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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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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 preferences 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.

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The Action of Lysozyme on Bacterial Electron Transport Systems

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(*Received* 12 *October* 1965)

SUMMARY

Oxidation of several substrates by whole or broken organisms of *Micrococcus lysodeikticus* was arrested following treatment with lysozyme, unless an osmotic stabilizer (sorbitol) were present. Whole organisms, which did not oxidize $NADH_2$, and broken organisms (which oxidized $NADH_2$ only slowly) oxidized $NADH_2$ readily after lysozyme treatment, though the activity was maintained only in presence of an osmotic stabilizer. Lysozyme treatment similarly allowed whole or broken *M. lysodeikticus* to oxidize (mammalian) cytochrome *c*, or to reduce this cytochrome with appropriate electron-donors, e.g. lactate, succinate, or $NADH_2$. Most of these observations held good also for the lysozyme-sensitive *Bacillus megaterium*, but not for several lysozyme-insensitive organisms (both Gram-positive and Gram-negative).

INTRODUCTION

Protoplasts formed by lysozyme treatment of sensitive bacteria in presence of an osmotic stabilizer may retain the respiratory activity of the whole organisms; but lysis of the protoplasts by osmotic shock or lysozyme treatment of bacteria in absence of a stabilizer may severely decrease the ability of the bacteria to oxidize common substrates. This has been shown for *Bacillus subtilis* (Wiame, Storck, & Vanderwinkel, 1955); *B. megaterium* (Weibull, 1953) and *Micrococcus lysodeikticus* (Pandya, 1963). The present paper describes an examination of the nature of the damage suffered by the respiratory enzyme systems.

METHODS

Organisms. The following bacteria were obtained from the National Collection of Industrial Bacteria: *Micrococcus lysodeikticus*, NCTC 2665 and *Bacillus megaterium*, NCIB 8291. The remainder were stock laboratory strains designated by our own numbers; *Escherichia coli*, E 106; *B. subtilis*, E 4; *Sarcina lutea*, E 7; *Proteus vulgaris*, L 10; *Pseudomonas pyocyanea* (*aeruginosa*), L 15. Organisms were maintained on nutrient agar slopes, grown in nutrient broth (Oxoid No. 2: Lab-Lemco beef extract, 1%; peptone, 1%; NaCl, 0·5%; in tap water) at 32–34° on a rotary shaker and at the end of the logarithmic phase (48 hr for *M. lysodeikticus*; overnight for other organisms) were harvested by centrifugation, washed twice on the centrifuge with distilled water; then suspended in water or buffer and stored at 4° till required. Suspensions of organisms were normally used the same day though the lysozyme

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sensitivity of *M. lysodeikticus* did not alter appreciably on storage for several days at 0°.

Turbidimetry. Concentrations of bacterial suspensions were estimated (without filter) in the EEL portable colorimeter (Evans Electroselenium Ltd., Halstead, Essex) and converted to equivalent mg. dry wt. organism/ml. by using factors based on dry weight (air-drying at 80°). The EEL colorimeter was also used for following changes in extinction during treatment with lysozyme and the results are recorded in terms of the scale readings of this instrument. (A reading of 3 corresponds to about 50% transmittance in a 1 cm. cuvette.)

Sonic disintegration. Bacteria were disintegrated in small batches (5 ml.) in a 60-W. 20-Kcyc./sec. M.S.E. sonic disintegrator with the large (18 mm. diam.) probe; temperatures were maintained below +5° by means of an ice-bath.

Materials. Crystalline lysozyme chloride, from egg-white (Koch–Light Laboratories Ltd., Colnbrook, Bucks.); reduced nicotinamide adenine dinucleotide ($NADH_2$); and mammalian cytochrome *c* as 1% solution, about 70% oxidized, 30% reduced (Boehringer Corporation (London) Ltd.). Other reagents were commercial products. Ubiquinone-O was a gift from Messrs Hoffmann-La Roche, Basel, Switzerland, through the courtesy of Dr O. Isler.

Buffer. Unless otherwise stated enzyme experiments were done in a mixture of Na_2HPO_4 and KH_2PO_4, the total phosphate concentration 0·067 M, at pH 7·4.

Succinoxidase was measured by recording the oxygen uptake in Warburg manometers at 37°, in presence of 0·05 M-succinate, with KOH in the centre wells. In most experiments succinate was included in the main flask and manometric measurements began after 10 min. equilibration; lysozyme or other additions were added from the side-bulb.

$NADH_2$ oxidase was measured by the decrease in extinction at 340 mμ at room temperature (19–20°). Reference and experimental cuvettes contained (in 3 ml. buffer): organism (equiv. 1 mg. dry wt.) and lysozyme (at stated concentration). After the interval of time required for lysozyme action $NADH_2$ ($7·5 \times 10^{-5}$ M) was added to the experimental cuvette, a corresponding volume of buffer being added to the reference cuvette. The cuvettes were placed in a recording spectrophotometer (Unicam Ltd., SP-700) set at constant wavelength (340 mμ). The $NADH_2$ oxidase activity was calculated from the steady initial decrease in extinction which was recorded over a period of 1–5 min. The molar extinction coefficient was taken as 6220 (Horecker & Kornberg, 1945). Any decrease in extinction of the cell material itself resulting from the action of lysozyme was compensated for by the presence of organisms and lysozyme in the reference cuvette. It was, nevertheless, possible that spurious 'oxidation' of $NADH_2$ might be recorded if the latter itself were to affect the rate of action of lysozyme on the bacteria. This, however, is not the case, since when the system was set up for measurement of $NADH_2$ oxidase but the spectrophotometer set for 450 mμ (at which wavelength there was no change in extinction arising from oxidation of $NADH_2$) the instrument recorded a zero trace. Lysozyme alone did not cause any oxidation of reduced $NADH_2$.

Cytochrome oxidase activity was measured by observing the decrease in extinction at 550 mμ in presence of cytochrome *c* and bacterial preparations; the details are given in the legends to the figures. No changes in extinction were observed when the

spectrophotometer was set at 500 mμ, an isosbestic point for oxidized and reduced cytochrome.

Electron transfer from lactate, succinate or $NADH_2$ to cytochrome *c* in *Micrococcus lysodeikticus* was followed by the increase in extinction at 550 mμ in presence of 0·007 M-cyanide which completely inhibited aerobic oxidation of these electron-donors in this organism; details are given in the legend to Fig. 7. The decrease in molar extinction coefficient at 550 mμ on oxidation of cytochrome *c* was taken as 19,700 (Hogeboom & Schneider, 1952). Electron transfer from $NADH_2$ to ubiquinone-O and menaquinone-O (vitamin K) was examined by setting up reference and experimental cuvettes containing (in 3 ml. buffer); organism, equivilent 5·4 mg. dry wt./ml.; KCN, 0·007 M; ubiquinone-O or menaquinone-O (6×10^{-5} M). $NADH_2$ was then added to the experimental cuvette only, and the rate of decrease in extinction at 340 mμ determined. To investigate the effect of lysozyme, 30 μg./ml. were added to experimental and reference cuvettes at the desired time before the addition of $NADH_2$.

Enzyme activities are expressed in international units/g. dry wt. of organism; 1 I.U. is the amount of enzyme which will transform 1 μmole of substrate/minute. For this purpose, the electron donor was regarded as the 'substrate' in all oxidation-reduction systems.

RESULTS

Lysis of Micrococcus lysodeikticus by lysozyme

Figures 1*a*, *b* show the percentage decrease of the extinction of a suspension of organisms following addition of lysozyme at 20° and 37°. At 37° lysis was about 60–70% complete within 5 min., and the extinction reaches its terminal value (10–15% of the initial value) after about 20 min. The decrease in extinction in

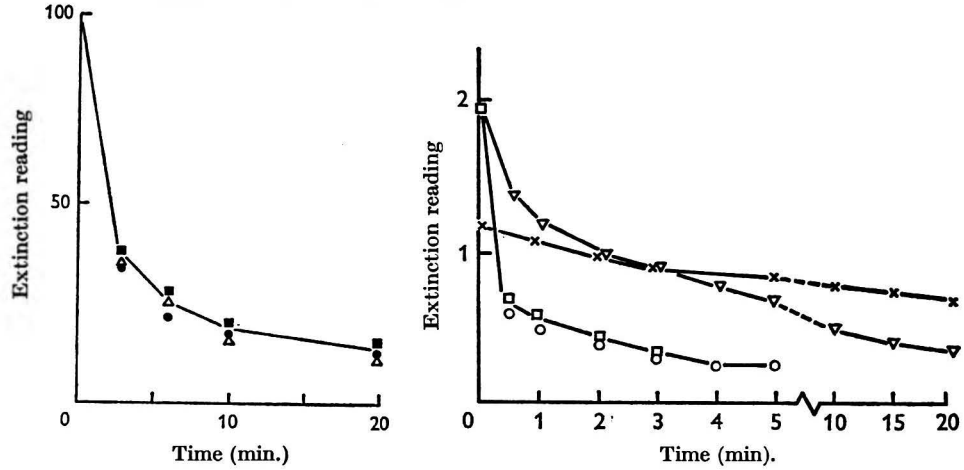

Fig. 1. Decrease in extinction reading of suspensions of *Micrococcus lysodeikticus* on treatment with lysozyme (*a*) 37°, lysozyme 1·6 μg./ml.; ■ in buffer, ● in buffer + 0·05 M-succinate. △ in buffer + 0·05 M-succinate, shaken in Warburg manometer. (*b*) at 20°; ▽, lysozyme, 0·33 μg./ml.; × lysozyme 0·33 μg./ml. with 1·5 M-sorbitol; □, ○, lysozyme 20 μg./ml., two separate experiments on different days.

presence of sorbitol was substantially less, and represents the difference between the intact organisms with their highly-refractile cell walls and the poorly refractile protoplasts. The course of the reaction was essentially as described by Weibull (1953) for *Bacillus megaterium*. We chose sorbitol as an osmotic stabilizer because, unlike sucrose, it is not metabolized at a significant rate by the organisms studied. Polyethyleneglycol did not protect *M. lysodeikticus* against lysis save at concentrations which gave excessively viscous solutions; NaCl was avoided because at concentrations adequate for osmotic stabilization it is itself an inhibitor of lysozyme action (Weibull, 1956).

Before decrease in extinction could be accepted as an index of lysis (in absence of sorbitol) it was necessary to decide at what stage actual lysis of the organisms occurred. Are some organisms rapidly lysed and others slowly, or does the progressive decrease in extinction represent a gradual removal of cell-wall material from all the organisms simultaneously? This was examined by treating organisms with lysozyme for different periods and then arresting both lysis and the action of the enzyme by adding M-NaCl. Surviving organisms and debris arising from lysis were centrifuged down (100,000 *g* for 30 min.) and nucleic acid in the clear supernatant fluid determined by extinction measurements at 260 mμ. Release of nucleic acid into the supernatant fluid ran closely parallel with the decrease in extinction of the suspension. Since little, if any, nucleic acid is present in the cell wall (Salton, 1953), we felt justified in using the decrease in extinction as a criterion of lysis in suspensions which did not contain an osmotic stabilizer.

Action of lysozyme on succinate and fumarate oxidations

Figures 2*a*, *b* show the effect of lysozyme on oxidation of succinate and fumarate. Comparison with Fig. 1 shows that respiration did not stop immediately on lysis; 5 min. after adding lysozyme (when lysis was about 70% complete) the respiration had not begun to decrease. Appreciable decrease in oxygen uptake was seen only after 10 min., when the extinction had already almost reached its final value. The situation was essentially similar with the oxidation of lactate. The possibility was considered that lysis might occur more slowly in the manometers than in the stationary tubes used for the extinction measurements; but when lysis was followed in shaken manometers in presence of succinate, the time-course was the same as in stationary tubes (Fig. 1*a*).

Sorbitol slightly retarded oxygen uptake, possibly because of a decreased diffusion of oxygen through the solution, but it protected the oxidation system against the action of lysozyme. When a system containing both lysozyme and sorbitol was diluted with buffer, thus causing lysis of the protoplasts, immediate arrest of succinate oxidation occurred (Fig. 2*a*).

Loss of enzyme activity was not due solely to disruption of the organisms. Sonic treatment for 5 min. disrupted about 80% of the organisms and increased succinate oxidase activity by some 20%; but on treatment with lysozyme, there was a decrease in activity very like that obtained with whole bacteria. The succinate oxidase of the disintegrated preparation was likewise protected by sorbitol (Fig. 3). We considered the possibility that our lysozyme preparation was acting as an unspecific enzyme inactivator, but a commercial $NADH_2$-cytochrome *c* reductase system, of mammalian origin, suffered no loss of activity on treatment with

lysozyme at 17 μg./ml. Lysozyme has been reported to have no lipase, protease or amyolytic activity (Meyer, Palmer, Thompson & Khorazo, 1936).

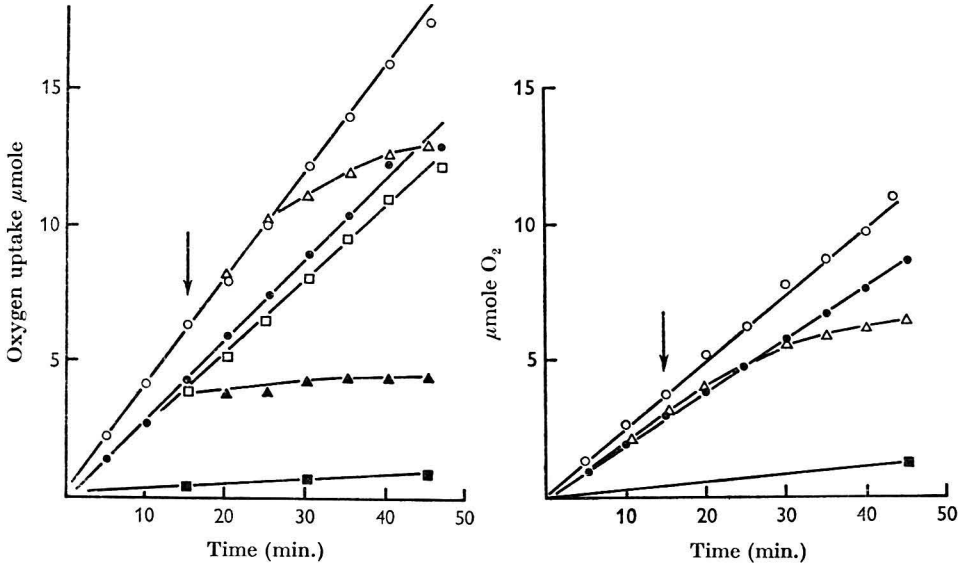

Fig. 2. Action of lysozyme on (*a*) succinate and (*b*) fumarate oxidation by suspension of *Micrococcus lysodeikticus*. Each manometer contained: equiv. 4 mg. dry wt. organisms suspended in 3 ml. buffer (NaOH in centre cup), with the following additions; ■, none (no-substrate control); ○, substrate (succinate or fumarate) 0·05 M; △, substrate with addition of lysozyme 1·66 μg./ml. at 15 min.; □, substrate + 1·5 M-sorbitol; ●, substrate + sorbitol, addition of lysozyme 1·66 μg./ml. at 15 min; ▲ substrate + 1·5 M-sorbitol + lysozyme in 3 ml.; 1 ml. buffer added at 15 min. to induce osmotic lysis.

Activation of $NADH_2$ oxidase in Micrococcus lysodeikticus

Whole *Micrococcus lysodeikticus* organisms, like those of many other bacteria, are unable to oxidize $NADH_2$ because of failure of the latter to obtain access to the enzyme sites within the organism. On sonic disintegration some degree of $NADH_2$ oxidase activity was developed (of the order of 10 I.U./g. dry wt.), unless treatment were so severe as to inactivate the system. On treating a cell suspension of *M. lysodeikticus* with lysozyme (Fig. 4*a*) the $NADH_2$ oxidase activity developed rapidly, roughly parallel with lysis, and then decreased again as (presumably) the system was inactivated, either directly through the action of the lysozyme or as a result of the physical damage of lysis. On the other hand, in the presence of sorbitol a stable activity at about 100 I.U./g. was obtained. Similar results were obtained with broken organisms (Fig. 4*b*).

Action of lysozyme on components of the electron transport chain

Neither whole nor broken *Micrococcus lysodeikticus* oxidized mammalian cytochrome *c*. On adding lysozyme, in both cases slight oxidase activity was observed but ceased within a few minutes. However, on adding lysozyme to a preparation of micrococci suspended in 1·5 M-sorbitol in presence of cytochrome *c*, oxidation of the latter occurred at a rate of about 60 I.U./g. dry wt. (Fig. 5). With broken

organisms lysozyme 0·33 μg./ml. (which causes relatively slow lysis) produced a gradual development of activity. When sufficient lysozyme (20 μg./ml.) to produce rapid lysis was used, a high initial value of activity was obtained, followed by rapid inactivation of the system.

