzoic acid	0	0	0
	Syringic acid	0·33*	70·0	0
	Vanillic acid	29·0	120·0	18·0*
6	Control	1·8	31·0	8·8
	Glucose	610·0	2200·0	1400·0
	o-Hydroxybenzoic acid	0	0	0
	m-Hydroxybenzoie acid	0	390·0	0
	p-Hydroxybenzoic acid	0	94·0	0

* Many pellets present, decreasing count.

comparative purposes. In the first four growth experiments the aromatic substrates were *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin. Growth on glucose started immediately, while on the aromatic substrates there was generally little growth after 1 day, the rate of growth increased between 1 and 2 days and reached a maximum after 2 days. It can be seen from Table 1 that the total growth on these aromatic substrates was always considerably greater than that of the controls without added substrate. In the fifth and sixth experiments the aromatic substrates (at 0·01 M) were benzoic, syringic and vanillic acids and *o*-, *m*- and *p*-hydroxybenzoic acids. Estimations of the number of viable particles were made only on the 5th day. The results are given in Table 1. With the exception of benzoic

Table 2. *Growth of* Pullularia pullulans, Margarinomyces heteromorpha *and* M. mutabilis *on different concentrations of* p-*hydroxybenzaldehyde*, p-*hydroxybenzoic acid*, *vanillin and vanillic acid*

Experimental details as in Table 1, but substrates added at concentrations shown and incubation was for 6 days.

Substrate	Concentration of substrate (M) 0·01	0·0025	0·0014	0·001
		P. pullulans		
p-Hydroxybenzaldehyde	−	−	(+)	(+)
p-Hydroxybenzoic acid	(+)	(+)	(+)	(+)
Vanillin	−	−	+	+
Vanillic acid	++++	++(+)	++	+
Control	−	.	.	.
		M. mutabilis		
p-Hydroxybenzaldehyde	−	+	++	++
p-Hydroxybenzoic acid	+	++	+++	++
Vanillin	−	(+)	++(+)	++(+)
Vanillic acid	−	++	+++	+++
Control	(+)	.	.	.
		M. heteromorpha		
p-Hydroxybenzaldehyde	−	+	+(+)	+(+)
p-Hydroxybenzoic acid	+++	++	++	++
Vanillin	(+)	(+)	++	++
Vanillic acid	+++	++	++	++
Control	(+)	.	.	.

− = no growth; (+) = small amount of growth; +, ++, ++++ = relatively greater amounts of growth.

and *o*-hydroxybenzoic acids all of these substrates supported growth of *M. heteromorpha*, while *P. pullulans* grew only on syringic and vanillic acids and *M. mutabilis* grew only on vanillic acid. A further series of growth experiments used *p*-hydroxybenzaldehyde and vanillin and their corresponding acids at 0·01 M, 0·025 M, 0·0014 M and 0·001 M, the results are given in Table 2. Counts were not taken, but a visual comparison of growth was made. *P. pullulans* was least, and *M. heteromorpha* most, tolerant of the higher concentrations. *P. pullulans* gave markedly greater growth on vanillic acid than on *p*-hydroxybenzoic acid, which did not occur with the other species. There was a tendency for the acids to support growth at a higher concentration than did their aldehydes.

DISCUSSION

The organisms isolated by the soil-enrichment technique used in the present work were all of the same basic morphological types, although some are classified with the yeasts and some with the Hyphomycetes. *Trichosporon cutaneum* var. *multisporum* is a yeast, while *Phialophora aurantiaca*, *Margarinomyces heteromorpha*, *M. mutabilis* and *Geotrichum candidum* are classified in the Hyphomycetes. *Pullularia pullulans* has been assigned to both groups.

The three isolates which were examined for their ability to grow on the lignin-related aromatic compounds *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin, were able to utilize them as sole sources of carbon. These compounds were not as readily available as glucose and there was usually a lag of about 2 days before the growth rates reached their maxima, which in each case were similar to the growth rates on glucose. Previous work (Henderson & Farmer, 1955) revealed the widespread ability of soil microfungi to grow on the above aromatic compounds, while di Menna (1959) found that a number of yeasts could also utilize them. The present studies therefore add to the list of organisms known to attack lignin-related aromatic compounds in soil and emphasize the possible role of micro-organisms in the decomposition of lignin under natural conditions.

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The Metabolism of Aromatic Compounds Related to Lignin by some Hyphomycetes and Yeast-like Fungi of Soil

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SUMMARY

The metabolism of various lignin-related aromatic compounds by several soil Hyphomycetes and yeast-like fungi was investigated. Adaptation studies with whole organisms and cell-free extracts confirmed previously proposed metabolic pathways (Henderson & Farmer, 1955; Henderson, 1960). It was shown that protocatechuic acid is an intermediate in the metabolism of vanillin and ferulic acid. Protocatechuic acid oxidase activity of cell-free extracts of *Pullularia pullulans* was found to be stimulated by ferrous ions and to depend on –SH groups.

INTRODUCTION

The studies reported in the previous paper (Henderson, 1961) were extended by investigation of the metabolism of several lignin-related aromatic compounds by yeast-like fungi of soil. Techniques previously employed with other soil microfungi for studying oxygen uptakes (Henderson, 1956) and for the isolation of intermediate products of metabolism (Henderson, 1957) were used. Cell-free extracts of *Pullularia pullulans* were found to possess protocatechuic acid oxidase activity, a property which proved to be very useful for indicating the adaptation to protocatechuic acid of organisms which had been exposed to several aromatic compounds. Thus these compounds could be associated in metabolic pathways which finally passed through protocatechuic acid.

METHODS

Organisms. These were isolated by a soil-enrichment technique (Henderson, 1961). They were *Pullularia pullans*, *Margarinomyces heteromorpha*, *Margarinomyces mutabilis* (2 isolates), *Phialophora aurantiaca*, *Geotrichum candidum*, *Fusarium* sp. and unidentified isolates no. 8 and 10.

Production of organisms. The fungi were grown on the basal mineral salts medium previously used (Henderson, 1961), to which were added glucose, Difco yeast extract and aromatic compounds as required. For metabolic experiments in the Warburg apparatus and for the large-scale metabolic experiments the growth medium contained 1 % (w/v) glucose and 0·5 % (w/v) yeast extract. The medium was dispensed in 40 ml. lots in 250 ml. conical flasks; incubation was for 3 days at 27° on a shaking machine (Webley & Duff, 1955). The growth was then harvested and washed three times with distilled water. When the organisms were to be adapted to different substrates the harvesting and washing were carried out aseptically, and the washed

organisms transferred to 250 ml. conical flasks containing 50 ml. 0·067 M-phosphate buffer (Clark, 1928) at pH 5·2, and substrate at 0·001 M. Control organisms were transferred to buffer only. After incubation overnight on the shaking machine the organisms were again harvested and washed.

In the production and adaptation of cells of *Pullularia pullulans* for preparation of crude cell-free extracts organisms were obtained from 160 ml. lots of medium containing 2% (w/v) glucose ± 0·001 M-aromatic compounds. The media were dispensed in 1 l. culture flasks (Jobling Cat. no. 1410). Since the fungus would not grow in the presence of catechol or *o*-hydroxybenzoic acid, when these substances were being investigated it was grown on the basal mineral salts medium + 2% (w/v) glucose, and the organisms were adapted by transferring them aseptically to phosphate buffer (pH 5·2) + 0·001 M solutions of these compounds and then incubating overnight. Control organisms were transferred to buffer only.

Manometry. Initially the respiration of *Pullularia pullulans* and *Margarinomyces mutabilis* in the presence of vanillin was tested over a range from pH 4 to pH 8 (McIlvaine's buffer). Since neither species was sensitive over this pH range it was decided to use 0·067 M-phosphate buffer (pH 5·2) as in previous experiments with soil fungi (Henderson, 1956). In experiments with whole organisms 1·0 ml. of suspension (containing about 7 mg. dry wt. organism/ml. and 0·5 ml. buffer were added to the main compartment of each Warburg flask, 0·2 ml. 5% (w/v) KOH to the centre cup and 0·5 ml. of 0·01 M solutions of substrates (except ferulic acid which was 0·003 M) to the side-arm. Acid substrates were neutralized with sodium hydroxide. Experiments were conducted at 30° in air. Readings were taken at 30 min. intervals for 3½ hr., substrates being tipped in from the side-arms after 30 min. In experiments with cell-free extracts 0·5 ml. of the extracts and 1·0 ml. of 0·067 M-phosphate buffer (pH 7) were added to the main compartment and 0·02 M solutions of substrates were used. Substrates were tipped from the side-arms at 0 hr. and experiments were run for 4 hr. before carrying out β-ketoadipic acid estimations. Otherwise conditions were the same as those for experiments with whole organisms.

Large-scale metabolic experiments. Suspensions containing about 11 mg. dry wt. *Pullularia pullulans*/ml., 10 mg. dry wt. *Margarinomyces heteromorpha*/ml. and 6 mg. dry wt. *M. mutabilis*/ml. were used. Three ml. of suspension were added to 25 ml. 0·01 M substrate solution in 100 ml. conical flasks (except in the case of ferulic acid when 25 ml. 0·003 M substrate solution was used). The aldehydes were sterilized by filtration through sintered glass and the acids by autoclaving at 120° for 20 min. at pH 6·5. After addition of the suspensions the flasks were incubated on the shaking machine and one flask of each substrate was removed after 5 and 22 hr. The growth was removed by centrifugation, the supernatant fluid acidified and extracted 3 times with 10 ml. ether. The ether was removed by evaporation and the extracts were dissolved in a few drops of absolute ethanol. Samples were applied to Whatman no. 1 filter papers which were developed with *n*-butanol + ammonia (sp.gr. 0·880) + water (80 + 5 + 15 vol.) for 16 hr. at 21° by the descending method. They were sprayed with diazotized sulphanilic acid (Bray, Thorpe & White, 1950).

Cell-free extracts. Crude extracts of organisms were prepared according to the method of McIlwain (1948). The organisms were harvested, washed three times and ground in a pestle and mortar with three times their wet weight of alumina (H fine Aloxite, The Carborundum Company Ltd., Manchester, 17). For this purpose

approximately 2 g. wet wt. organism were used. The crushed organisms were extracted with 3 ml. of 0·067 M-phosphate buffer (pH 7). The crude extract was freed from alumina by centrifugation at 1600 *g* for 10 min. After the crude extract had been centrifuged at 20,000 *g* for 30 min. precipitation with $(NH_4)_2SO_4$ was sometimes carried out according to Dagley & Patel (1957). The precipitates obtained were separated by centrifugation at 20,000 *g* for 30 min. The supernatant fluids were removed and the precipitates dissolved in a volume of 0·02 M-phosphate buffer (pH 7) equal to the volumes of crude extract from which they were prepared.

β-Ketoadipic acid estimation. The presence of β-ketoadipic acid was detected by the Rothera test (Rothera, 1908). The amount of acid present was estimated by decarboxylation with 4-aminoantipyrine and measuring the carbon dioxide evolved (Sistrom & Stanier, 1953).

RESULTS

Oxygen uptake by fungi in the presence of aromatic substrates

In experiment 1 (Table 1) oxygen uptakes in the presence of *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin were measured. All species metabolized these substrates to some extent, but there was considerable variation

Table 1. *Oxygen uptake by fungi in presence of aromatic compounds*

Added to each flask were: 1·0 ml. cell suspension (equiv. about 7 mg./ml. dry wt.) + 0·5 ml. phosphate buffer (pH 5·2) in the main compartment; 0·2 ml. 5 % (w/v) KOH in centre cup; 0·5 ml. 0·01 M solutions of substrates (except ferulic acid which was 3 × 0·001 M) or 0·5 ml. water in side-arm. In Expt. 2 the cells were starved by shaking overnight in phosphate buffer (pH 5·2). The figures refer to oxygen uptake over the 3 hr. period following the addition of substrates.

Expt. 1.

Fungus	Substrate: *p*-Hydroxybenzaldehyde	Ferulic acid	Syringaldehyde	Vanillin	Control
	(oxygen uptake, μl.)				
Fusarium sp.	400	589	314	251	213
G. candidum	174	94	131	142	88
M. heteromorpha	349	344	432	526	199
M. mutabilis (*a*)	489	328	558	635	240
M. mutabilis (*b*)	444	263	403	508	191
P. aurantiaca	265	307	455	553	267
P. pullulans	198	330	157	287	93
Isolate no. 8	651	298	541	564	201
Isolate no. 10	142	101	92	92	85

Expt. 2.