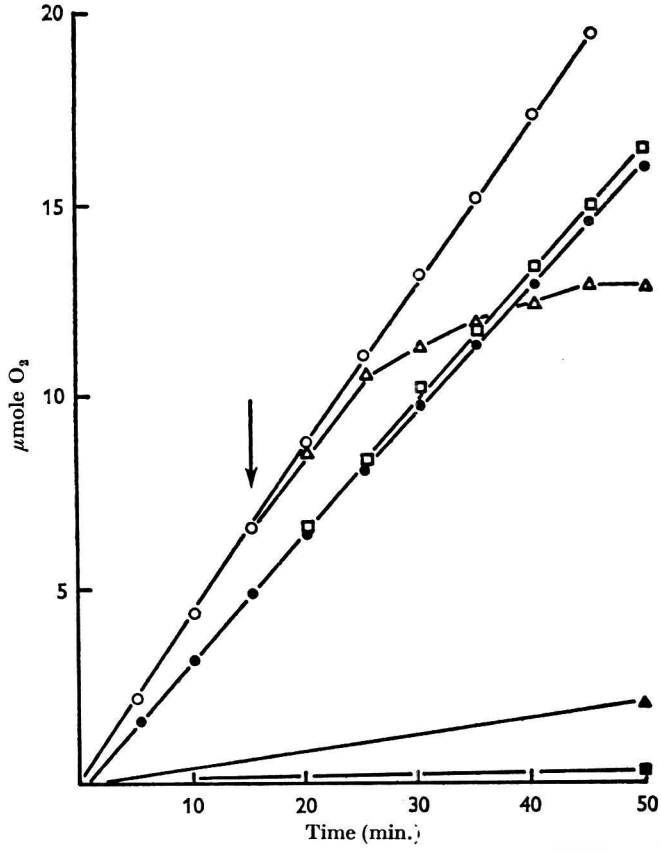

Fig. 3. Action of lysozyme on succinate oxidation by sonically broken *Micrococcus lysodeikticus*. Each manometer contained broken cocci equivalent to 4 mg. dry wt. in 3 ml. buffer (NaOH in centre cup) with the following additions; ■, none (no-substrate control); ▲, 1·5 M-sorbitol; ○, 0·05 M-succinate; △, 0·05 M-succinate with addition of lysozyme 1·66 μg./ml. at 15 min.; □, 0·05 M-succinate + 1·5 M-sorbitol; ●, 0·05 M-succinate – 1·5 M-sorbitol, with addition of lysozyme 1·66 μg./ml. at 15 min.

Added cytochrome *c* was not reduced by succinate, lactate or $NADH_2$ in presence of whole or broken *Micrococcus lysodeikticus*. On adding lysozyme to whole organisms reduction of the cytochrome occurred with all three substrates (Fig. 6). In experiments in which micrococci (whole or broken) were treated with lysozyme (20 μg./ml.) and the cytochrome c reductase activity measured after various times relatively rapid destruction of the reductase system was observed.

Whole or broken *Micrococcus lysodeikticus* showed only low activity for oxidation of $NADH_2$ by ubiquinone-O (2,3-dimethoxy-5-methyl-1, 4-benzoquinone) or by menaquinone-O (2-methyl-1,4-naphthaquinone: vitamin K) in a system in which aerobic oxidation was blocked by 0·007 M-cyanide; but on treating with lysozyme,

20 μg./ml., rapid oxidation took place. Though inactivation ultimately occurred, the system was considerably more stable in the presence of lysozyme than was the $NADH_2$-cytochrome *c* reductase system.

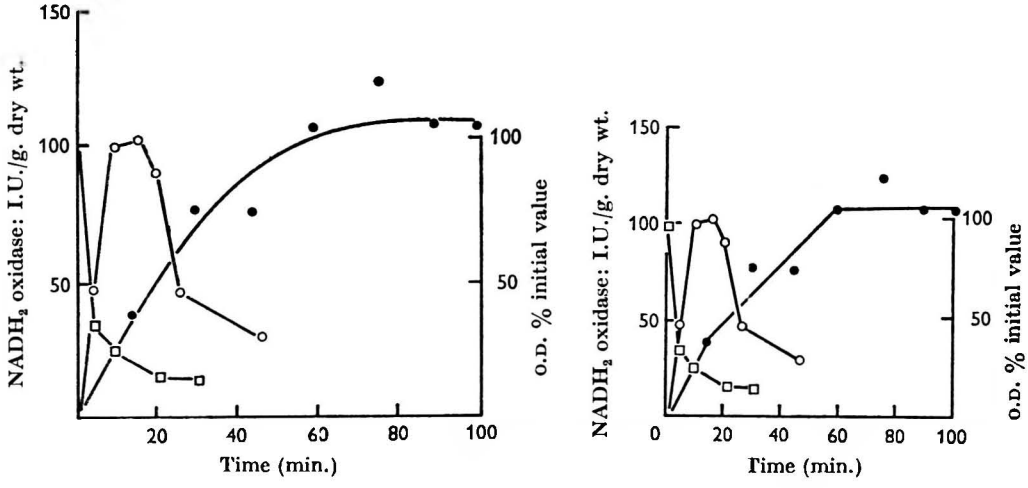

Fig. 4. Development of $NADH_2$ oxidase in suspensions of (*a*) whole organisms, (*b*) broken organisms of *Micrococcus lysodeikticus* on treatment with lysozyme 0·33 μg./ml. ○, without sorbitol; ●, in 1·5 M-sorbitol. □, extinction reading of suspension (without sorbitol) expressed as % of initial value.

Bacillus megaterium: action of lysozyme on respiratory system

Bacillus megaterium is lysozyme-sensitive but only at concentrations of lysozyme 50–100 times higher than required for *Micrococcus lysodeikticus*. The effect on succinate oxidation was very similar in the two organisms (Fig. 7) although the high endogenous respiration in *B. megaterium* made interpretation of the results more difficult. Lysozyme treatment much increased the low $NADH_2$ oxidase activity of intact *B. megaterium*, roughly *pari passu* with lysis. In absence of an osmotic stabilizer prolonged treatment with lysozyme inactivated the oxidase system of *B. megaterium* as in *M. lysodeikticus*; when lysis was prevented by sorbitol the system was more stable though the maximum activity attained is lower (Fig. 8). The $NADH_2$ oxidase activity in sonically-broken material was about double that of whole *B. megaterium*, but was not appreciably increased by lysozyme. This was quite different from the results obtained with *M. lysodeikticus* (Fig. 4*b*), but the difference may be more apparent than real since concentrations of lysozyme in excess of those required to expose the enzyme systems may, in some cases, cause rapid inactivation. It was possible that in *B. megaterium* destruction of the enzyme occurred as rapidly as its release (see Fig. 8).

Neither whole nor broken *Bacillus megaterium* preparations oxidized (mammalian) cytochrome *c*; addition of lysozyme had little effect, any activity liberated being, presumably, rapidly lost. In presence of sorbitol, however, after some delay, a stable cytochrome oxidase activity was developed (Fig. 9). The delay may perhaps represent the time required to dissolve away cell wall down to the layers at which

the external cytochrome *c* can make contact with the enzyme systems. When these results are compared with the corresponding data for *Micrococcus lysodeikticus*, there are indications of a similar though less clear-cut lag when the lysozyme concentration used was sufficient to produce only a moderate rate of lysis (Fig. 5, curves B and E).

The activation of the cytochrome c reductase system of *Bacillus megaterium* by lysozyme is shown in Fig. 10. At a relatively high lysozyme concentration (50 μg./ml.) broken bacilli developed reductase activity to a high value with very little lag; succinate, lactate or $NADH_2$ were equally effective as electron donors. The system was stable even in absence of sorbitol. With less lysozyme (20 μg./ml.) the activity was much lower and there were marked differences between the electron donors; curiously, $NADH_2$ gave a higher activity than lactate or succinate. Whole bacilli developed cytochrome *c* reductase activity only relatively slowly on treatment with lysozyme, and to a much lower degree than was attained with lysozyme-treated broken cell preparations.

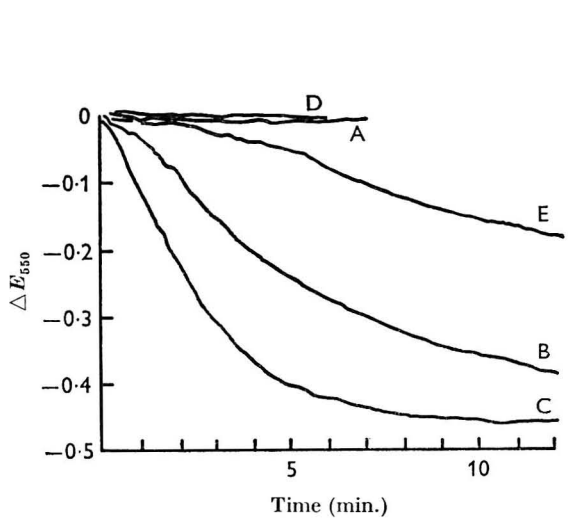

Fig. 5

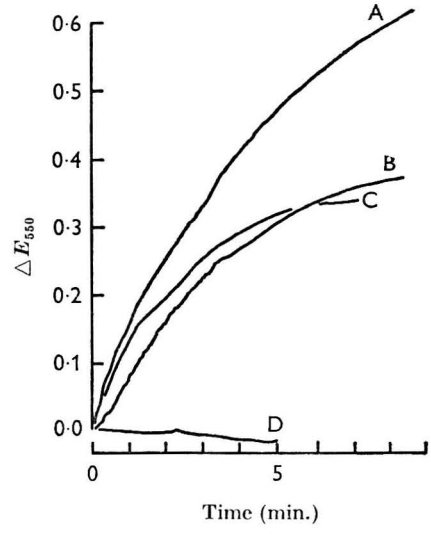

Fig. 6

Fig. 5. Release of cytochrome oxidase activity in *Micrococcus lysodeikticus* by lysozyme. Reference and experimental cuvettes contained equivalent of 0·27 mg. dry wt. of organisms/ml. in 1·5 M-sorbitol; 2 mg. cytochrome *c* in experimental cuvette only. Lysozyme added to both cuvettes at 0 min. Oxidation of cytochrome followed by decrease in extinction at 550 mμ. Experiments with whole organisms in presence of 1·5 M-sorbitol; A, control, no lysozyme; B, lysozyme 0·33 μg./ml.; C, lysozyme 20 μg./ml. Experiments with broken bacteria in presence of 1·5 M-sorbitol; D, control, no lysozyme; E, lysozyme 0·33 μg./ml. No change in extinction occurs when cytochrome is treated with lysozyme in absence of the organism.

Fig. 6. Development of electron transfer for lactate, succinate or $NADH_2$ to cytochrome *c* on treatment of whole *Micrococcus lysodeikticus* organisms with lysozyme 20 μg./ml. Both cuvettes contained (in 3 ml. buffer); organisms equivalent to 0·8 mg. dry wt.: cytochrome *c*, 2 mg.; KCN, 0·007 M. Experimental cuvettes contained also; A, lactate, 150 μmole; B, succinate, 150 μmole; C and D, $NADH_2$, 0·255 μmole. Lysozyme 20 μg./ml. added to both cuvettes at $t = 0$ in A, B, and C. D served as control showing that no reduction of cytochrome took place in absence of lysozyme.

Bacillus megaterium can transfer electrons from $NADH_2$ to ubiquinone-O in a system in which aerobic oxidation is blocked by 0·007 M-cyanide, with an activity of the order of 35 I.U./g. dry wt. On sonic treatment this activity increased to 90 I.U./g., and was not further increased by lysozyme treatment. When, however, whole bacilli were treated with lysozyme (30 μg./ml.) the activity increased, roughly parallel with lysis, and to a value of 310 I.U./g. Continued contact with lysozyme up to 1 hr caused only a slight loss in activity. Very similar results were obtained with menaquinone-O as electron acceptor.

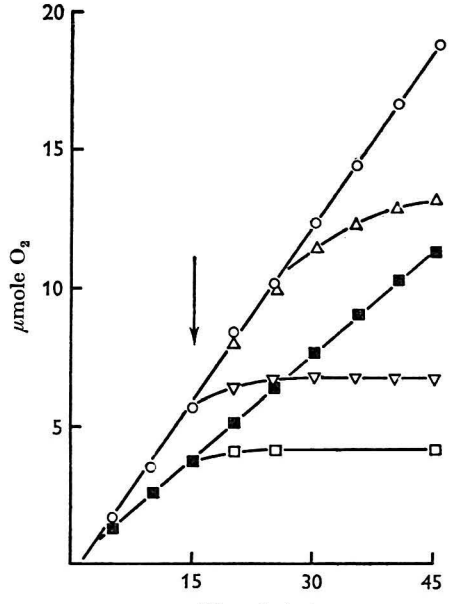

Fig. 7

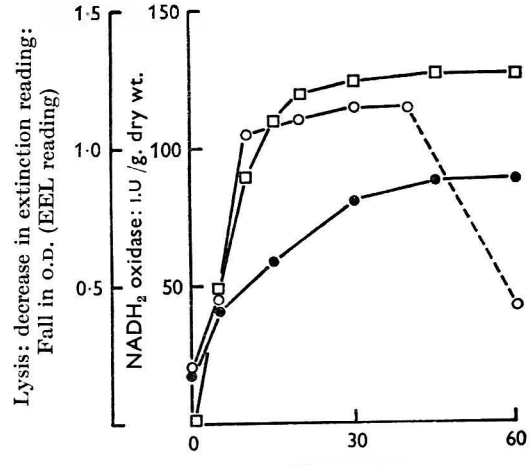

Fig. 8

Fig. 7. Effect of lysozyme on succinate oxidation and endogenous respiration in *Bacillus megaterium*. Manometers contained the equivalent of 6 mg. dry wt. organisms in 3 ml. buffer. ■, no additions (endogenous respiration; □, lysozyme 33 μg./ml. added at 15 min; ○, 0·05 M-succinate; △, lysozyme, 33 μg./ml. or ▽ lysozyme 50 μg./ml. added at 15 min.

Fig. 8. Development of $NADH_2$ oxidase activity in suspensions of *Bacillus megaterium* following treatment with lysozyme 10 μg./ml. ○ without sorbitol; ● in 1·5 M-sorbitol. □ fall in extinction of suspension (without sorbitol) as index of lysis.

Effect of lysozyme on respiratory system in organisms which are not lysed

The following organisms were resistant to lysozyme in so far as no decrease in the turbidity of suspensions occurred when treated at the lysozyme concentrations stated: *Bacillus subtilis*, 1·5 mg./ml.; *B. mycoides* and *Proteus vulgaris*, 0·5 mg./ml.; *Sarcina lutea* and *Escherichia coli*, 1·0 mg./ml.; *Pseudomonas pyocyanea* (*aeruginosa*), 0·25 mg./ml. The lysozyme concentrations used were thus up to 1000 times greater than that which lysed *Micrococcus lysodeikticus* readily. Lysozyme was also allowed to act on sonically-treated suspensions of these organisms; again, no decrease in turbidity was observed, except for a 25% decrease with *P. pyocyanea*.

Succinate oxidation by whole organisms was not affected by lysozyme treatment

in *Bacillus subtilis* (Fig. 11 *a*), *Escherichia coli* (Fig. 11 *b*), *B. mycoides* or *Pseudomonas pyocyanea*. The strain of *Sarcina lutea* used was apparently not able to oxidize succinate. *Proteus vulgaris* showed an appreciable increase in succinate oxidation on addition of lysozyme (Fig. 12). No action of lysozyme was observed on broken organisms, so it is presumed that some surface layer was being removed. This need not necessarily have been mucopeptide, since the β-glucosaminidase activity of lysozyme extends, for example, to chitin (Berger & Weiser, 1957) and also possibly to some constitutent of the mucoids of hen-egg white (Meyer *et al.* 1936).

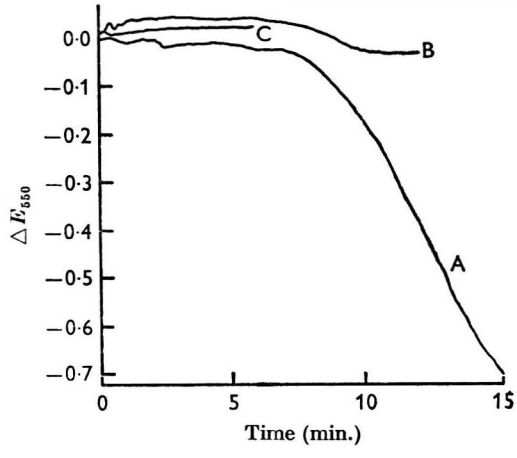

Fig. 9

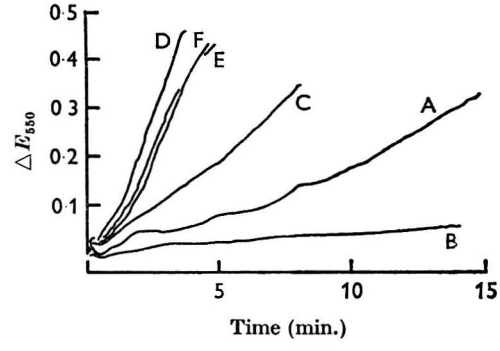

Fig. 10

Fig. 9. Cytochrome oxidase activity in lysozyme-treated *Bacillus megaterium*. Reference and experimental cuvettes contained (in 3 ml. buffer); equivalent of 1·8 mg. dry wt. organisms; also (Expt. A only) 1·5 M-sorbitol. Experimental cuvettes contained 2 mg. (mammalian) cytochrome *c*. Lysozyme 30 μg./ml. added at $t = 0$ and decrease in E_{550} recorded. A, sorbitol present; B, no sorbitol; C buffer added in place of lysozyme.

Fig. 10. Reduction of (mammalian) cytochrome *c* by lysozyme-treated sonically treated *Bacillus megaterium*. Reference and experimental cuvettes contained equivalent of 1·8 mg. dry wt. organisms, sonically treated for 2 min.; cytochrome c, 2 mg.; KCN, 0·007 M; buffer to 3 ml. Experimental cuvettes contained also electron-donors: 0·05 M-succinate (A, D); 0·05 M-lactate (B, E); $7{\cdot}5 \times 10^{-5}$ M-$NADH_2$ (C, F). No change in E_{550} was observed until addition ($t = 0$) of lysozyme (20 μg./ml., A, B, C; 50 μg./ml., D, E, F).

The lysozyme-susceptible mucopeptide is believed to be present in many Gram-negative bacteria but at some distance below the surface of the organism. It was therefore thought possible that lysozyme might have an effect on broken organisms when it was not active against whole organisms. This was examined in *Escherchia coli* and *Proteus vulgaris*, since with the other organisms sonic treatment caused inactivation of the succinate oxidase system. With *P. vulgaris* lysozyme did not affect the rate of succinate oxidation (Fig. 12); in *E. coli* (Fig. 11 *c*) a slow inactivation was observed. In comparing these observations with the corresponding results for *Micrococcus lysodeikticus* (Fig. 3) it should be remembered that a 300-fold greater lysozyme concentration was used with *E. coli* and *P. vulgaris*.

None of the lysozyme-resistant organisms examined showed any appreciable $NADH_2$ oxidase activity in whole organisms, and exposure to lysozyme (for 60 min. at 37°; 17 μg./ml., *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*; 33 μg./ml., *B. mycoides*; 300 μg./ml. *Sarcina lutea*) did not expose any activity.

Sonic treatment for 5 min. (causing over 90% cell breakage) yielded $NADH_2$ oxidase activities of 54 I.U./g. in *B. subtilis*, 105 in *E. coli*, 50 in *B. mycoides*, 16 in *P. vulgaris* and no activity in *S. lutea*. In the first two of these organisms exposure to lysozyme (conditions as above) caused slight loss in activity; *B. mycoides* and *P. vulgaris* were unaffected. No activity was developed in *S. lutea*. None of the organisms studied showed an increase in $NADH_2$ oxidase activity at any stage of lysozyme treatment.

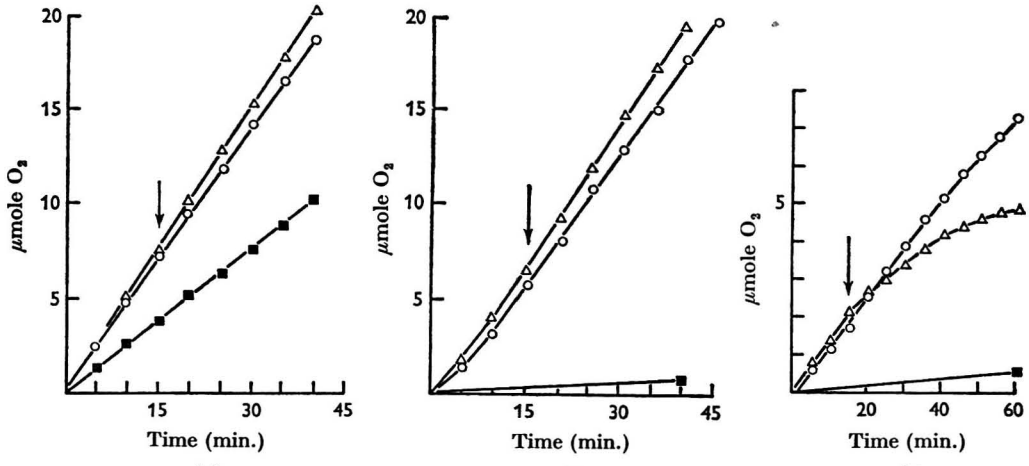

Fig. 11. Effect of lysozyme on succinate oxidation by whole organisms of (*a*) *Bacillus subtilis*, equivalent to 9 mg. dry wt. organism; (*b*) *Escherichia coli*, equivalent to 10 mg. dry wt. organisms; and (*c*) *E. coli* equivalent to 10 mg. dry wt. organism subjected to 5 min. sonic treatment. ■, organisms suspended in (phosphate) buffer (endogenous respiration); ○, 0·05 M-succinate; △, 0·05 M-succinate with lysozyme 0·5 mg./ml. added at 15 min.

DISCUSSION

The present work confirms previous reports that bacterial respiratory systems are largely inactivated on lysis with lysozyme but suffer little or no damage when the cells are protected against lysis by addition of an osmotic stabilizer. It is well known that mechanical breakage of the cell (e.g. by sonic treatment, the Hughes press, or shaking with glass beads) can lead to severe loss of respiratory activity, and it might seem plausible that the action of lysozyme is only another form of mechanical breakage. Two factors suggest that the action of lysozyme is more subtle than this. (1) There is an appreciable time-lag between actual lysis of the organisms, and the loss of succinate oxidation. (2) Succinate oxidation which has survived sonic breakage of the organisms is destroyed by lysozyme. This might suggest that the cell membrane (if we assume the latter to be the site of the oxidizing enzymes) is embedded within the cell wall rather than lying between the cell wall and the cytoplasm, as is generally believed (see Salton, 1964). Alternatively, we might assume that the cell membrane is stuck firmly to the cell wall; when the cell is mechanically disrupted, this attachment will allow a portion of the membrane material, at least, to retain its morphological integrity despite exposure to a hypotonic medium. Lysozyme treatment would then remove this mechanical protection.

The concept of a respiratory system embedded in cell wall (or at least in some lysozyme-sensitive material) is supported by the results of the experiments with $NADH_2$ oxidase. Removal of the cell wall without lysis (by lysozyme treatment in presence of sorbitol) allows access of $NADH_2$ (and also cytochrome *c*) to the respiratory system. The cell wall thus appears as an organ of selective permeability as well as a mechanical structure. Sonic disintegration exposes only a

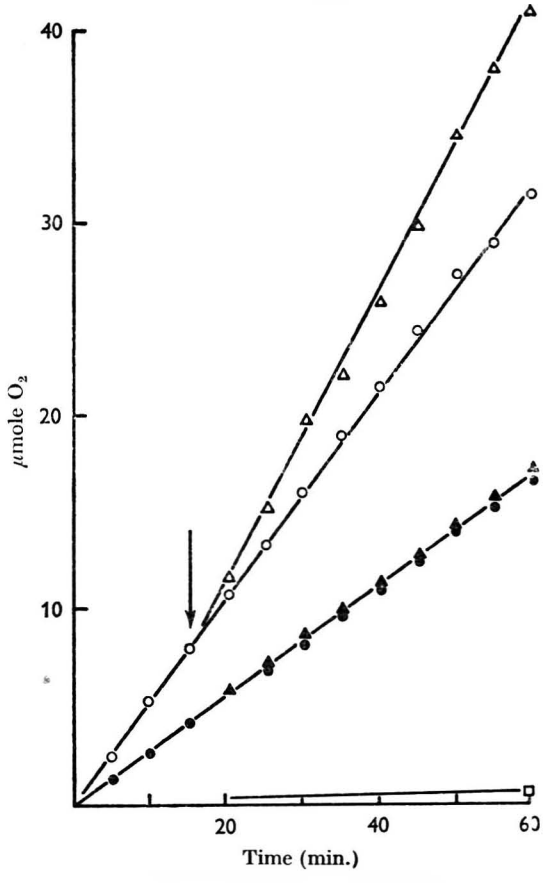

Fig. 12. Action of lysozyme on succinate oxidation in *Proteus vulgaris*. Each manometer contained equivalent of 7·5 mg. dry wt. organism (whole, or sonically treated for 5 min.) in 3 ml. buffer. □, no additions (endogenous respiration); ○, ●, 0·05 M-succinate, with whole or sonically treated organisms respectively; △, ▲; 0·05 M-succinate with addition of lysozyme 0·5 mg /ml. at $t = 15$ min., whole or sonically treated organisms, respectively.

fraction of the total potential $NADH_2$ oxidase; greater activity is attained by lysozyme treatment of the sonically-fragmented material. The oxidase system must be either embedded in 'cell wall', or accessible to $NADH_2$ only from its outer aspect. The report by Cole & Hughes (1965) that ATPase activity in *Lactobacillus arabinosus* was released on prolonged sonic treatment, to a much higher degree than that obtained merely by breaking of the organisms in the Hughes press, presents an analogy with our findings.

Ability to react with cytochrome *c* after lysozyme treatment is also of some interest

in view of the almost uniformly negative results obtained on numerous occasions when bacteria or bacterial extracts have been examined for 'cytochrome oxidase' (see Smith, 1954, 1961). The highest reaction rates obtained in the present studies with *Micrococcus lysodeikticus* were of the order of 30 I.U./g. (based on two-electron transfer; data calculated from Figs. 6 and 7) compared with figures of the order of 150 I.U./g. for $NADH_2$ or succinate oxidation. It is possible, however, that we had not achieved the conditions for maximal cytochrome *c* reaction.

Lysozyme ultimately inactivated the $NADH_2$ oxidase system in disintegrated or lysed organisms though the loss of activity was much less rapid than with succinate oxidase. It is not possible to decide whether the respiratory systems possess lysozyme-sensitive components, or whether the membrane itself is liable to osmotic destruction as distinct from the lysis of the organism. Suspensions of broken organisms can retain their oxidase activities after treatment with lysozyme in presence of osmotic stabilizers; but loss of activity will occur when lysozyme is added at concentrations much in excess of that required for lysis.

Treatment of the lysozyme-insensitive organisms, even with relatively high concentrations of lysozyme, neither inhibited succinate oxidation nor enhanced $NADH_2$ oxidase. In only one case did lysozyme produce (in an organism which it did not lyse) effects comparable with those obtained with *Micrococcus lysodeikticus* or *Bacillus megaterium*; this was the inhibition of succinate oxidation on treatment of broken *Escherichia coli* with lysozyme. No effect was observed with whole *E. coli* organisms, so this is consistent with the idea that Gram-negative bacteria possess a layer of the lysozyme-sensitive mucopeptide located at or near the inner surface of the cell wall (Salton, 1964).

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The Use of Phage to Study Causes of Loss of Viability of *Escherichia coli* in Aerosols

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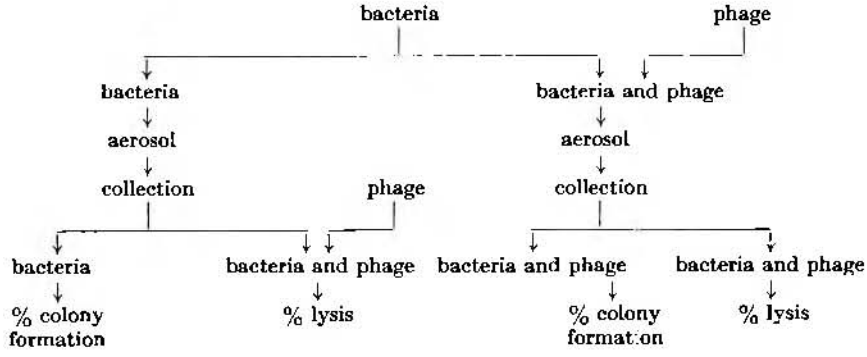
SUMMARY

This paper presents data to show that loss of viability of *Escherichia coli* and phage in an aerosol was the consequence of at least three mechanisms. Phage T7 was used and shown to be unstable when sprayed into air. When previously adsorbed to *E. coli* the aerosol stability of phage T7 appeared to be dependent upon its stage of development in its host. The host *E. coli* strain B in an aerosol was itself subject to two stresses which operated through different mechanisms within the bacterium: (i) a relative humidity stress having its locus of action such that colony formation and the production of phage T7 were both impaired; (ii) an air stress such that colony formation was impaired but that the bacterium was able to produce phage T7.

INTRODUCTION

Investigations (Cox, 1966) have shown that the survival of bacteria in an aerosol depends upon a number of factors. In assessing degree of survival the usual test for deciding whether bacteria are alive or dead depends upon the appearance of visible colonies on an agar plate, but this method does not allow a determination of the cause of death. The use of a suitable phage (Cox & Baldwin, 1964) enables a distinction to be made between death resulting from breakdown of the phage productive capacity of a bacterium (measured as its ability to produce phage) and from loss of the reproductive capacity of the bacterium (reactions other than those involved in phage production). By using the following scheme, deductions can be made as to which capacities have been damaged by the processes involved in aerosol experiments:



For example, two simple cases can be considered.

(i) When the bacterium loses reproductive capacity only, then colony formation will be less than 100 %. However, the ability of these bacteria to produce phage will be unaffected and provided that an aerosol-stable phage is used, subjecting the bacteria to phage after or before 'aerosolization' and collection will result in all of the bacteria undergoing lysis.

(ii) When the bacterium loses phage productive capacity only, then the ability to form colonies and to produce phage will be equally affected. Values will be the same for % colony formation and % lysis for bacteria infected with phage after collection. This will also be so for bacteria infected with phage before aerosol formation, provided that an aerosol-stable phage is used and provided that no phage development is allowed to occur before aerosol formation. This paper presents data obtained by the application of these techniques.

METHODS

Organisms. Cultures of *Escherichia coli* strain B, were grown in a 2% tryptone medium (pH 7·2) for 16 hr at 37° in shaken flasks. Coliphage T7 was prepared in a conventional manner by inoculating a log-phase culture of *E. coli* B with phage T7 and allowing the suspension to clear. Centrifugation removed unlysed bacteria.

Suspensions of bacteria and phage. Two types of suspension were used. Type 1 suspensions were of *Escherichia coli* B infected with phage T7 + *Bacillus subtilis* var. *niger* spores. The spores were added so that allowance could be made for physical loss of aerosol particles (Harper, Hood & Morton, 1958). The suspensions of *E. coli* B were prepared by separating bacteria of the 16 hr culture from the culture medium by centrifugation. These bacteria were re-suspended at 37° in aerated tryptic meat broth, containing 0·004 M-potassium cyanide. After 2 min. phage T7 was added in an amount which depended upon the required ratio of phage to bacteria. The mixture was shaken and maintained at 37° for 10 min. to allow phage adsorption and injection of phage DNA. After this time bacteria and free phage were separated by centrifugation, the bacteria being re-suspended in tryptic meat broth. For those experiments in which phage development was allowed to occur before aerosolization, the resuspended bacteria in tryptic meat broth were maintained at 37° for the required time and then were rapidly chilled. Following this, tracer spores of *B. subtilis* var. *niger* in tryptic meat broth were added and the suspension aerosolized immediately. A control suspension differing only in that tryptic meat broth replaced the phage suspension was similarly prepared.

Type 2 suspensions were prepared by centrifuging down bacteria from culture fluid, washing them in glass-distilled water, re-centrifuging and suspending in glass-distilled water. Type 2 suspensions, without tracer spores, were used in experiments for estimating % lysis of bacteria infected with phage T7 after collection from the aerosol.

Aerosol storage. A rotating drum apparatus was used, as described by Cox, (1966).