Fungus	Substrate: *p*-Hydroxybenzoate	Syringate	Vanillate	Protocatechuate	Control
	(oxygen uptake, μl.)				
Fusarium sp.	151	53	129	137	40
G. candidum	81	110	91	79	87
M. heteromorpha	373	149	290	276	39
M. mutabilis (*a*)	232	206	241	250	88
M. mutabilis (*b*)	208	187	213	213	79
P. aurantiaca	155	195	197	302	142
P. pullulans	144	129	352	324	62
Isolate no. 8	118	67	64	132	63
Isolate no. 10	161	90	82	142	67

in the oxygen uptakes by different isolates on different substrates. These substrates were metabolized without any lag period. In Expt. 2 (Table 1) oxygen uptakes in the presence of sodium *p*-hydroxybenzoate, syringate, vanillate and protocatechuate were recorded. Since the rates of oxygen uptake with this group of substrates were considerably slower than with the first group, overnight starvation of the organisms in buffer was necessary to decrease the endogenous respiration. This made the differences in oxygen uptakes between the control and experimental flasks more distinct. In contrast to Expt. 1 there were lag periods for all these substrates before the oxygen uptakes reached their maximum rate, as found previously for other soil fungi (Henderson, 1956). The lag period with *Pullularia pullulans* on protocatechuic acid was eliminated by previous incubation with vanillin, vanillic acid, *p*-hydroxybenzaldehyde or *p*-hydroxybenzoic acid (Fig. 1, *a*, *b*). Incubation with syringic acid did not appear to lead to adaptation to protocatechuic acid. Previous incubation with ferulic acid eliminated the lag period with vanillic acid.

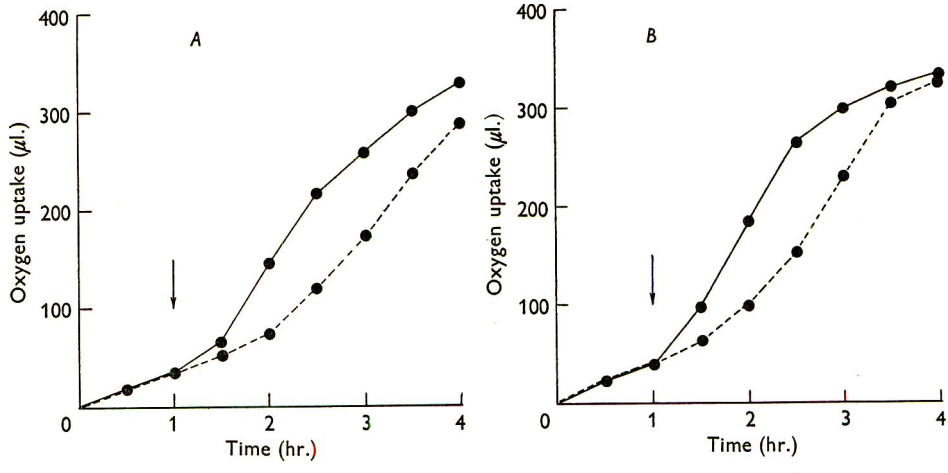


Fig. 1, *A*, *B*. Oxygen uptake by *Pullularia pullulans* in the presence of protocatechuic acid. Added to each flask were : 1·0 ml. organism suspension (equiv. about 7 mg. dry wt./ml.)+ 0·5 ml. phosphate buffer (pH 5·2) in main compartment; 0·2 ml. 5 % (w/v) KOH in centre cup; 0·5 ml. 0·01 M solution of protocatechuic acid in side arm. — = adapted cells; --- = non-adapted cells. *A*: cells adapted to vanillin; *B*: cells adapted to *p*-hydroxybenzaldehyde. Protocatechuic acid added where indicated by arrow.

Intermediate products of metabolism detected in large-scale metabolic experiments

All of the aromatic compounds tested were attacked by the fungi and some were so rapidly metabolized that no trace of them could be found when incubation proceeded beyond 2 days. The principal intermediate products detected (see Table 2) included those found previously (Henderson & Farmer, 1955; Henderson, 1957): *p*-hydroxybenzoic acid from *p*-hydroxybenzaldehyde, vanillic acid from vanillin and ferulic acid, syringic acid from syringaldehyde, the hydroxybenzoic acids from the corresponding mono-methoxybenzoic acids, protocatechuic acid from *p*-hydroxybenzoic acid, *p*-hydroxybenzoic and protocatechuic acids from benzoic acid and vanillic acid from 3:4-dimethoxybenzoic acid. No intermediates

Table 2. *Principal intermediate products of metabolism of aromatic compounds detected by paper chromatography*

Suspensions of organisms were added to 25 ml. of 0·01 M solutions of the substrates and incubated for 5 or 22 hr. The cell material was removed and the supernatant fluids extracted with ether. The extracts were examined by paper chromatography. Papers were developed with *n*-butanol + ammonia (sp.gr. 0·880) + water (80 + 5 + 15) for 16 hr. at 21°, by the descending method. They were sprayed with diazotized sulphanilic acid. R_F values were calculated from leading edge of spots.

Substrate	Organism					
	P. pullulans, product or colour	R_F	*M. heteromorpha*, product or colour	R_F	*M. mutabilis*, product or colour	R_F
Benzoic acid	*p*-Hydroxybenzoic acid	0·267	*p*-Hydroxybenzoic acid	0·262	*p*-Hydroxybenzoic acid	0·283
	Protocatechuic acid	0·057			Protocatechuic acid	0·058
Ferulic acid	Vanillic acid	0·133	Vanillic acid	0·125	Vanillic acid	0·117
p-Hydroxybenzaldehyde	*p*-Hydroxybenzoic acid	0·141	*p*-Hydroxybenzoic acid	0·131	*p*-Hydroxybenzoic acid	0·175
	? Yellow	0·885			? Yellow	0·836
	? Yellow	0·784				
o-Hydroxybenzoic acid	? Yellow	0·183	? Yellow	0·152	Gentisic acid	0·239
					? Yellow	0·162
m-Hydroxybenzoic acid	—		Protocatechuic acid	0·042	—	
p-Hydroxybenzoic acid	Protocatechuic acid	0·046	Protocatechuic acid	0·062	Protocatechuic acid	0·025
o-Methoxybenzoic acid	*o*-Hydroxybenzoic acid	0·584	*o*-Hydroxybenzoic acid	0·474	*o*-Hydroxybenzoic acid	0·492
m-Methoxybenzoic acid	*m*-Hydroxybenzoic acid	0·223	—		*m*-Hydroxybenzoic acid	0·283
p-Methoxybenzoic acid	*p*-Hydroxybenzoic acid	0·179	*p*-Hydroxybenzoic acid	0·140	*p*-Hydroxybenzoic acid	0·173
3:4-Dimethoxybenzoic acid	Vanillic acid	0·122	—		Vanillic acid	0·126
2:4-Dimethoxybenzoic acid	? Orange-Yellow	0·107	? Yellow	0·272	? Orange	0·320
			? Orange	0·129	? Pink-Orange	0·113
Syringaldehyde	Syringic acid	0·104				
	? Red	0·827				
Vanillin	Vanillic acid	0·129	Vanillic acid	0·143	Vanillic acid	0·179
	Vanillyl alcohol	0·797			? Orange	0·774