Aerosol collection and assay. Type 1 suspension. Samples were collected in raised impingers (May & Harper, 1957) at 0·3 sec., 3 sec., 2 min., 15 min. and 30 min. aerosol ages. The phage-infected bacteria were assayed by plaque formation at 37° in freshly prepared media of soft tryptic meat 0·5% agar layered on to a 1% tryptone agar, and the bacillus spores were assayed on 1% tryptone agar at 37°

(Miles & Misra, 1938). Each sample was assayed on six plates, and the ratio of the number of plaques to the number of *Bacillus subtilis* var. *niger* colonies per sample was used to calculate % lysis, based on the ratio in the spray fluid as being 100%. Percentage survival of the control phage-free bacteria was measured as for *B. subtilis* var. *niger*. Since *Escherichia coli* B formed white colonies and *B. subtilis* var. *niger* formed orange colonies differential counting was possible.

Type 2 suspension. Samples were collected directly on to Postgate chambers (Postgate, Crumpton & Hunter, 1961) containing 1% tryptone agar, by means of a slit sampler (Bourdillon, Lidwell & Thomas, 1941). Percentage colony formation was calculated by observing the proportion of the total number of bacteria per microscope field which formed microcolonies at 37°. At least six fields per sample were examined under the microscope before growth began, and at intervals these same fields were observed for $4\frac{1}{2}$ hr.

The % lysis was determined by collecting bacteria on to Postgate chambers (Postgate *et al.* 1961) previously inoculated with phage T7 and observing the proportion of the initial number of bacteria per microscope field which lysed. Other details were as for % colony formation. Included in the assay were the % lysis (usually 100) and % colony formation values for unsprayed bacteria taken from the type 2 suspension.

Samples were also collected in raised impingers (May & Harper, 1957) at times as for a type 1 suspension. Since no tracer spores were present, physical decay was assumed to be as for experiments reported by Cox (1966).

RESULTS

Experiments with Escherichia coli strain B infected with phage T7 before aerosol formation in air at 23° and 50% relative humidity

A type 1 suspension of *Escherichia coli* B, prepared and assayed as described, gave the results shown in Fig. 1. Development times greater than 12 min. were not used since the latent period of phage T7 at 37° was 15 min. (Fig. 2). The highest value of % lysis was obtained at 12 min. but did not exceed the value of bacterial survival. Increasing the phage:bacteria ratio to 10:1 gave even less satisfactory results (Fig. 3). The results showed two things. (i) Phage T7 stability was increased by adsorption to bacteria even when no development was allowed to occur. For zero development time initial values for % lysis of 9% (Fig. 1) and 6% (Fig. 3) contrast with 0·4% survival of phage T7 when sprayed alone from the same medium. (ii) Since % survival of bacteria was invariably greater than % lysis, loss of viability of *E. coli* B was not responsible for the inability of phage T7 to mature and form plaques.

Included in Figs. 1 and 3 are the arithmetic means of the % survival values and the standard deviations for the control experiments conducted with non-infected bacteria. The magnitude of the standard deviations, especially for the 30 min. aerosol age samples, may have been because, as the development time for the phage-free control bacteria increased, so the degree of *Escherichia coli* B survival decreased. This was confirmed in that for a 40 min. development time for *E. coli* B, 5% survival was obtained at an aerosol age of 30 min. as compared to 49% survival for zero development time.

To stabilize phage T7 to a greater degree than that described above, the procedure for obtaining phage-infected bacteria needed to be modified. A suspension of 16 hr *Escherichia coli* B in aerated tryptic meat broth at 37° was prepared without cyanide and this suspension was inoculated with phage T7 to give a ratio of phage: bacteria of 10:1. For the next 10 min. the suspension was allowed to cool while adsorption and phage development occurred. After 10 min. the suspension was centrifuged to remove unadsorbed phage and the deposited infected bacteria were resuspended in tryptic meat broth at room temperature. Such infected bacteria gave 100 % lysis after 30 min. in the aerosol, as previously reported (Cox & Baldwin, 1964). Electron micrographs of these bacteria showed that complete maturation of phage had not occurred.

These results suggest that the aerosol stability of phage T7 inside the bacteria critically depended upon the state of development of the phage.

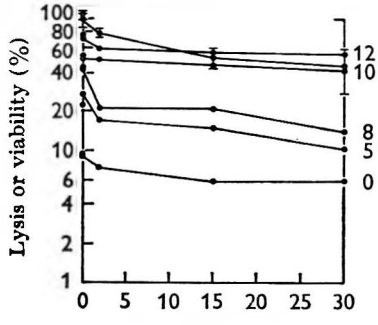


Fig. 1

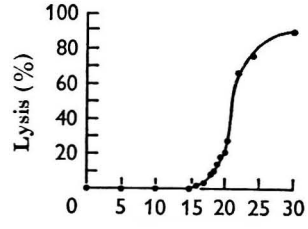


Fig. 2

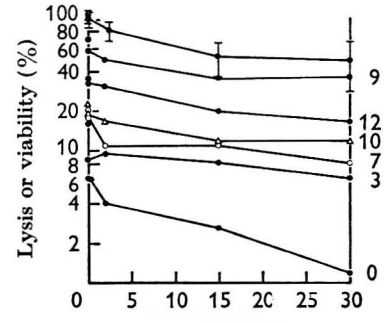


Fig. 3

Fig. 1. Aerosol survival of phage T7 as a function of development time (min). in *Escherichia coli* strain B. Temperature 23°. Phage:bacteria ratio of 1:1.

Fig. 2. Latent period of phage T7 at 37°; host *Escherichia coli* strain B.

Fig. 3. Aerosol survival of phage T7 as a function of development time (min.) in *Escherichia coli* strain B. Temperature 23°. Phage:bacteria ratio of 10:1.

Experiments with Escherichia coli strain B infected with phage T7 before and after collection from air at 23° and 50 % relative humidity

A type 1 suspension (phage:bacteria ratio of 5:1) without *Bacillus subtilis* var. *niger* spores was prepared, allowing zero development time. After aerosol formation samples were collected at 0·05, 1 and 15 min. by raised impinger (May & Harper, 1957) and at 15 min. only by slit sampler (Bourdillon, Lidwell & Thomas, 1941) on to phage-free and phage-treated Postgate chambers (Postgate *et al.* 1961). The principle of the experiment was that if only inactivation of the phage occurred, then after collection the phage-infected bacteria should form colonies, and also support phage growth when re-infected. The results of the experiment, including the aerosol survival of a phage-free control, are given in Table 1. The data suggest the following conclusions. (*a*) Since only 28 % of bacteria infected with phage T7 before aerosol formation were able to lyse after collection, then 72 % of these bacteria as a result of aerosolization and collection developed a condition which prevented phage T7 growth. Samples collected by impinger show that this condition quickly developed

and that, concomitant with loss of ability to lyse was the appearance of colonies (53 %), i.e. bacteria forming phage plaques before aerosol formation became colony producers as a result of aerosolization and collection. (*b*) The % colony formation of uninfected bacteria was 50 %, yet 61 % of bacteria lysed when infected with phage T7 after collection; therefore 11 % of the bacteria must have had an impaired bacterial reproductive capacity. (*c*) Following re-infection by phage T7 of bacteria infected with this phage before aerosol formation, only 61 % were able to lyse. Hence 39 % of these bacteria must have had an impaired phage productive capacity. The results given in Table 1 were therefore the consequence of at least one mechanism responsible for loss of viability of phage T7 and two mechanisms for *Escherichia coli* B.

Table 1. *Percentage lysis for Escherichia coli strain B infected with phage T7 before spraying and after aerosol collection, and % colony formation*

Type 1 suspension of *E. coli* B infected with phage T7 (phage:bacteria ratio of 5:1) sprayed from culture medium into air at 50 % relative humidity and 23°

Aerosol age (min.)	% colony formation* of control	% lysis†	% colony formation‡	% colony formation‡	% lysis†	% lysis§
0	100	96	4	—	—	—
0·05	100	4	53	—	—	—
1	100	6	41	—	—	—
15	50	4	23	9	28	61
Collector		Impinger		Slit sampler		

* % colony formation = % colony formation of phage-free control.

† % lysis = % lysis of bacteria infected with phage before aerosol formation.

‡ % colony formation (owing to death of phage) = % colony formation of bacteria infected with phage before aerosol formation.

§ % lysis (owing to death of phage) = % lysis of bacteria re-infected with phage before aerosol formation.

Experiments with Escherichia coli strain B infected with phage T7 after collection of an aerosol stored in nitrogen or air at various relative humidities

Other work has shown (Cox, 1966) that three stresses could influence the survival of *Escherichia coli* in the aerosol, namely, an air stress, a relative humidity stress and a collection stress. Of these, the first and second were investigated by using phage T7 in an attempt to demonstrate the locus of action of the stresses. Bacterial suspensions type 2 were prepared with a spray fluid of distilled water and were aerosolized into atmospheres of nitrogen (> 99·9 %) or air at several values of relative humidity. Percentage colony formation and % lysis values are given in Table 2 for an aerosol age of 30 min. The % colony formation is represented by two values, that in normal type is the % actually dividing, while the values in bold type include bacteria which showed swelling or filamentous growth but did not divide during the period of observation. The large difference in the two values at low, but not at high, relative humidity, suggest that the process of cell division may have been slightly impaired at low relative humidity. The results also show that the relative humidity stress and the air stress operated at different loci of the bacteria.

Table 2. *Escherichia coli strain* B. *% colony formation and %, lysis values for aerosol samples subjected to phage T7 after collection*

Aerosol age 30 min. Temp. = 26·5°. Values in parenthesis are % colony formation for impinger samples. Values in bold type are % non-static bacteria. Spray fluid: distilled water.

Relative humidity (%)	atmosphere	colony formation (%)	lysis (%)
100	nitrogen	7·0	20
		9·0 (7.0)	
90	nitrogen	3·0	24
		3·7 (10)	
85	nitrogen	1·6	8
		2·4 (1.2)	
82	nitrogen	3·0	11
		3·4 (3.5)	
50	nitrogen	4·7	78
		5·7 (37)	
50	air	4	63
		24 (1.3)	
25	nitrogen	42	82
		84 (77)	
25	air	1·7	59
		30 (1·1)	

DISCUSSION

Causes of death of phage T7. Phage T7 was not stable as an aerosol when sprayed in the free state. When allowed to infect a suspension of *Escherichia coli* B without permitting development of the phage to occur, nearly 100% plaques were produced; but after aerosol generation, storage and collection, many of the bacteria were able to form colonies and were also capable of supporting phage T7 growth upon re-infection. It would seem then, that phage T7 DNA became biologically inactivated at 50% relative humidity in times comparable to those for droplet evaporation. If phage infection had not occurred, then damage to the process of infection is likely to have produced the observed result. DNA inactivation would also account for the results (Figs 1, 3) when phage T7 development was allowed to occur before aerosol formation. If this explanation is correct then phage T7 DNA can be inactivated in the aerosol and its inherent stability can be increased by allowing the phage to develop to a certain degree; a similar result was found by Webb, Dumasia & Singh Bhorjee (1965). The actual processes responsible for enhancing the stability are unknown, but would appear to be of two kinds: (i) processes involved during phage adsorption and injection of phage DNA; (ii) processes which occur late in phage T7 development.

Causes of death of Escherichia coli B. Collected bacterial aerosol samples did not show 100% colony formation, and did not produce 100% lysis when infected with phage T7 after collection. Provided that phage adsorption and infection processes are not impaired, the difference between colony formation and % lysis represents the % of bacteria with an impairment which occurs off the phage production pathway, i.e. the % with an impaired bacterial reproductive capacity. The difference between % lysis and 100% represents the bacteria with an impairment on a path-

way common to the reproduction of bacteria and the production of phage, i.e. an impaired phage productive capacity. At high and low relative humidities for aerosols in atmospheres of nitrogen (Table 2) the main reason for loss of bacterial viability is an impaired phage productive capacity. At intermediate relative humidities for aerosols in atmospheres of nitrogen the results suggest that loss of bacterial viability is the result of impaired phage productive and bacterial reproductive capacities. Therefore the degree and the nature of impairment is related to the relative humidity. For the conditions tested the degree of impairment of phage productive capacity appeared to be greatest at 85 % relative humidity.

For aerosols in air (Table 2) at relative humidity values other than those at which a substantial impairment of phage productive capacity occurs, the main cause of loss of bacterial viability was through an impaired bacterial reproductive capacity. For aerosols in air at relative humidity values (e.g. 85 %) where substantial impairment of phage productive capacity occurs, then loss of viability of *Escherichia coli* B was mainly from an impaired phage productive capacity. If the adsorption processes of phage were impaired when bacteria collected from the aerosol were subjected to phage, then the argument is similar to that presented above except that the loss of *E. coli* B viability attributed to loss of phage productive capacity is now attributed to an impairment of phage adsorption processes, the degrees of which are related to the loss of bacterial viability caused by loss of bacterial reproductive capacity. A combination of the two postulates is possible, in that the difference between % lysis and 100 % can be attributed to loss of phage productive capacity together with some impairment of the adsorption processes of phage multiplication.

By using an aerosol-stable phage it should be possible to determine the position in the phage productive pathway at which impairment of bacterial viability occurs through aerosol formation, storage and collection. The procedure would be to use different development times prior to aerosol formation and determine the development time required to give 100 % lysis. Provided that sufficient is known about the timing of the stages of phage development then the position of the impairment in the metabolic pathway of *Escherichia coli* B could be determined.

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The Morphology of *Alcaligenes faecalis* bacteriophages

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SUMMARY

The morphology of seven temperate phages from lysogenic strains of *Alcaligenes faecalis* and two virulent phages obtained from sewage and active on this species was examined by a negative-staining technique. The phages showed a variety of morphological forms and two of them have unusual combinations of features. One (A11/A79) has a contractile tail sheath around a hollow core, but lacks the base plate, pins and tail fibres usually associated with this type of tail. The other (A5/A6) resembles the C1, F1 group of coliphages but has an octahedral head and a collar. The five remaining temperate phages are similar to the C1, F1 and Providence 9000/9402 group of phages. The sewage phages differ in many respects from one another and also from the temperate phages examined. At least three of the nine phages have octahedral heads.

INTRODUCTION

Temperate phages active on *Alcaligenes faecalis* were isolated by Moore & Pickett (1960), but the morphology of these phages has not been described. Another series of *A. faecalis* phages derived from lysogenic strains and from sewage was reported by Maré & Coetzee (1963), who determined the host ranges of these phages. The differentiation of *A. faecalis* from other non-sugar-fermenting bacteria is largely based on morphology and a few biochemical reactions (*Bergey's Manual*, 1957). The classification of *A. faecalis* is regarded as unsatisfactory by many workers (Conn, 1942; Sarkar, Choudhury & Tribedi, 1959; Moore & Pickett, 1960). As an approach to this problem it was decided to study the morphology of the *A. faecalis* phages previously isolated in an attempt to contribute to their taxonomy and possibly to that of their hosts.

METHODS

Media. The liquid medium contained (%, w/v): Bacto-tryptone, 1·3; NaCl, 0·8; glucose, 0·15. The solid medium contained 1·1% (w/v) agar or 0·6% (w/v) agar when used as a top layer.

Bacteriophages and host organisms. The isolation of the seven temperate phages from lysogenic strains of *Alcaligenes faecalis* and the two virulent phages from sewage was described by Maré & Coetzee (1963). The seven temperate phages A5/415, A20/415, A5/A6, A11/A79, A64/A62, A74/A3 and A86/A88 were propagated on *A. faecalis* strains NCTC 415, A6, A79, A62, A3, A88, respectively. Lysates of the two sewage phages A6 and 8764 were prepared on *A. faecalis* strains A6 and NCTC 8764. The phages can all be differentiated by means of their host range and three of the lysogenic strains (A5, A20, A11) can at the moment be differentiated

by their reaction to a series of typing phages (Maré, unpublished). The general phage techniques were those of Adams (1959) and cultures were incubated at 37°. High titre lysates of all the phages were prepared by the modification (Adams, 1959) of the double agar layer method of Hershey, Kalmanson & Bronfenbrenner (1943).

Electron microscopy. The phages were purified and concentrated by differential centrifugation (de Klerk, Coetzee & Fourie, 1965). Samples of the purified phages (plaque-forming units about 1×10^{11}/ml.) suspended in 0·1 M-ammonium acetate (pH 7·2) were mixed with equal volumes of neutral 2% (w/v) potassium phosphotungstate and spread on carbon support films (Bradley, 1962). These preparations were examined with a Philips EM 200 electron microscope.

RESULTS

The dimensions of the phages are presented in Table 1. Five of the temperate phages A5/415, A20/415, A64/A62, A74/A3 and A86/A88 are similar in morphology; phage A64/A62 is shown in Pl. 1, fig. 1. Their heads have regular hexagonal outlines and their tails are long, thin, non-contractile, with pointed ends, and they lack cross-striations. They have no necks or collars; no tail fibres were seen. Phage A86/A88 is larger than the others of this group. These phages are larger than the providence phage 9000/9402 (Prozesky, de Klerk & Coetzee, 1965) and smaller

Table 1. *Dimensions of alcaligenes bacteriophages*

Phage	Head* (Å)	Tail length (Å)	Tail width (Å)	Overall length (Å)
A5/415	750	2400	100	3150
A20/415	750	2400	100	3150
A5/A6	750	2400	90	3150
A11/A79	650	1300	190	1950
A64/A62	750	2500	100	3250
A74/A3	750	2500	120	3250
A86/A88	800	2500	170	3300
A6	900	1100	160	2000
8764	600	1700	110	2300

* Dimensions between apices are given. Figures are the mean of six to twelve measurements.

than the C1, F1 group of coliphages (Bradley, 1963*a*), both of which they resemble. Phage A5/A6 (Pl. 1, fig. 2) differs from the first group of five phages in that it has a neck and a collar about 120 Å in width. Its head is larger than most of those of the first group of phages and is octahedral in shape (Pl. 1, fig. 3). The remaining temperate phage, A11/A79 (Pl. 1, figs. 4, 5) has a small head with a regular hexagonal outline. The tail consists of a hollow core surrounded by a contractile sheath and it is attached to the head by a short neck which has no collar. This phage lacks a base plate; no tail pins or fibres were seen. It resembles typhoid phage $\phi 2$ (Bradley & Kay, 1960) which has, however, a distinct base plate without pins. The sewage phage A6 (Pl. 1, figs. 6, 7) has a large octahedral head. Its other features also resemble those of coli-typhoid phages $\phi 2$ and E1 (Bradley, 1963*a*) and pseudomonas phage 12S (Bradley, 1963*b*). The tail has a hollow core and a contractile sheath in which subunits are visible; no cross-striations. No base plate is evident,

but an indistinct cluster of fibres like those of phage 12S (Bradley, 1963*b*) moves with the contractile sheath. The other sewage phage 8764 (Pl. 1, fig. 8) has a small octahedral head and a long, thin, non-contractile tail without cross-striations. No neck or collar is present and the pointed tail tip has a cluster of delicate fibres around it. This phage resembles coliphages $\beta 4$ and $\gamma 2$ (Bradley, 1963*a*). Three of the phages (A5/A6, A6, 8764) have octahedral heads. The head shapes of the remaining phages could not be determined. Many of the capsids have an uneven rough appearance suggestive of arrays of capsomeres, but neither their shape nor their packing arrangements could be resolved. None of the tails show cross-striations and they appear to consist of small tightly packed subunits.

DISCUSSION

Most of the phages examined resemble certain coli, typhoid, pseudomonas or providence phages, but two phages show unusual combinations of features. Phage A11/A79 has a tail with a contractile sheath but lacks all the appendages usually associated with this type of tail structure (Bradley & Kay, 1960; Prozesky *et al.* 1965). Phage A5/A6 has a single collar and an octahedral head-shape. This combination differs from phage ZG/3A (Bradley, 1964*b*) which has a collar and a head like that of coliphage T2, and also from phage PS4 (Matthews & Bradley, 1964) which has a double collar. Other phages with long, thin, non-contractile tails do not have collars (Bradley, 1964*b*). All the phages are large and three have octahedral heads. The micrographs do not reveal the geometrical form of the other heads, but their size favours the octahedral shape (Matthews & Bradley, 1964). A striking feature of these phages, both with and without tail sheaths, is the absence of cross-striation of the tails. Many phages previously described with the same general morphology possess cross-striations (Bradley & Kay, 1960; Bradley, 1963*a*, *b*; Prozesky *et al.* 1965). The techniques employed were those of Prozesky *et al.* (1965) and the absence of cross-striations may be due to the packing of the small subunits, so that striations are below the resolution obtainable. Five of the temperate phages described here are similar to one another but originated in different strains of *Alcaligenes faecalis*. This may be of some importance in the taxonomy of these phages, although as mentioned above phages which attack this family are basically typical of 'enterophages'. Unlike the filamentous phages of *Escherichia coli* which are specific for male strains of this species (Bradley, 1964*a*, *b*) the phages studied here failed to show a morphology peculiar to *A. faecalis* and in this respect do not contribute to its classification.

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EXPLANATION OF PLATE

The magnification in figs. 1–7 is ×270,000 and in fig. 8 is ×350,000. All phages in ammonium acetate and phosphotungstate.

Fig. 1. Phage A64/A62.

Fig. 2. Phage A5/A6 showing collar.

Fig. 3. Phage A5/A6 showing octahedral head and collar.

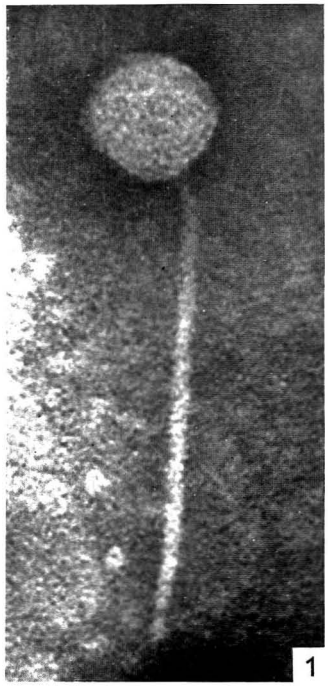
Fig. 4. Phage A11/A79.

Fig. 5. Phage A11/A79 with contracted tail sheath.

Fig. 6. Phage A6.

Fig. 7. Phage A6 with contracted tail sheath.

Fig. 8. Phage 8764.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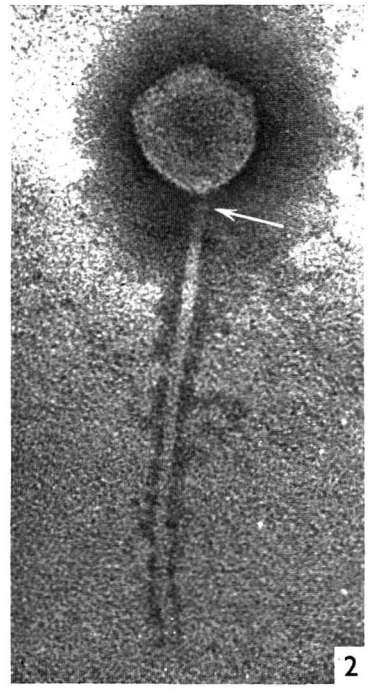
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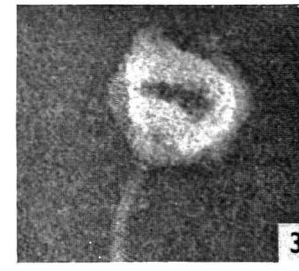
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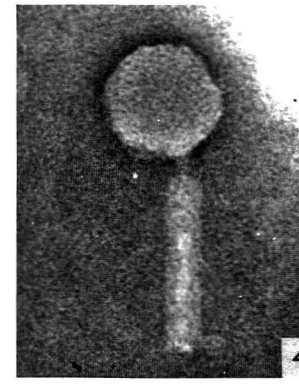
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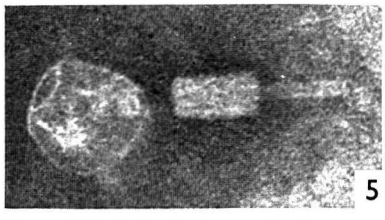
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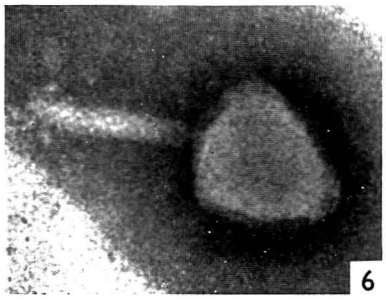
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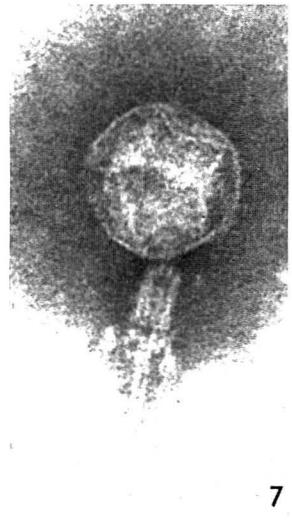
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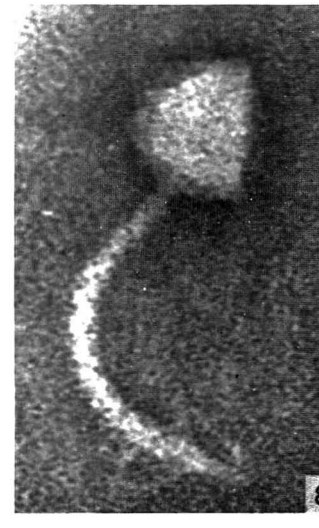
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Flagella-Shape Mutants in Salmonella

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SUMMARY

Seven flagella-shape mutants were isolated from a curly-flagella strain of *Salmonella abortusequi*. The motility in broth and spreading ability on semi-solid medium of these mutants as well as flagellar morphology were examined by dark field and electron microscopy. They were classified into the following five mutant types, heteromorphous, small-amplitude, para-curly, short and hooked-curly.

Each of these mutants manifests its characteristic flagellar shape and motility, and the spreading ability of these mutants on semi-solid medium decreases in the order: normal, heteromorphous, small-amplitude, para-curly, short, hooked-curly, curly.

Except in the small-amplitude and short mutants, the shape of a flagellar bundle of living bacteria under the dark-field microscope corresponded in each mutant to a spiral of the flagella observed under the electron microscope. In the small-amplitude mutant, transconformation of the flagellar bundle from small-amplitude to curly was observed in organisms suspended in 0·5% (w/v) methylcellulose solution. In the short mutant, the flagellar bundle was not seen in the dark-field microscope.

Difference of antigenic specificities between the flagella of the parental curly and each of the mutants were not detected when examined by absorption-agglutination tests.

From transduction analyses with P 22 phage, it was found that the traits of each of the four mutants heteromorphous, small-amplitude, para-curly, short were transferred with the structural gene of phase-2 flagellin and had no effect on the flagellar shape and motility in phase-1. From this, it is inferred that the characteristic flagellar shape and motility of these mutants are primarily to be attributed to the conformation of the flagellin which composes the mutant flagella.

INTRODUCTION

Bacterial flagella are becoming recognized as unique material for the study of cytomorphogenesis. It has been occasionally noticed that more than two different shapes of flagella appear in a bacterial strain. The presence of both normal and curly are the most common. The phenomenon was called flagellar 'biplicity' (Pijper, 1957; Leifson, 1960). In salmonella, curly flagella were found to appear by mutation of the normal (Iino, 1962*a*). A typical curly mutant does not have the ability to spread in semi-solid medium and move rotationally in broth and its wavelength is about one-half the normal. Curly character of the mutant is flagellar phase-specific, and genetical evidence showed that the site of mutation maps in the structural gene of the flagellin concerned. By the fingerprinting analysis, it was demonstrated that the tryptic peptides of the curly flagella were different from the normal in one

peptide (Enomoto & Iino, 1963). Thus genetic analyses of curly mutants are disclosing the correlation between flagellar shape and primary structure of the component protein flagellin. On the other hand, the change from normal to curly was found to occur in a single organism under the dark-field microscope (Pijper, 1957). From this observation it was suggested that the biplicity could occur by transconformation of already formed flagella even if their chemical composition was the same. *In vitro* reconstitution experiments have examined the process of polymerization of flagellin into flagella (Abram & Koffler, 1964; Asakura, Eguchi & Iino, 1964; Lowy & McDonough, 1964). and flagellar regeneration experiments have been concerned with the chemical nature of flagellum-forming apparatus (Kerridge, 1960). The present paper reports a part of an investigation into the genetic system which controls 'flagellar morphogenesis'. It deals with the characterization and preliminary genetic analysis of five types of flagella-shape mutants isolated from a strain of *Salmonella abortusequi*.

METHODS

The curly strain SJ 30 used in the present experiments was a mutant of *Salmonella abortusequi* SL 23 (Iino, 1962*a*). This strain is stable in antigenic phase-2 (antigen-*e*, *n*, *x*). Genetic analyses have shown that the curly character of this strain occurs by a mutation in a site of the structural gene of phase-2 flagellin, *H2*, of strain SL 23.

For the selection of flagellar mutants, tests for spreading ability and transduction experiments, semi-solid medium containing 0·2% (w/v) agar and 8% (w/v) gelatin in broth was used throughout. On the semi-solid medium, a normal clone spreads and forms a swarm, while a curly clone grows confined at the region of inoculation. For antiserum selection, 0·2% (w/v) final concentration of anti-H serum of agglutination titre 5000 was added to semi-solid medium before pouring into Petri dishes.

Cellular motility was observed by phase-contrast microscopy of hanging drops of broth culture. To observe the flagella of living bacteria, dark-field microscopy of bacterial suspensions in methyl cellulose (Wako Pure Chemical Co., Ltd., Tokyo) solution was adopted following the procedure of Pijper (1957). Bacteria were grown in broth at 37° for 2 hr and the organisms at the middle log phase were used for the observation. Bacterial samples were prepared by mixing a drop of culture with the same volume of saline containing 0·5% (w/v) methylcellulose at the final viscosity of $2{\cdot}8 \times 10^{-2}$ poise (20°). The mixture was dropped on a 1·4 mm thick glass slide and 0·25 mm coverslip put on. Manicure enamel was used for sealing the coverslip. The dark-field microscope used was a 7-volt Ernst Leitz Wetzler Nr. 457210, in combination with a Chiyoda paraboloid condenser of aplanat 1·4 N.A., and an immersion objective ×90 (with iris) with an eyepiece ×10.

Flagellar shape was observed by electron microscopy. Bacteria were grown in Penassay broth (Difco Lab., Inc., Detroit, Michigan) for 8 hr at 37° with shaking, harvested by centrifugation at 3000 rev./min. for 10 min. and finally resuspended in distilled water at pH 6·2. This suspension was ordinarily used without any fixative for the electron microscope observations of flagella. For the preparation of samples for electron microscopy, droplets of bacterial suspension were placed on collodion-coated grids, thereafter extra drops were dried in vacuum and shadowed with chromium at an angle of 20°. For negative staining the method of Brenner & Horne (1959) was used. The grids were examined in a JEM T 6S electron

microscope (Japan Electron Optics Laboratory Co., Ltd.) with a single condenser system and accelerating voltage of 60 kV. Micrographs were taken at initial magnification of ×5000 on Sakura process hard plates. One micron diam. Bacto latex particles (Difco Lab., Inc., Detroit, Michigan) were used for the determination of magnification.

To obtain average and standard deviation values for wave-length, amplitude and spiral unit length, several waves in a single flagellum were observed on different organisms and a total 60 to 90 waves were measured per clone.

RESULTS

Detection and isolation of mutants

Curly type organisms of *Salmonella abortusequi* strain SJ 30 cannot spread on plates of semi-solid medium and when they are incubated for 10–20 hr at 37° grow at the area where the organisms were inoculated (Iino, 1962*a*). Occasionally it was found that spreading mutants which were present in the inoculum started to form swarms in this period (Pl. 1, fig. 1). After continued incubation, the number and size of the swarms increased until at 2 days at least some mutant swarms were invariably found on the plates; further increase was not observed, probably because spreading of the organisms was inhibited by changes of the media. The sizes of the developed swarms ranged from that of the normal motile type to that of the small satellite type.

The edges of seven swarms of independent origin and of types apparently different from each other and from the normal type, were isolated and streaked on nutrient agar plates, and one of the colonies which developed from each isolate was transferred to nutrient broth. Each broth culture was spotted on a plate of semi-solid medium and incubated for 8 hr at 37°. The developed swarms from the seven clones varied in size and texture in parallel with the mode of spreading of the swarms formed by each of the clones on the initial selective media (Table 1, column 3; Pl. 1, fig. 2). The spreading of clone 1 was about 70% of that of the original normal type, while clone 7 formed a compact colony surrounded by many tiny dotted colonies like satellite colonies reported by Quadling (1958). The remaining five colonies were intermediate types between clones 1 and 7. Clones 2 and 3 spread slower than the normal type and formed swarms having about half the diameter as compared to the normal. Clones 4, 5 and 6 spread more slowly than clones 2 and 3; the swarms formed by these clones were compact, and their diameters were one-sixth to one-seventh of the normal type.