Colours. Hydroxybenzoic acids: yellow; protocatechuic acid: pink-white; vanillyl groups: orange; genitisic acid: grey-white; syringic acid: red. — = No spots obtained.

from 2:4-dimethoxybenzoic acid were identified. Gentisic acid was formed by *Margarinomyces mutabilis* from *o*-hydroxybenzoic acid. It was identified as follows. A solution of *o*-hydroxybenzoic acid was incubated in the presence of *M. mutabilis* for 2 days, the organisms removed and the supernatant fluids acidified and extracted with ether. The extract, after application to paper chromatograms, was developed with *n*-propanol + ammonia (sp.gr. 0·880) + water (80 + 5 + 15 vol.) and sprayed with diazotized sulphanilic acid. Pure gentisic acid was run as a standard along with the extract. Strips containing standard spots were cut from each side of the paper and the position of gentisic acid in them was located by spraying with diazotized sulphanilic acid. Strips from the centre of the paper, one containing the

extract and the other gentisic acid, were cut out and the areas in them, corresponding to the location of gentisic acid in the test strips, were removed and eluted with 2·5 ml. 0·067 M-phosphate buffer (pH 7). The solutions obtained were examined by ultraviolet spectrometry and were found to have corresponding maxima at 328 mμ in acid solution, 317 and 259 mμ in alkaline solution and 320 mμ in neutral solution, confirming that gentisic acid was present in the extract from the experimental solution.

A number of spots of high R_F values which had not been obtained previously were noted. These included vanillyl alcohol which was identified in an extract obtained after incubating a solution of vanillin in the presence of a suspension of *Pullularia pullulans* for 2 days. After removal of the organisms the supernatant fluid was extracted with ether without previous acidification. The extract yielded a large orange spot of R_F 0·812 and a faint orange spot of R_F 0·085 when developed with butanol + ammonia + water (80 + 5 + 15) and sprayed with diazotized sulphanilic acid. The former spot corresponded to an unidentified spot obtained in extracts after incubation for 5 and 22 hr.; the latter spot was vanillic acid. Since the extract contained no residual vanillin it could be used directly for analysis by infrared spectrometry; its spectrum was found to be identical with that of vanillyl alcohol. A spot corresponding to vanillyl alcohol was also obtained on developing the extract from *Margarinomyces mutabilis* cultures on vanillin. Spots of high R_F value from extracts of cultures of the three species on *p*-hydroxybenzaldehyde and of *P. pullulans* on syringaldehyde may also have been the corresponding alcohols, but standards were not available for comparison.

These results indicate that the metabolism was basically similar to that of the filamentous fungi investigated previously, but there was evidence of a strong reducing mechanism in these organisms which led to the formation of alcohols from aldehydes. Vanillyl alcohol was produced even when a stream of air was bubbled through a flask containing *Pullularia pullulans* and vanillin, but no spot of high R_F value was obtained when *p*-hydroxybenzaldehyde was similarly treated.

Protocatechuic acid oxidase activity in cell-free extracts

Preliminary Warburg experiments showed that cell-free extracts from adapted organisms took up oxygen in the presence of protocatechuic acid and that β-ketoadipic acid was produced, as indicated by the Rothera test (Rothera, 1908). Extracts from non-adapted organisms showed negligible protocatechuic acid oxidase activity. In subsequent experiments protocatechuic acid oxidase activity was determined by estimating the β-ketoadipic acid which accumulated after incubation of extracts with protocatechuic acid.

Table 3 shows that protocatechuic acid oxidase activity was well developed in extracts of those organisms which had been exposed to *m*- and *p*-hydroxybenzoic and vanillic acids, as well as to protocatechuic acid. These results indicate that protocatechuic acid was an intermediate product in the metabolism of these acids. Activity was very low in organisms which had been exposed to syringic acid, signifying that protocatechuic acid was not involved in its metabolism. It can also be seen that exposure to *o*-hydroxybenzoic acid and to catechol did not lead to the adaptation of organisms to protocatechuic acid. However, exposure to these substrates led to adaptation to catechol and *cis-cis*-muconic acid, while organisms which had been exposed to protocatechuic acid were negative in this respect.

Table 3. *β-Ketoadipic acid production by crude extracts of* Pullularia pullulans *from protocatechuic acid,* cis-cis-*muconic acid and catechol*

Before extraction half of the organisms were adapted to various substrates. Added to each flask were: 1·0 ml. phosphate buffer (pH 7)+ 0·5 ml. cell extract in the main compartment; 0·2 ml. 5 % (w/v) KOH in the centre cup; 0·5 ml. 0·02 M solutions of substrates (10 μmole) in the side-arm. Experiments were run for 4 hr. at 30°. β-Ketoadipic acid was estimated by decarboxylation with aminoantipyrine.

	Substrate					
	Protocatechuic acid		*cis-cis*-Muconic acid		Catechol	
	Extract: from adapted (A) or non-adapted (NA) organism					
	A	NA	A	NA	A	NA
Adapting substrate	β-ketoadipic acid produced (μmole)					
Catechol	1·4	1·4	6·5	1·8	8·8	1·4
o-Hydroxybenzoic acid	1·2	0·9	5·0	0·9	8·8	1·1
m-Hydroxybenzoic acid	6·3	1·5	—	—	—	—
p-Hydroxybenzoic acid	5·9	0·7	—	—	—	—
Protocatechuic acid	8·2	0·7	0·8	0·7	0·4	0·5
Syringic acid	1·9	1·3	—	—	—	—
Vanillic acid	7·5	1·8	—	—	—	—

Protocatechuic acid oxidase activity in crude cell-free extracts was partly inhibited by $1{\cdot}4 \times 10^{-4}$ M-*p*-chloromercuribenzoate (CMB), but activity was retained when glutathione at $1{\cdot}3 \times 10^{-3}$ M was present. When the concentration of CMB was increased to $5{\cdot}6 \times 10^{-4}$M the enzyme activity was completely inhibited, but was restored by $1{\cdot}3 \times 10^{-3}$ M glutathione (see Table 4). When crude cell-free extracts were precipitated with $(NH_4)_2SO_4$ and the precipitates dissolved in phosphate buffer according to Dagley & Patel (1957), the resulting solutions showed considerable protocatechuic acid oxidase activity, which was inhibited by $5{\cdot}6 \times 10^{-4}$ M-CMB and