Motility in broth

The mutant clones described in the foregoing section differed from each other with regard to the predominant type of cellular movement in broth culture (Table 1, column 4). Most of the organisms in clone 1 moved translationally and a few wriggled as shown in Fig. 1. In clone 2, the predominant organisms also moved translationally and some rotated. The translational movement in clone 2 was slower than that of the normal clone. The predominant type of movement in clone 3 was a wriggle, but this clone differed from the following clones in showing a circular movement, that is, the bacteria travelled in small circles with diameter of 2–3 μ (Fig. 1). Clones 4, 5 and 6

Table 1. *Characteristics of the flagella-shape mutants obtained from a curly strain, SJ 30, of Salmonella abortusequi.*

Type	Representative clone	Spreading ability*	Major type of movement	Flagellar shape observed by E.M.†	Flagellar shape observed by D.M.	Other remarks
Normal	SL 23	1·00	Translation	Normal	Normal	—
Heteromorphous	Mutant 1	0·67	Translation and wriggle	Normal and curly	Normal	Tendency to stretch
Small-amplitude	Mutant 2	0·53	Translation and rotation	Small-amplitude	Small-amplitude and curly	—
Para-curly	Mutant 3	0·44	Wriggle and circular	Curly	Curly	Strong tendency of bundling
Short	Mutant 4, 5, 6	0·15	Wriggle and rotation	Uncertain	Invisible	Short and small number of flagella
Hooked-curly	Mutant 7	0·08	Rotation and wriggle	Hooked-curly	Hooked-curly	Aggregation in small amount as compared to SJ 30
Curly	SJ 30	0·00	Rotation	Curly	Curly	Aggregation in broth

* Diameter of a swarm grown on semi-solid medium after 8 hr incubation at 37°; the value of SL 23 was taken as 1·00.
† E.M. = electron microscopy; D.M. = dark field microscopy.

Table 2. *Wavelength, amplitude and spiral unit length of flagella-shape mutants of Salmonella abortusequi observed with electron microscope*

Type	Strain	WL* (μ)	SD	Amp. (μ)	SD	WL/amp.	s.u.l. (μ)	SD
Normal	SL 23	2·90	0·30	0·51	0·11	5·9	3·00	0·38
Heteromorphous	Clone 1 L†	2·85	0·34	0·45	0·07	6·5	3·01	0·36
	Clone 1 S	1·51	0·07	0·38	0·02	4·0	1·83	0·19
Small-amplitude	Clone 2	2·31	0·24	0·30	0·07	7·8	2·44	0·31
Para-curly	Clone 3	1·52	0·24	0·50	0·09	3·1	1·92	0·22
Hooked-curly	Clone 7	1·56	0·27	0·45	0·09	3·8	1·97	0·21
Curly	SJ 30	1·12	0·08	0·29	0·05	3·9	1·86	0·19

* WL = wavelength; Amp. = amplitude; s.u.l. = spiral unit length; SD = standard deviation;
† L = larger flagellar wave; S = smaller flagellar wave.

were indistinguishable from each other by their motility. The organisms of these clones mostly wriggled but rotation and translation were also observed in them. Clone 7 was characterized by rotation in most of the organisms, each spinning around on itself; the rest wriggled. The organisms of clone 7 aggregate in broth, but in smaller amount compared with those of strain SJ 30.

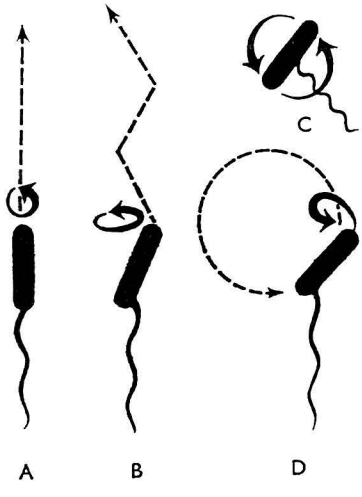

Fig. 1

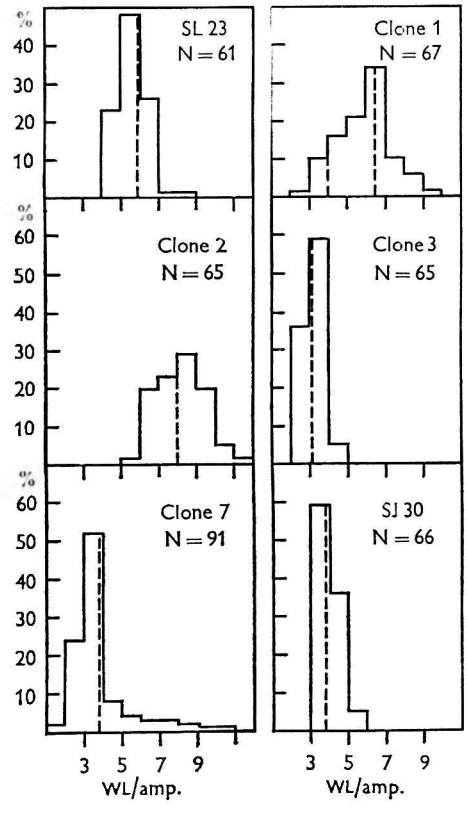

Fig. 2

Fig 1. Four representative types of movement of Salmonella organisms in liquid medium. A = translation; B = wriggle; C = rotation; D = circulation.

Fig. 2. Distribution diagrams of the ratio of wavelength to amplitude of flagellar waves observed by electron microscope on each flagella-shape mutant. The broken lines denote the average ratios and N denotes the number of flagellar waves measured. WL = wavelength; amp. = amplitude.

Stability of the mutant clones

For the examination of the stability of the mutant clones, each of them was subcultured through broth. In each subculture, 0·01 ml. of the culture at the late log-phase of growth (10^7 organisms/ml.) was transferred to 10 ml. of fresh broth. Thus bacteria passed about 6·2 divisions in a single-step culture. The subcultivation was repeated five times for each clone. At the time of each subculture, a drop of culture was sampled from each clone, and cellular and clonal motilities were observed in broth and on semi-solid medium. Even after five successive subcultures,

each clone showed its characteristic cellular and clonal motility, indicating that the mutant characters were so stable that they could be maintained through successive subculture.

Flagellar shape observed by electron microscopy

Normal flagella of the wild strain SL 23 observed by the electron microscope have wavelength of 2·20 μ and amplitude of 0·53 μ (Pl. 2, fig. 11). The curly mutant, SJ 30, has flagella about one-half of the normal in wavelength. Consequently its

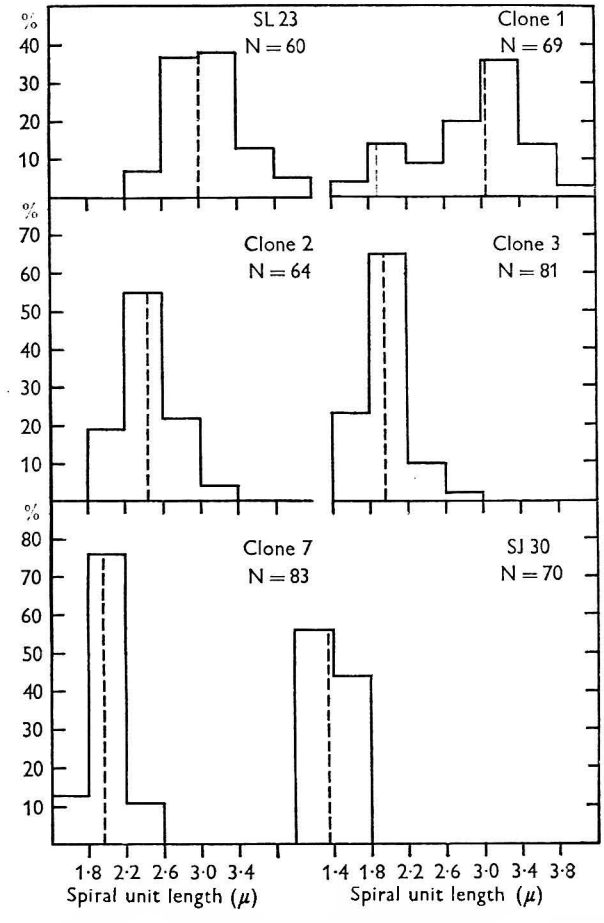

Fig. 3. Distribution diagrams of spiral unit length of flagellar waves observed by electron microscope on each flagella-shape mutant. The broken lines denote the average spiral unit length and N denotes the number of flagellar waves measured.

flagella are more curled compared with that of the normal (Pl. 3, fig. 18). Mutant clones characterized by motility were found to have characteristic flagellar shapes (Pls. 2 and 3). They were classified into five morphological types (Table 1, column 5). Wavelength, amplitude and spiral unit length of the representative strain of each type are listed in Table 2, and histograms of the ratio of wavelength to amplitude and spiral unit length are shown in Figs. 2 and 3. The five morphological types are:

(1) Heteromorphous (Pl. 2, fig. 13), represented by clone 1. Flagella of this type were mostly normal. Curly waves were observed mixed with normal ones in one flagellum in 2% of the organisms in the clone. Bacteria with only curly flagella have never been seen. The spiral unit length, flagellar wavelength and amplitude of normal flagella were the same as those of wild-type flagella (Pl. 2, fig. 11). With the curly mutant, its wave resembled para-curly. Irregular-shaped flagella with wavelength longer than the normal were often observed.

(2) Small-amplitude (Pl. 2, fig. 12), represented by clone 2. The small-amplitude mutant is characterized by flagellar shape with the ratio of wavelength to amplitude higher than the normal type (Leifson, 1960); the ratio is the highest among the flagella-shape mutants. Amplitude and wavelength of this type of flagella were about 60 and 30% of the normal, respectively (Table 2).

(3) Para-curly (Pl. 3, figs. 15, 16), represented by clone 3. Organisms of this type produce curly flagella having larger wavelength, amplitude and spiral unit length compared with the original curly strain (Pl. 3, fig. 18). Moreover, flagella of this type have more tendency to retain bundles than the curly (Mitani & Iino, 1965), and the bundle has a specific wavelength 60% longer than that of the component flagella.

(4) Short (Pl. 2, fig. 14), to which clones 4, 5 and 6 belong. As described in the foregoing paragraph, the motility behaviour of the clones belonging to this type were the same as each other. Organisms of this type carried short flagella and the number of flagella per bacterium was small. Consequently, the flagellar shape cannot be identified. The presence of many fragmented flagella in the field of the electron microscope suggests that these flagella were more fragile than those of other types.

(5) Hooked-curly (Pl. 3, fig. 17), represented by clone 7. Flagella of this type are curly but have longer spiral unit length and a curvature more bent than the original curly; consequently their waves are hook-shaped. They tend to coil with each other and to form bundles. From the measurements of wavelength, amplitude and spiral unit length, the difference between flagellar shapes of para-curly and hooked-curly were not clear, but the discrimination of these two types is shown by observation of flagellar curvature and the mode of spreading on semi-solid medium (Table 1; Pl. 1, fig. 2). The ratio of wavelength to amplitude of hooked-curly flagella was diversely distributed compared to that of other mutants, while their spiral unit length was almost constant (Figs. 2, 3). This may indicate that the hook-shaped flagella have a tendency to stretch.

Dark-field microscopy

Under the dark-field microscope, most of the bacteria were motile when observed immediately after mounting on slides. But the number of motile bacteria decreased gradually with the passage of time. When the organisms were moving rapidly bundled flagella were blurred and had the appearance of a smooth straight tail as reported by Pijper (1957). While the organisms were moving slowly the spirals of bundled flagella were clearly seen. The organisms moved forward by making the bundled flagella as the axis (Fig. 1). The shape of bundled flagella showed a wavelength of 2·20 μ and an amplitude 0·53 μ in normal bacteria while in the curly mutant the respective figures were 0·91 and 0·29 μ (Pl. 1, figs. 3, 10). The average wave-

lengths and amplitudes of the bundles of each mutant clone are listed in Table 3. The characteristic type of flagellar shape of each mutant was the same, between electron microscopy and dark-field microscopy, for all clones. One of the remarkable differences between the figures seen by electron microscopy and dark-field microscopy was that dimorphism was observed in clone 2 in the dark field but not by the electron microscope (Pl. 1, figs. 5, 6). During 30–60 min. after preparation of the bacterial suspension in 0·5% methylcellulose solution, flagellar bundles observed in clone 2 were mostly those with the longer wavelength. Thereafter the number of the bundles with the shorter wavelength gradually increased to more than 50% of the observed bundles. By continuous observation of single organisms having bundles of long wavelength, change from long type to short type was observed to occur in a single bundle. The detail of this change will be reported elsewhere. The shape of a flagellum of clone 2 by the electron microscope was comparable to that of the bundle with long wavelength (Tables 2, 3).

Table 3. *Wavelength and amplitude of flagella-shape mutants of Salmonella abortusequi observed with dark-field microscope*

Type	Strain		WL* (μ)	SD	Amp. (μ)	SD	WL/amp.
Normal	sJ 23		2·20	0·09	0·53	0·06	4·2
Heteromorphous	Clone 1		2·24	0·12	0·46	0·03	4·9
Small-amplitude	Clone 2	L†	1·69	0·14	0·32	0·02	5·3
		S	0·95	0·10	0·45	0·09	2·1
Para-curly	Clone 3		1·08	0·06	0·31	0·04	3·5
Hooked-curly	Clone 7		0·98	0·06	0·42	0·03	2·4
Curly	sJ 30		0·91	0·06	0·29	0·08	3·1

* WL = wavelength; Amp. = amplitude; SD = standard deviation; † L = larger flagellar wave; S = smaller flagellar wave.

In contrast to clone 2, dimorphism observed with clone 1 by the electron microscope was not seen by dark-field microscopy: the shape of flagellar bundle in clone 1 by dark field was like with that of a normal flagellum of the clone as seen by the electron microscope. The bundles of clones 3 and 7 corresponded to spiral of each flagellar wave as observed on the bundle formation of curly flagella of *Salmonella abortusequi* strain sJ 30 (Mitani & Iino, 1965). The spiral of the flagellar bundles of clone 3 by dark-field microscopy did not resemble the curvature of the bundle as observed by the electron microscope but that of a unit flagellum.

The ratio of wavelength observed with the electron microscope to that by dark-field microscopy was 1·3 to 1·6 in all clones. On the contrary, the ratio of amplitude by electron microscope to that by dark-field microscopy was not significantly different except in clone 3 (Tables 2, 3). In clone 3 the ratios of wavelength and of amplitude by electron microscope to those by dark-field microscopy was greater than 1. Clone 7 organisms formed a spiral of bundled flagella with the same wavelength and longer amplitude compared with that of clone 3; while after flattening on a grid, vertical extension was not seen as it was with clone 3. This may explain the overbending of the wave in a flagellum of clone 7. Organisms of strain sJ 30 and of clone 7, which aggregated in broth, were very often found to bind each other with their flagellar bundles. Bundled flagella were not detected in clones 4, 5 or 6 by dark-field microscopy (Pl. 1, fig. 8).

Flagellar antigens of the mutants

By the slide agglutination test, the H-antigens of all the seven mutants examined were identified as *e*, *n*, *x*. For further details of their antigenicity, anti-*e*, *n*, *x* sera were obtained against *Salmonella abortusequi* strain SJ 30 and each of the five mutant clones, representing each flagellar type. The agglutination titre of the anti-*e*, *n*, *x* sera to the *e*, *n*, *x* type organisms was examined before and after reciprocal absorption of all pairs of mixtures. The titres with the antisera before absorption were all 2^{14}; after absorption, agglutination was not observed at the dilution of $1/2^{3}$ to $1/2^{16}$. That is, the *e*, *n*, *x* antigens of strain SJ 30 and its five mutant strains behaved identically in the absorption-agglutination test.

Genetic analysis

Transductions were done with P 22 phage from the five mutant clones, representing each flagellar type, to a phase-1 monophasic strain of *Salmonella typhimurium* SW 1166 *i*: (genotype $H1^{i}H2^{1\cdot2}ah2^{-}$; Iino, 1962*b*). Antigen type recombinants were screened on semi-solid medium containing anti-*i* serum. The developing transductional clones, i.e. swarms, were isolated on nutrient agar plates and their flagellar antigen typed by slide agglutination. They were further transferred to broth and

Table 4. *Transduction from the flagella-shape mutants of Salmonella abortusequi* SJ *30* (*a*)*: e, n, x to S. typhimurium* SW *1166 i: (1,2)*

SJ 30 is curly in both phase-1 and phase-2. The culture used for the present experiment is stable in phase-2. SW 1166 is a phase-1 monophasic (*ah2*$^{-}$) type. Recombinants were selected on plates of semi-solid medium containing anti-*i* serum.

	No. of phase-2 type transductional clone				
Donor	Total	M-*e, n, x*	N-*e, n, x*	M-*1, 2*	N-*1, 2*
Mutant: clone 1	12	11	0	0	1
Mutant: clone 2	14	13	1	0	0
Mutant: clone 3	6	6	0	0	1
Mutant: clone 4	5	5	0	0	0
Wild: SL 23	12	0	10	0	2
None	0	0	0	0	0

M = mutant type corresponding to phase-2 of donor; N = normal type.

semi-solid medium, and the motility of the component organisms examined; the results are listed in Table 4. The major types developed were *a* and *i*: *e, n, x*. Movement of the former in broth was rotational as expected from the genotype of the donor (Iino, 1962*a*). The latter showed motility in *e, n, x*-phase as that of the phase-2 organisms of the donor strain. In transduction from clone 7, *i*: *e, n, x* type recombinant was not detected, probably because the *e, n, x* types among the transductional clones were as poorly motile as the donor strain and could not form distinctive swarms on the screening media. In transduction from SJ 30 to clone 7 no swarms developed, on semi-solid medium. These results indicate that each characteristic flagellar shape of the mutant clones 1, 2, 3 and 4 was co-transduced with the phase-2 antigen, *e, n, x*. As a minor type, *i*: *1, 2* swarms were detected on the screening

plates. The motility of the component organisms were normal without exception. In the control plate, to which only recipient organisms were inoculated, *i*: *1, 2* swarms did not grow. Moreover, the mutation of strain SW 1166 to diphasic, *ah2*$^+$, type was not observed. The *i*: *1, 2* swarms may, therefore, represent the recombinant between *ah2* and *H2*. Normal flagellar type in phase-2 of these recombinants indicated that the flagellar shape determinant of the corresponding donor mutants was more closely associated with phase-2 antigen type determinant with *ah2*.

Transductions were next done from the four mutant clones 1, 2, 3 and 4, to a phase-1 curly mutant strain of *Salmonella typhimurium* SW 577 *i*: *1, 2* (Iino, 1962*a*). The purpose of this experiment was to examine the possibility of modification of phase-1 curly character by the mutant genes of these clones. Transductional clones were isolated, inoculated into broth and their motilities examined; further subculture to broth was made. To check whether phase-1 antigen-*i* remained as curly or was modified, the cultures were diluted appropriately and plated together with anti-*e, n, x* serum on semi-solid medium. The results are summarized in Table 5; the alternative phase of *e, n, x* was curly with antigen type *i*. That is, the mutation affected only flagellar type in phase-2.

Table 5. *Flagellar type of the recombinants obtained by transductions from the flagella-shape mutants of Salmonella abortusequi* SJ *30* (*a*)*: e, n, x to S. typhimurium sw 577 i: 1, 2*

SW 577 is curly in phase-1 and normal in phase-2. The recombinants in which phase-2 flagellin gene, $H2^{1,2}$, was replaced by $H2^{e,n,x}$ were selected on plates of semi-solid medium containing anti-*i* serum.

Donor	No. of phase-2 type transductional clone examined: Total	Normal-*e, n, x*	Mutant-*e, n, x*	Flagellar type in phase-1
Mutant: clone 1	21	0	21	Curly-*i*
Mutant: clone 2	98	0	98	
Mutant: clone 3	63	0	63	
Mutant: clone 4	5	0	5	
Wild: SJ 23	39	39	0	

DISCUSSION

Bacterial flagella have been known to have characteristic shapes for each bacterial strain. The flagellar morphology of various bacteria has been studied extensively with the light microscope with stained materials; the accumulated information was reviewed by Leifson (1960). The flagellar shapes which have been reported are normal, curly, small-amplitude, coiled semi-coiled, and straight. The occurrence of subclones with flagellar shape different from that of the parental clone, probably because of mutation, has sometimes been noticed (Leifson & Palen, 1955). The present investigation has shown that a curly flagellar strain of *Salmonella abortusequi* can mutate at least to five different flagellar types, namely, heteromorphous, small-amplitude, para-curly, short and hooked-curly. The small-amplitude mutant appears to correspond in shape to that described by Leifson (1960) by this name. Para-curly and hooked-curly are to be regarded as subtypes of curly: they have not been differentiated by the foregoing light-microscope study. Among the curly types reported in a previous paper (Iino, 1962*a*), curly-*i* flagella of *Salmonella typhimurium*

belong to the original curly and curly-*a* flagella of *S. abortusequi* to hooked-curly. The curly type observed by Pijper, Nefer & Abraham (1956) and Leifson, Carhart & Fulton (1955) under dark-field or light microscope may correspond to para-curly or hooked-curly in the present report: in all of them the ratios of normal wavelength/curly wavelength were in the range 1·8–2·2 to 1 while in the curly type flagella of SJ 30 it was 2·4–2·5 to 1.

The comparative observation of flagellar shape with the electron microscope and by dark-field microscopy has indicated that the characteristics of the shape of flagella on living organisms are preserved well in samples dried for electron microscopy. Although after being dried the spiral of a flagellum is generally flattened to form a wave with wavelength about 30–50% longer than that of the spiral, the amplitude of the wave is not significantly different in the two preparations. An exceptionally greater value of the amplitude after flattening as compared with that of the flagellar spiral was observed in the para-curly mutant of *Salmonella abortusequi*. This may reflect a physico-chemical character of the flagella of this type. Irregularly shaped flagella of heteromorphous clone observed by electron microscopy may be also due to the tendency to stretch during the preparation of the bacterial samples.

By dark-field microscopy, dimorphism in the spiral of flagellar bundles was observed by Pijper & Abraham (1954) in *Sarcina urea* and *S. agilis*. The dimorphous spirals they reported were normal and curly. Similar type of dimorphism was observed in the present work in clone 2 of *Salmonella abortusequi* in which both small-amplitude and curly flagellar bundles appeared. Further, this dimorphism occurred by transition from small-amplitude to curly in already-formed bundles. The dimorphism in clone 2 was not seen with the electron microscope: the flagella were all of small-amplitude, even in the sample prepared from organisms showing curly bundled flagella by dark-field microscopy.

The characteristic modes of movement of the organisms of each mutant of *Salmonella abortusequi* indicate that the flagellar shape influences the mode of locomotion. Generally speaking, curly type organisms manifested abnormal movement, such as rotation and circulation in broth, and their speed of spreading in semi-solid medium was slower than that of the normal type. The motility behaviour of the small-amplitude type was intermediate of normal and curly. Whether or not the minor differences in flagellar shape are directly reflected in differences of spreading ability among three curly types is uncertain. It may be worth noting that curly type organisms tended to aggregate by the linking of flagella in liquid medium, and the degree of aggregation differed among the different curly types: the higher the degree of aggregation, the lower the ability to spread.

Based on current information, we may summarize the sequential steps of flagellar formation as follows: (1) synthesis of flagellin polypeptide from the component amino acids; (2) folding of the flagellin polypeptide into flagellin monomer; (3) polymerization of flagellin monomer to flagellar fibre. By analogy with other protein-forming systems, the first step is presumed to occur on the active ribosomes, where messenger-RNA transcribed from the structural gene of flagellin determines the whole amino acid sequence of flagellin polypeptide, and immediately following the first step, the second step may occur on or near the site of the first step reaction (Rich, Warner & Goodman, 1963). The third step must proceed at the basal

granule of each flagellum. Theoretically, reactions involved in all of these three steps can contribute to the determination of the specific shape of the flagella finally produced. Genetic analysis of curly mutants in *Salmonella abortusequi* indicates that the genetic change of flagellar shape from normal to curly occurred by one step mutation in the structural gene of flagellin; that is, the genetic information of the flagellar shapes are primarily tendered in the form of amino acid sequence of the component flagellin (Iino, 1962*a*; Enomoto & Iino, 1963). On the other hand, the change of flagellar shape has been known to be caused by certain environmental factors, for example, the change of pH value (Leifson, 1960). Further, it has been observed that flagella reconstituted from normal flagellin monomers are changed to curly flagellar fibres *in vitro* when they are stored in certain environmental conditions (Asakura *et al.* 1964). These observations suggest that flagellin of the same amino acid composition can form more than one type of flagella-shape under different conditions. Preliminary genetic analysis on the five flagellar shape mutants used in the present work has indicated that at least in four, the mutant characters are phase-2 flagella specific and transduced together with the phase-2 H-antigen type. In transduction with the remaining one, hooked-curly, the recombinants of the mutant character were not obtained, probably because they spread slowly on semi-solid medium used for the screening. The most plausible explanation of these results is that the change of flagellar shape occurred by mutation in the structural gene of phase-2 flagellin, consequently by the replacement of an amino acid in flagellin polypeptide leading to the alteration of the conformation of flagellin monomer and of the mode of polymerization. The clear-cut examples of such second site reversion were given in *td* locus of *Escherichia coli* by Helinski & Yanofsky (1962).

In the heteromorphous mutant of *Salmonella abortusequi*, dimorphism of flagellar shape was observed in a clone: curly flagella appeared occasionally among the normal. The strain used is stable as regards the flagellar phase (Iino, 1962*b*). Therefore the involvement of flagellar phase variation in the dimorphism is excluded. When the curly waves appear they are always mixed with the normal in an organism or even in a single flagellum. Consequently, the possibility is also excluded that curly flagella are formed by organisms mutated from the clone. The following two possibilities remain to explain this flagella-shape dimorphism. The first one is that flagellin produced by the heteromorphous organisms is a single kind but can make two kinds of polymer, normal and curly, under the environmental condition provided by the basal granules of flagella in the mutant strain, though the choice of the alternative is greatly biased to the former. The second is that curly flagellin is occasionally produced by the degeneracy of the genetic code responsible for the heteromorphism.

When the flagella were visible by dark-field microscopy, they were always presumed to be bundled (Mitani & Iino, 1965), as a single Salmonella flagellum would scatter insufficient light to make it visible (Stocker, 1956). The difference of curvature of flagellar bundles between dark-field microscopy and electron microscopy was observed in the para-curly mutant. In this type, the difference in the curvature was also present between flagellar bundle and its component flagella as seen with the electron microscope. The curvature of the flagella is more like that of the flagellar bundle observed with dark-field microscopy. This type of flagella-shape variation in a clone indicates that certain types of the already formed flagella transform their

curvature when they are dried and flattened on a collodion membrane for the electron microscope observation. The treatment of drying and flattening may cause tension between the unit flagellum and this may make the flagella stretch.

Flagella of the short mutants of *Salmonella abortusequi* are pronounced by the decrease of both number per organism and length. As regards the interrelation between the type of flagellin and the ability of polymerization, marked differences of the speed of polymerization among the different types of flagellin has been observed *in vitro* (Asakura, Eguchi & Iino, unpublished data). Therefore, there is a possibility that the flagellin of these mutants has exceedingly poor ability of polymerization among the mutant flagellins.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. A plate of semi-solid medium on which the organisms of curly flagellar strain SJ 30 of *Salmonella abortusequi* were brushed and incubated for 2 days at 37°. The organisms of SJ 30 grew only at the site of inoculation, from where flagella-shape mutants appeared as various types of swarms.

Fig. 2. Spreading ability of representative clones of flagella-shape mutants shown by the growth on a plate of semi-solid medium. (*a*) Clone 1, (*b*) clone 3, (*c*) clone 2, (*d*) clone 4, (*e*) clone 7, (*f*) SL 23: normal type, (*g*) SJ 30: parental curly type.

Fig. 3. Normal flagellar bundle of SL 23 in 0·5 % methylcellulose solution observed under dark-field microscope. × 2160.

Fig. 4. Flagellar bundle of clone 1 observed as in Fig. 3.

Fig. 5. Small-amplitude flagellar bundle of clone 2 observed as in Fig. 3.

Fig. 6. Curly flagellar bundle transformed from the small-amplitude in clone 2 observed as in Fig. 3.

Fig. 7. Para-curly flagellar bundle of clone 3, observed as in Fig. 3.

Fig. 8. Flagellar bundle cannot be observed on clone 4 under dark-field microscope. × 2160.

Fig. 9. Hooked-curly flagellar bundle of clone 7, observed as in Fig. 3.

Fig. 10. Flagellar bundle of parental curly strain SJ 30 observed as in Fig. 3. Linking of flagellar bundles is seen.

PLATE 2

Fig. 11. Flagella of wild strain SL 23 observed under electron microscope, after being stained by PTA for 10 min. and shadowed by chromium. × 14,400

Fig. 12. Small-amplitude flagella of clone 2 observed under electron microscope, after being stained by PTA for 10 min. × 14,400.

Fig. 13. Flagella of clone 1 observed as in Fig. 12.

Fig. 14. Short flagella of clone 4 observed as in Fig. 12.

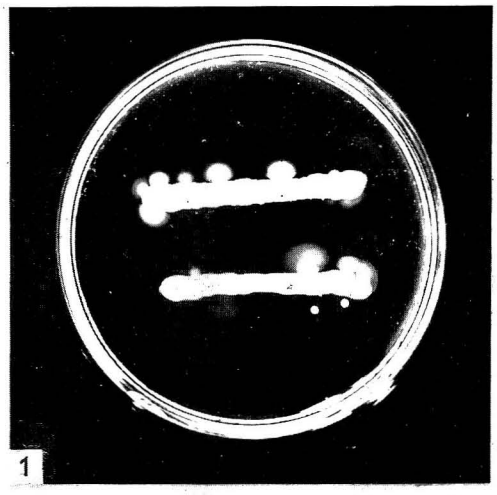
PLATE 3

Fig. 15. Flagellar bundle of clone 3, which has specific wavelength longer than the unit flagellum, observed as in Fig. 12.

Fig. 16. Flagellar bundle and its component flagella of clone 3, observed as in Fig. 12. Notice the difference between the wavelength of bundled flagella and a component flagellum.

Fig. 17. Hooked-curly flagella of clone 7, observed as in Fig. 12.

Fig. 18. Flagella of parental curly strain SJ 30 observed under electron microscope after chromium shadow was given on the unfixed and dried organisms. × 14,400.



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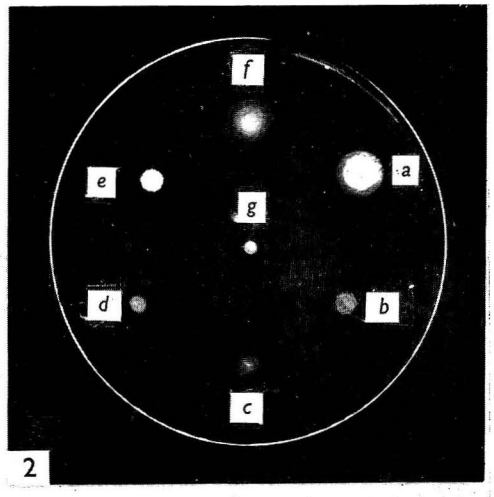

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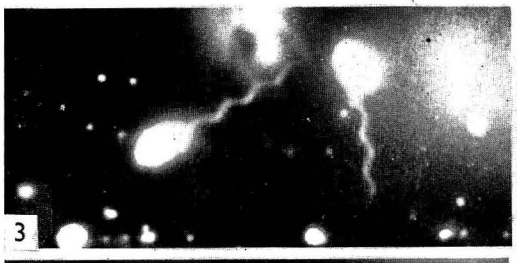

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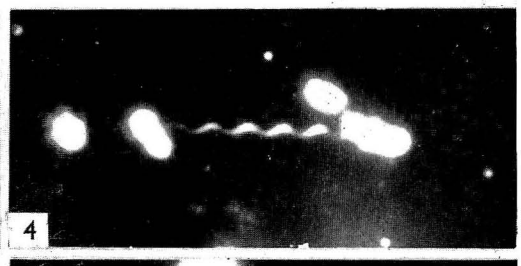

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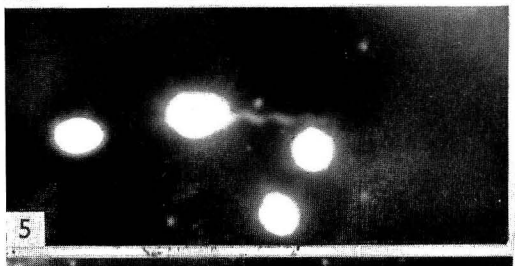

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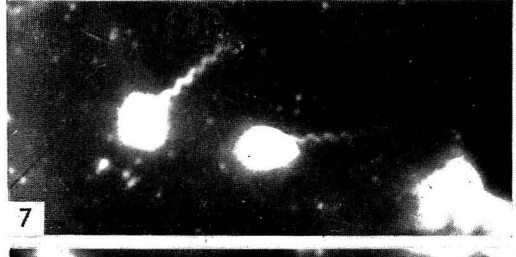