Table 4. *Influence of* p-*chloromercuribenzoate and glutathione on protocatechuic acid oxidase activity in extracts of* Pullularia pullulans

Activity was measured by β-ketoadipic acid production. Organisms were adapted to protocatechuic acid before extraction (crude extract) and precipitation with $(NH_4)_2SO_4$. The precipitate was dissolved in 0·02 M-phosphate buffer (pH 7; $(NH_4)_2SO_4$ precipitate). Added to each flask were: 1·0 ml. phosphate buffer (pH 7)+ 0·5 ml. cell extract+ 0·5 ml. *p*-chloromercuribenzoate (CMB), to give a final concentration of $1{\cdot}4 \times 10^{-4}$ M or $5{\cdot}6 \times 10^{-4}$ M, or 0·5 ml. water + 0·5 ml. glutathione, to give a final concentration of $1{\cdot}3 \times 10^{-3}$ M in the main compartment, or 0·5 ml. water; 0·2 ml. 5 % (w/v) KOH in the centre cup; 0·5 ml. 0·02 M-protocatechuic acid (10 μmole) in the side arm. Experiments were run for 4 hr. at 30°. β-Ketoadipic acid was estimated by decarboxylation with aminoantipyrine.

	Crude extract		$(NH_4)_2SO_4$ precipitate
	Expt. 1	Expt. 2	
	β-ketoadipic acid produced (μmole)		
Addition			
None	4·5	2·3	1·2
CMB $1{\cdot}4 \times 10^{-4}$ M	2·2	—	—
CMB $5{\cdot}6 \times 10^{-4}$ M	—	0	0
CMB+ Glutathione	5·4	3·4	1·4

— = Not tested

restored by $1{\cdot}3 \times 10^{-3}$ M-glutathione (Table 4). Dialysis against 0·022 M-phosphate buffer (pH 6·9) for 16 hr. decreased the activity of crude extracts and was partly restored by 0·001 M-$FeSO_4$. The results were as follows (μmole β-ketoadipic acid): crude extract, 4·1; dialysed extract, 1·2; dialysed extract + $FeSO_4$, 2·3; dialysed extract control, 0. No consistent results were obtained about the influence of glutathione or ferrous ion on the activity of untreated crude extracts, but the activity of solutions of ammonium sulphate precipitates was increased by the presence of $1{\cdot}6 \times 10^{-3}$ M-glutathione, or 0·001 M-$FeSO_4$ or $FeCl_3$ (see Table 5).

Table 5. *Influence of glutathione and various ions on protocatechuic acid oxidase activity in extracts of* Pullularia pullulans *after treatment with* $(NH_4)_2SO_4$

Activity was measured by β-ketoadipic acid production. Organisms were adapted to protocatechuic acid before extraction which was followed by precipitation with $(NH_4)_2SO_4$ and solution of the precipitates in 0·02 M-phosphate buffer, (pH7). Added to each flask were: 1·0 ml. phosphate buffer (pH7) + 0·5 ml. cell extract + 0·5 ml. glutathione (to give a final concentration of $1{\cdot}6 \times 10^{-3}$ M) or 0·5 ml. $FeSO_4.7H_2O$, $FeCl_3.6H_2O$, $MnSO_4.5H_2O$, or $ZnSO_4.7H_2O$ solution (to give a final concentration of 0·001 M) in the main compartment; 0·2 ml. 5% (w/v) KOH in the centre cup; 0·5 ml. 0·02 M-protocatechuic acid (10 μmole) in the side arm. Experiments were run for 4 hr. at 30°. β-Ketoadipic acid was estimated by decarboxylation with aminoantipyrine.

Addition	Expt. 1	Expt. 2
	β-ketoadipic acid produced (μmole)	
None	0·3	1·5
Glutathione	2·0	—
$FeSO_4$	—	4·5
$FeCl_3$	—	3·0
$MnSO_4$	—	1·1
$ZnSO_4$	—	1·3

— = Not tested

DISCUSSION

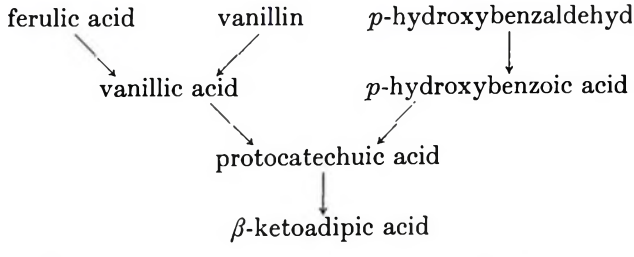
The replacement experiments showed that the soil organisms used in the present work metabolize aromatic compounds in essentially the same way as did those studied previously (Henderson & Farmer, 1955; Henderson, 1960). The principal difference lay in the reduction of vanillin to vanillyl alcohol and possibly also of *p*-hydroxybenzaldehyde and syringaldehyde to their corresponding alcohols. At the same time, oxidation of the aldehydes with the formation of the corresponding acids was proceeding. This may be compared with the anaerobic dismutation of aldehydes by *Acetobacter* spp. investigated by Molinari (1929). He found that benzaldehyde, cinnamaldehyde and anisaldehyde were dismuted to their respective acids and alcohols. In contrast Bachman, Dragoon & John (1960) obtained quantitative conversion of *o*-hydroxybenzoic to the corresponding alcohol by *Neurospora crassa*, while *Polystictus versicolor* reduced certain aromatic acids to the corresponding aldehydes and alcohols (Farmer, Henderson & Russell, 1959). The acids and alcohols found in the present work were intermediate products of metabolism and with time all trace of phenolic compounds disappeared. A further difference from previous findings was the metabolism of *o*-hydroxybenzoic acid through gentisic acid by *Margarinomyces mutabilis*. Catechol was previously detected as an intermediate

product of the metabolism of *o*-hydroxybenzoic acid by fungi (Henderson, 1960) and is the most common intermediate in bacterial metabolism. However, Mitoma, Posner, Reitz & Udenfriend (1956) obtained gentisic acid from *o*-hydroxybenzoic acid when it was subjected to a hydrolysing system found in liver microsomes.

The results from the present experiments showed that protocatechuic acid was an intermediate product in the metabolism of *p*-hydroxybenzoic, *m*-hydroxybenzoic and benzoic acids, while *p*-hydroxybenzoic acid is itself an intermediate in the metabolism of *p*-hydroxybenzaldehyde and *p*-methoxybenzoic acid. Previously the formation of protocatechuic acid, by other fungi, from *m*-hydroxybenzoic acid (Henderson, 1960) and from *p*-hydroxybenzoic acid (Henderson, 1957) was demonstrated; protocatechuic acid is also well known as an intermediate in the metabolism of various aromatic compounds by bacteria. Thus the metabolism of protocatechuic acid itself appears to be a focal point in the metabolism of aromatic compounds. The principal aim of the present respiration studies with whole organisms and with cell-free extracts was, therefore, directed towards linking the metabolism of various compounds with protocatechuic acid and studying the metabolism of this acid itself.

The morphology of the organisms used here made them very suitable for respiration experiments. In addition, *Pullularia pullulans*, which was selected for further work with cell-free extracts, lent itself very well to this type of work. Adaptation studies with whole organisms confirmed the postulated pathways of metabolism leading eventually to protocatechuic and β-ketoadipic acids. Previous incubation with vanillin, vanillic acid, ferulic acid, *p*-hydroxybenzaldehyde or *p*-hydroxybenzoic acid resulted in the elimination of the lag period which preceded the oxidation of protocatechuic acid, thus linking the metabolism of the compounds in a common pathway. Although it was not demonstrated by isolation from cultures on vanillin or vanillic acid that protocatechuic acid was an intermediate in their metabolism, in previous experiments with *Aspergillus niger* (Henderson, 1960) an indication of its production from vanillic acid was obtained. Also, vanillic acid is known to induce the synthesis of protocatechuic acid oxidase in *Neurospora crassa* (Gross & Tatum, 1955). These authors found, however, that vanillic acid was not metabolized by their organism, in contrast to the fungi studied in the present work which not only oxidized it but could grow on it as sole source of carbon.

Experiments with crude cell-free extracts, in which β-ketoadipic acid formation was used to indicate protocatechuic acid oxidase activity, confirmed the above results. They can be summarized as follows:



o-Hydroxybenzoic acid is frequently metabolized through catechol (Walker & Evans, 1952; Henderson, 1960) which is further metabolized to yield *cis-cis*-muconic

acid (Stanier & Hayaishi, 1951) and β-ketoadipic acid (Evans & Smith, 1951). The results obtained with *Pullularia pullulans* indicated that it follows the *o*-hydroxybenzoic acid → catechol → *cis-cis*-muconic acid → β-ketoadipic acid pathway. The course of metabolism of syringic acid was not elucidated. Protocatechuic acid oxidase activity in organisms which had been exposed to syringic acid was very low indeed, indicating that protocatechuic acid is not produced during metabolism of syringic acid. In being stimulated by $FeSO_4$ this protocatechuic acid oxidase resembles those of *Neurospora* sp. (Ottey & Tatum, 1956) and of *Pseudomonas* sp. (Dagley & Patel, 1957) and not that of a soil pseudomonad studied by Ribbons & Evans (1960) or that of liver (Stanier & Ingraham, 1954). The occurrence of –SH groups in the enzyme was demonstrated by its inhibition by *p*-chloromercuribenzoate, which was annulled by glutathione. Protocatechuic acid oxidase of liver (Stanier & Ingraham, 1954) was also inhibited by *p*-chloromercuribenzoate.

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Bacteriophages of *Bacillus cereus* and of Crystal-forming Insect Pathogens Related to *B. cereus*

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SUMMARY

Four phages isolated from bacteria of the *Bacillus cereus* group are described. They show cross-reactions between crystal-forming and non-crystal-forming strains which are deficient in ability to produce a lecithinase C. The implications of this finding are discussed.

INTRODUCTION

Hannay's rediscovery of the parasporal body in the insect pathogen *Bacillus thuringiensis* (Hannay, 1953) and the subsequent identification of this crystalline protein as the toxin of the organism (Angus, 1954) have stimulated considerable interest in crystal-synthesizing aerobic spore-formers and several workers have discussed the relationship between these organisms and *B. cereus* (Toumanoff, 1956; Le Corroller, 1958; Heimpel & Angus, 1958). Most of the crystal-formers produce an active lecithinase C, as do the majority of strains of *B. cereus*; but two strains, the so-called *B. entomocidus* var. *entomocidus* and *B. entomocidus* var. *subtoxicus* of Heimpel & Angus (1958) are distinguished from the rest by absence of lecithinase activity and by failure to produce acetylmethylcarbinol.

During work with *Bacillus entomocidus* var. *entomocidus* phage plaques appeared spontaneously in slope cultures and a study of the phage responsible, and subsequently of others, was undertaken in an attempt to throw some light on the interrelationships of this group of bacteria.

METHODS

Bacteria. The work involved 21 typical lecithinase-producing strains of *Bacillus cereus*, 12 lecithinase-producing strains of *B. cereus* var. *mycoides* and 13 lecithinase-producing crystal-formers. Two lecithinase-negative crystal-formers, *B. entomocidus* var. *entomocidus* and *B. entomocidus* var. *subtoxicus* were supplied by Dr C. L. Hannay and a further strain, G2, was isolated from larvae of *Galleria mellonella*. A crystal-forming organism which produced a very small amount of lecithinase C, G1, was isolated from the same stock of larvae at an earlier date. Three lecithinase-negative strains of *B. cereus* (strains 634, 826, 827) were obtained from Dr Ellen Garvie (see Stone, 1952) and had originally been isolated from milk. A lecithinase-negative strain of *B. cereus* var. *mycoides*, strain A.C., was a laboratory stock culture of uncertain origin.

Lecithinase. Lecithinase production was detected by growth on egg-yolk agar. Most of the organisms studied were active lipase producers and this may lead to

confusion when egg-yolk agar is used to detect lecithinase activity; for this reason results were checked with the lecithin agar described by Willis (1960).

Acetylmethylcarbinol production. Ability to produce acetylmethylcarbinol was examined by the method described by Smith, Gordon & Clark (1952).

Production of acid from carbohydrates. Ability to produce acid from carbohydrates was tested in the presence of ammonia-N on the solid medium of Smith *et al.* (1952) as used by Heimpel & Angus (1958) when studying these organisms.

Media. Cultures for the production of phage stocks and for phage sensitivity testing were grown, usually from spore inocula, on 1% (w/v) nutrient agar plates containing 1% (w/v) peptone and 0·5% (w/v) NaCl. Comparison with several other growth media showed that this relatively poor medium gave the most readily visible plaques.

Isolation of phages. Four phages were isolated and used in the investigation. When crude filtrates of *Bacillus entomocidus* var. *entomocidus* were spotted on to lawns of the same organism the plaques formed were all clear and indistinguishable from one another, but on lawns of the subtoxicus variety two types of plaque were formed: (i) clear plaques from which were isolated phage A which gave similar clear plaques on the variety entomocidus; (ii) turbid plaques yielding phage B which also gave clear plaques on the entomocidus variety.

When 7-day-old broth cultures of *Bacillus entomocidus* var. *subtoxicus* are streaked on to agar plates many of the resulting colonies are phantom forms (Nungester, 1929) collapsing after incubation for 2 days to form thin flat transparent 'ghost' colonies. The organisms remain in this form indefinitely on subculture and the effect is probably due to phage although attempts to demonstrate this conclusively have so far failed. When filtrates of broth cultures of a phantom variant of this organism were spotted on to lawns of organism G1, a few discrete phage plaques were formed from which a further phage, phage C, was isolated.

Cultures of the lecithinase-negative *Bacillus cereus* strain 826 (see Stone, 1952) often showed spontaneously developing phage plaques which yielded the fourth phage, phage D.

Phage A was routinely propagated on *Bacillus entomocidus* var. *entomocidus*, phage B on *B. entomocidus* var. *subtoxicus*, phage C on organism G1 and phage D on *B. cereus* 826. The propagating strains were also used as indicator strains when titres of phage preparations were being determined.

Phage sensitivity tests. As mentioned by McCloy (1951) some strains of *Bacillus cereus* produce colicine-like agents whose effects can simulate confluent phage lysis at low dilutions. This is also true of many crystal-formers and the effects of these agents were readily seen when undiluted filtrates of phage preparations were dropped on to plates inoculated with test organisms. Such non-specific lysis was never seen when phage preparations were diluted 1/10 before use; sensitivity tests were therefore carried out at this dilution. Positive results observed at phage concentrations which gave confluent lysis were always confirmed by the production of separate phage plaques at higher dilutions.

Phage stocks. Phages were purified by picking from single plaques on the routine propagating strain at least four successive times and grown by spreading a few drops of a sterile-filtered preparation on agar plates the surfaces of which were inoculated with cultures of phage-sensitive organisms, and incubating overnight at

30°. The resulting growth was harvested into a few ml. of peptone water and the suspension sterilized by passing it through a Hemming centrifugal filter (Beaumaris Instrument Co. Ltd., Rosemary Lane, Beaumaris, Anglesey). Phage preparations containing 10^{10} or 10^{11} plaque-forming units/ml. on the corresponding indicator strains were readily prepared by this method which gave, on the whole, higher titres than fluid culture techniques.

Titration of phage. Rough estimates of numbers of phage particles were made by the modified Miles & Misra technique described by McCloy (1958). Accurate titrations were made by the soft agar-layer method described by Adams (1959). In both methods the plates were inoculated with spore suspensions of the indicator organism produced by harvesting 10-day-old agar slope cultures in sterile distilled water and pasteurizing the resulting suspensions at 75° for 15 min. Control plates without added phage were always set up as a check against spontaneous phage lysis. With some organisms indicator plates prepared in this way always showed spontaneous phage plaques, but phage was usually absent from 16 hr. broth cultures grown from single plaque-free colonies; such cultures were used to inoculate titration plates instead of the usual spore suspensions.

RESULTS

Acetylmethylcarbinol production

Acetylmethylcarbinol was produced by all the lecithinase-positive strains of *Bacillus cereus*, *B. cereus* var. *mycoides* and the crystal-formers. Steinhaus (1951) and Heimpel & Angus (1958) reported that the two varieties of *B. entomocidus* did not produce acetylmethylcarbinol and Dr Ellen Garvie (personal communication) has found the same for *B. cereus* 634. These negative reactions were confirmed. However, the lecithinase negative *B. cereus* var. *mycoides* strain A.C., *B. cereus* strains 826 and 827 and crystal-formers G1 and G2 were all strongly positive.

Lecithinase activities

The majority of strains of *Bacillus cereus*, *B. cereus* var. *mycoides* and of the crystal-formers were strong lecithinase producers, the zones of turbidity spreading well beyond the margins of colonies on egg-yolk and lecithin agars. *B. cereus* 634 and 826, *B. cereus* var. *mycoides* A.C., the two varieties of *B. entomocidus* and crystal-former G2 were negative in the lecithinase test. Feeble lecithinase production leading to a weak zone of turbidity below the colony and scarcely extending beyond its margin was detected with *B. cereus* 827 and with crystal-former G1.

Phage sensitivities

None of the strongly lecithinase-positive organisms (*Bacillus cereus*, *B. cereus* var. *mycoides* or crystal-formers) was susceptible to any of the four phages. The sensitivity patterns of the other strains are summarized in Table 1, which also shows the results of tests for the production of lecithinase and acetylmethylcarbinol and certain sugar fermentations, since these characters are used by Heimpel & Angus (1958) in the classification of the group. Table 2 shows the plating efficiencies of the phages when tested against the different strains as compared with a standard figure of 100 plaque-forming units for the reaction between each phage and the organism on which it was grown.

Table 1. *Phage sensitivities and other characteristics of* Bacillus *strains*

Organism	Sensitivity to phage				Protein crystal formation	Lecithinase production	Acetylmethyl-carbinol production	Production of acid from				
	A	B	C	D				Xylose	Arabinose	Glucose	Trehalose	Laevulose
B. entomocidus var. *entomocidus*	+	+	+	+	+	−	−	−	−	+	+	+
B. entomocidus var. *subtoxicus*	+	+	+	+	+	−	−	−	−	w	w	−
G 1	−	−	+	−	+	w	+	−	−	+	+	w
G 2	+	−	−	−	+	−	+	−	−	+	+	+
B. cereus 634	+	+	+	+	−	−	−	−	−	w	w	−
B. cereus 826	+	+	+	+	−	−	+	−	−	+	+	+
B. cereus 827	−	−	−	−	−	w	+	−	−	+	+	+
B. cereus var. *mycoides* A.C.	−	+	+	+	−	−	+	−	−	+	+	+

w = weak reaction.

Table 2. *Plating efficiencies of phages A, B, C and D on the susceptible* Bacillus *strains*

Test organism	Phage			
	A	B	C	D
	Plating efficiency			
B. entomocidus var. *entomocidus*	100	50	150	200
B. entomocidus var. *subtoxicus*	500	100	180	200
G 1	0	0	100	0
G 2	5	0	0	0
B. cereus 634	100	5	1	1
B. cereus 826	100	5	100	100
B. cereus var. *mycoides* A.C.	0	0·5	0·1	0·3

Plaque morphologies

Plaque morphology showed considerable variation which was clearly dependent on both the phage and the test organism. Plaques ranged from completely clear areas about 1 mm. in diameter (e.g. phage A acting on *Bacillus entomocidus* var. *entomocidus*; phage B acting on the variety *subtoxicus*) to faintly discernible plaques in which there was no complete clearing of the lawn (e.g. most of the plaques formed on *B. cereus* 634 and on *B. cereus* var. *mycoides* A.C.). In general, phage D tended to produce smaller and less well-defined plaques than the others; phage A behaved characteristically in producing turbid plaques on lawns of *B. entomocidus* var. *subtoxicus*. The plaque morphologies were not sufficiently characteristic to be of taxonomic value.

Phage resistance

Colonies of phage-resistant organisms frequently developed in areas of confluent lysis resulting from the action of high phage concentrations. Some of these colonies

were picked and tested for sensitivity to the different phages and for ability to produce lecithinase, acetylmethylcarbinol and protein crystals. In some cases organisms resistant to one phage were treated with a second phage and doubly resistant variants isolated.

Resistance to phage B was frequently accompanied by the development of resistance to phages C and D but was not associated with resistance to phage A except in the case of *Bacillus entomocidus* var. *subtoxicus*. Selection for resistance to phage A did not normally yield strains resistant to the other phages, although results were not always clear cut and increased resistance was occasionally seen with several strains. It was possible to obtain organisms showing a wide variety of phage sensitivity patterns and by selection in several steps to derive strains completely resistant to the four phages from originally susceptible strains.

In all cases changed susceptibility to the phages had no effect on the abilities of the organisms to produce lecithinase or acetylmethylcarbinol. Neither were there any instances of gain or loss of ability to form crystals, but strains of *B. entomocidus* var. *subtoxicus*, which normally produce rather slender delicate bi-pyramidal crystals, produced larger fatter bi-pyramids when they developed resistance to phage B.

DISCUSSION

Apart from the detailed study of phages derived from *Bacillus cereus* strain W reported by McCloy (1951, 1958) and a brief note by Földes, Meretey & Varga (1961) our knowledge of phages in the *B. cereus* group is scant. Only one short communication concerns phages of the crystal-formers. Gochnauer (1960) isolated phages from lysogenic strains which were active against *B. entomocidus* var. *entomocidus* and against the 'terminalis' strain of *B. cereus* but he made no mention of lecithinase production by his strains. The 'terminalis' strain in my collection is strongly lecithinase positive and resistant to all four of my phages.

Many strains of *Bacillus cereus* carry temperate phage and mutation of such phage to a form capable of attacking the lysogenic host appears to be common so that spontaneous phage lysis is frequently seen in bacteria of this group. The most thorough investigation of this type of behaviour is that of McCloy (1951, 1958) who showed that a strain of *B. cereus* (strain W) regularly produced a phage (Wα) which formed plaques on strain W. Strain W was lysogenic with a temperate phage, Wβ, of which Wα was a virulent mutant. A further non-lysogenizing phage, Wγ, was also found in cultures of strain W. The three phages were identical in serological and other characters and McCloy (1958) studied the close relationship which exists between them. In the present work cultures of *B. entomocidus* var. *entomocidus* and of *B. cereus* 826 behaved like McCloy's strain W in that they regularly produced phages (A and D, respectively) which were able to produce plaques on the host strain. Spontaneous plaques appeared even when the cultures were derived from single spores and these bacteria presumably carry temperate phages and are susceptible to virulent variants of them.

Studies of *Bacillus cereus* phages show patterns of group specificity at subspecies level. Földes *et al.* (1961) isolated phages active against the streptomycin-resistant *B. cereus* strain 569 from soil and found them to be active only against certain *B. cereus* strains. The phages did not attack *B. anthracis* or strains of *B. cereus* which

produced the 'anthrax wall polysaccharide'. Autolysates of phage-resistant strains gave a precipitation reaction with anti-anthrax polysaccharide sera while autolysates of sensitive strains did not give this reaction. The *B. cereus* cell wall polysaccharide which precipitates anthrax antibody consists of galactose and glucosamine; the corresponding polysaccharide of phage-sensitive strains contains glucose, glucosamine and an unknown hexosamine. The phages described by McCloy (1951) attacked certain *B. cereus* strains and the anthrax bacillus. The group of phage susceptible organisms described in the present communication consists of strains with little or no ability to produce lecithinase and includes both crystal-forming and non-crystal-forming bacteria.

Heimpel & Angus (1958) suggested a scheme of classification of crystal-formers, recognizing parasporal body formation as an essential criterion for separating them from *Bacillus cereus* and *B. anthracis* and then further subdividing them into *B. thuringiensis* and *B. entomocidus* on a basis of acetylmethylcarbinol and lecithinase production. *B. finitimus* constitutes a third species in which the parasporal body does not separate from the spore at maturity. It produces both lecithinase and acetylmethylcarbinol and was not susceptible to any of my phages. The Heimpel & Angus scheme of classification is of considerable value since it brings order into the group but it does not accommodate lecithinase-negative strains of *B. cereus* (which vary in their abilities to produce acetylmethylcarbinol) or lecithinase-negative acetylmethylcarbinol-producing organisms such as *B. cereus* var. *mycoides* A.C. and crystal-former G2. It is generally accepted that the crystal-formers are very similar to *B. cereus*. I have observed a common spore precipitinogen present in all the crystal-formers and *B. cereus* strains in my collection. The close relationship between crystal-formers and non-crystal-formers is further supported by the present findings in which phage sensitivity cuts across several of the characters used for subdivision in these organisms.

There seem to be two types of '*Bacillus cereus*': one group of strains which produces large amounts of lecithinase and another group which does not do so. Both groups contain strains with the typical rhizoid colonial morphology of the mycoides variety and both groups contain crystal-forming strains, many of which are pathogenic for lepidopterous larvae. The characters discussed above could be used as the basis for a scheme of classification of these organisms. But it is clear that our knowledge of the group is still fragmentary; the present observations may perhaps serve better as a stimulus to further work than as the basis for a taxonomic concept of doubtful validity.

Much remains to be learned about the ecology of crystal-formers. Toumanoff (1960) showed that the disease 'flacherie' caused by a crystal-former in the silkmoth *Bombyx mori* can be transmitted from one generation of larvae to the next. My own closed colony of *Galleria mellonella* is normally free from visible disease but crystal-forming bacteria (strains G1, G2) have been isolated from diseased larvae on occasions separated by a year during which there were no signs of disease. Further isolations of organism G2 have been made from excreta of the colony but infected larvae have remained rare. Such observations emphasize the complexity of the host/parasite relationship. The finding that the two organisms G1 and G2 have a different pattern of phage susceptibility suggests that phage typing might play an important part in its elucidation.

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