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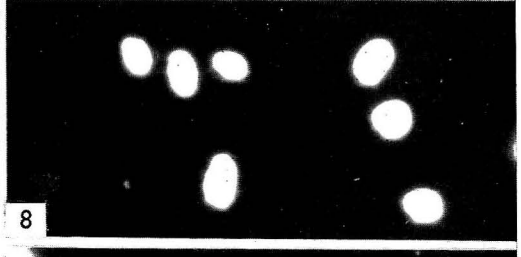

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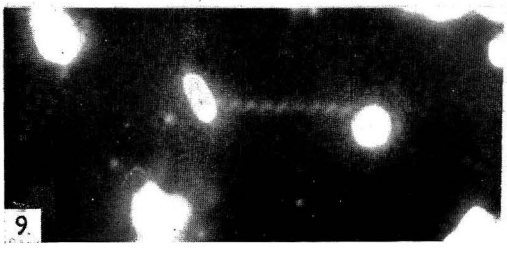

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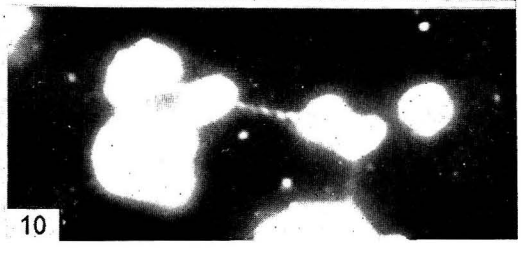

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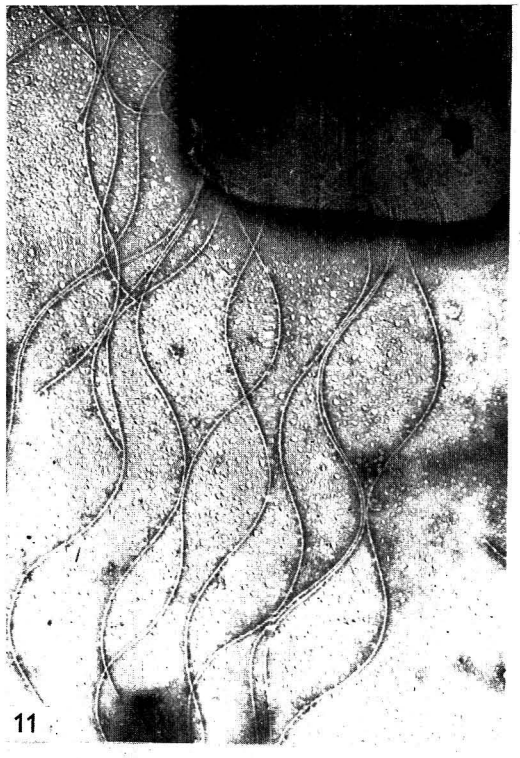
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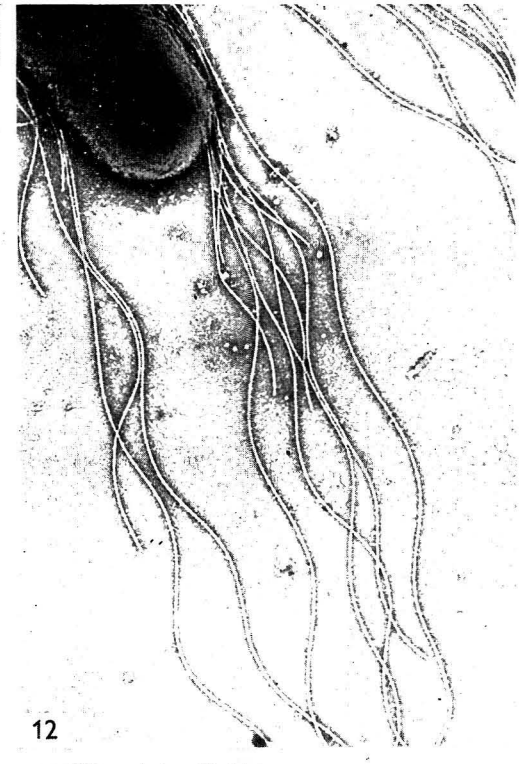
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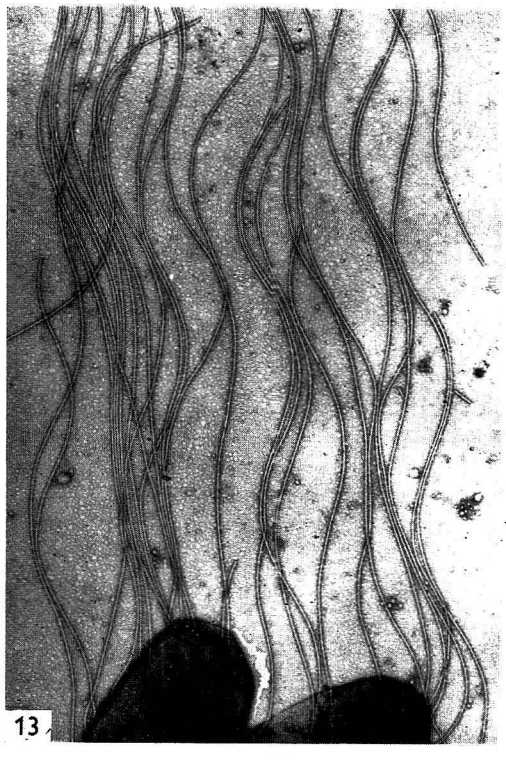
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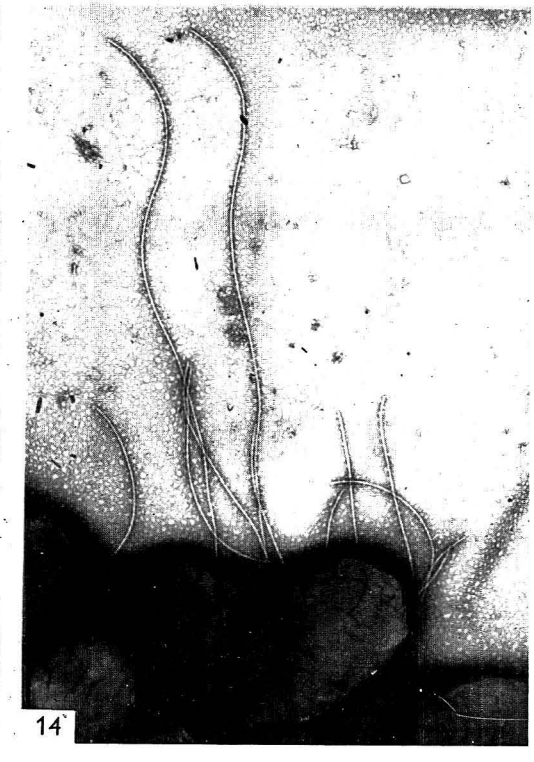
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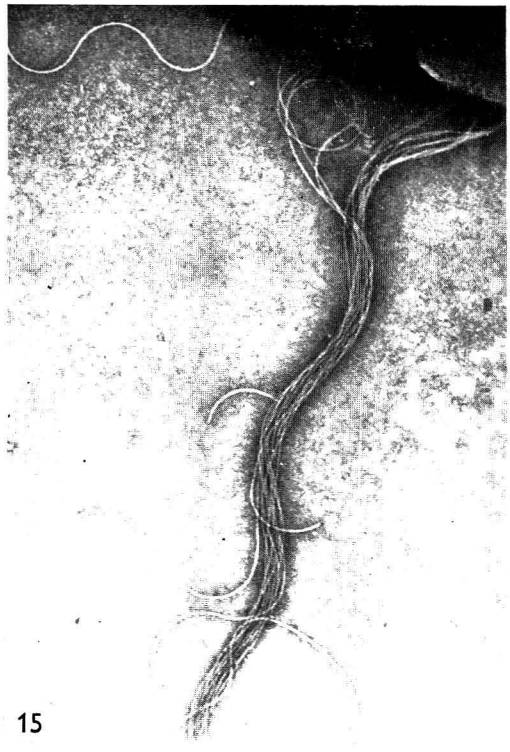

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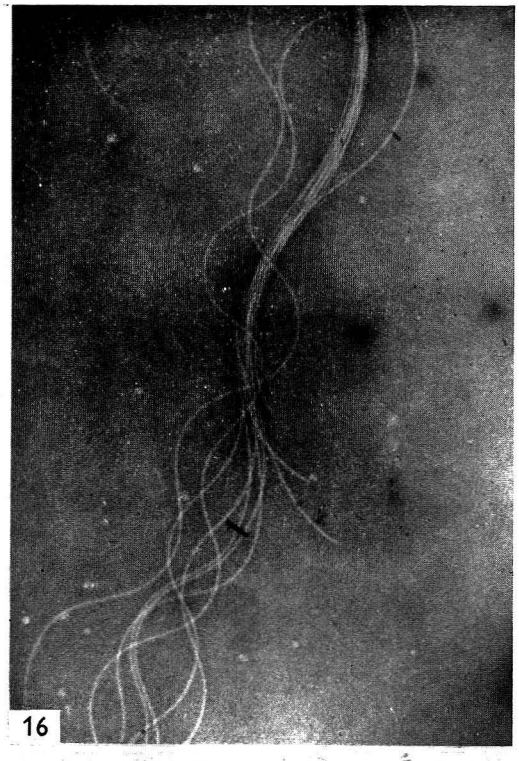

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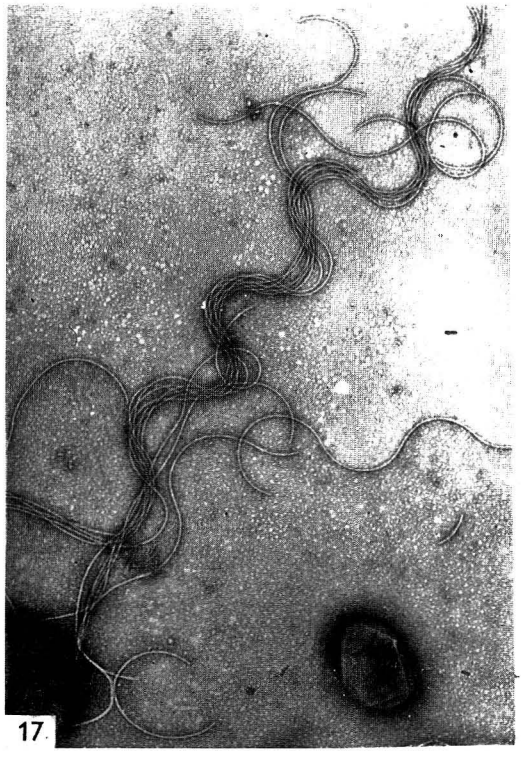

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With 1 plate
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Bacillus macquariensis n.sp., a Psychrotrophic Bacterium from Sub-Antarctic Soil

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SUMMARY

A new species of the genus *Bacillus* was isolated from Macquarie Island soil. The organism is psychrotrophic, producing spores at temperatures down to and including 0°. The maximum temperature for growth is 25°. Apart from its temperature relations, it most closely resembles *B. pulvifaciens*, but differs from that organism in its ability to produce acid from carbohydrates with ammonium salts as sole nitrogen source and in its inability to hydrolyse casein or gelatin, to grow on soybean agar or to grow in 5% NaCl. The name *Bacillus macquariensis* is proposed.

Vegetative organisms stained Gram-negative at all stages of growth. Electron micrographs revealed cell-wall structures typical of Gram-positive bacteria; on the other hand, chemical analyses demonstrated in cell-wall preparations a wide range of amino acids and relatively low amounts of amino sugars, as commonly found in Gram-negative species.

INTRODUCTION

The range of bacteria capable of growth at low temperatures (psychrotrophs; Eddy, 1960) is wide, and includes Gram-positive and Gram-negative rods and cocci. There appear to have been no reports of the isolation of aerobic bacteria sporing at 0°, although Sinclair & Stokes (1964) have reported the isolation of strictly anaerobic psychrotrophic spore-forming bacteria from soil, mud and sewage. This report describes the isolation and properties of a facultatively anaerobic bacterium, capable of growth and sporulation at 0°, from sub-Antarctic soil.

METHODS

Isolation of the organism. Suspensions (10%, w/v) of soil from Macquarie Island (latitude 54° 29′ S.; longitude 158° 58′ E) were prepared in saline and heated for 10 min. at 80° to destroy vegetative forms. Suitable dilutions were plated on brain + heart and nutrient agars, and the plates incubated at 20° and at 1·1°. Colonies were more discrete on nutrient agar, and after 3 weeks at 1·1° were picked from this medium and transferred to nutrient broth. After growth at 20° in this medium each culture was streaked on nutrient agar again and re-isolated.

Physiological tests. Growth in nutrient broth was poor, and for the determination of growth and sporulation at various temperatures isolates were inoculated onto agar slopes of potato infusion plus inorganic salts (%, w/v: $MnSO_4.5H_2O$, 0·002; $MgSO_4.7H_2O$, 0·04; $ZnSO_4$, 0·003; $CuSO_4.5H_2O$, 0·001; $FeSO_4.7H_2O$, 0·001; $CaCl_2$, 0·01; K_2HPO_4, 0·05).

In studying the morphological and growth characteristics of the isolates, the methods and media described by Smith, Gordon & Clark (1946) were used. The incubation temperature for these tests was 20°.

To prepare spore suspensions for the determination of heat resistance, vegetative organisms were removed by incubation with papain (1 mg./ml. of suspension) at 7·5° for 10 days.

Cell-wall analyses

Preparation. Organisms were grown in brain + heart broth with aeration by shaking for 4 days at 20°. No sporulation occurred in this medium. After harvesting and washing twice with saline, organisms were disintegrated by treatment for about 80 min. with ballotini beads (0·1 mm. diam.) in a reciprocating shaker at 1·1°. When heated organisms were used, contaminating cytoplasm and debris from the wall preparation could not all be removed. Hence viable organisms were disintegrated without previous heating. For heated and unheated organisms, the amino sugars present in the supernatant fluid fractions after disintegration and centrifugation were 0·06 and 0·08% dry wt., respectively, thus indicating no significant autolysis of the unheated cell walls during this process. The crude wall preparations were purified by the method of Salton (1964), and freeze-dried when electron micrographs showed that the preparations were free from cytoplasm. Chemical analyses were made on these freeze-dried cell walls.

Nitrogen content was determined on triplicate samples by the method of Johnson (1941) after digestion by the Kjeldahl procedure.

Phosphorus was estimated colorimetrically by the method of Allen (1940).

Lipid content was determined by ether extraction after hydrolysis of the walls with 6N-HCl for 2 hr at 100°.

Amino sugars were estimated by the colorimetric method of Rondle & Morgan (1955) after hydrolysis of the wall preparations in sealed ampoules with 2N-HCl for 2 hr at 100°.

Identification of cell-wall constituents by paper chromatography. Amino acids and amino sugars were identified after two-dimensional paper chromatographic separation (Whatman paper no. 1, pyridine + water; 4 + 1 by vol.; *n*-butanol + glacial acetic acid + water; 6 + 1 + 2, by vol.) of 6N-HCl hydrolysates (24 hr at 100°). To estimate relative molecular proportions of amino acids, chromatograms were dipped in ninhydrin solution (5%, w/v, in acetone + phosphate buffer; 9·5 + 0·5, by vol.), the colours eluted with a mixture of acetone + water (3 + 1, by vol.) and the extinctions measured at 570 mμ.

Electron microscopy. Samples were fixed overnight in 1% osmium tetroxide (or for 2 hr in 0·6% $KMnO_4$), soaked in uranyl acetate for 2 hr, dehydrated by passage through graded concentrations of ethanol up to 100%, and embedded in Araldite. Sections cut with a Porter–Blum microtome fitted with a diamond knife were examined with a Siemens Elmiskop 1 electron microscope.

RESULTS

Three of the four samples of Macquarie Island soil yielded psychrotrophic aerobic spore-forming bacteria. On primary isolation, similar numbers of colonies developed in 3 weeks at 1·1° as in 4 days at 20°, and they represented about 1% of the viable

bacteria present in the soils before the heat treatment. The twelve isolates studied appeared to be the same organism, a slender Gram-negative rod with a subterminal ellipsoidal spore which swelled the sporangium (Pl. 1, fig. 1).

The bacteria grew at 0° within 3 weeks. Cultures incubated at 0° were immersed in a water+ice bath which controlled the temperature to within 0·1°. The optimum temperature for growth was 15–20° and the maximum about 25°; no isolate grew at 30°. On agar slopes, sporulation began after 4 days at 20°, yielding 80–90% of mature spores after 10 days. In aerated broth cultures only 10% of the organisms produced mature spores, although 90% showed early stages of sporulation. At 0°, sporulation began after 4–5 weeks; 5–10% of mature spores were present after 7·5 weeks on potato infusion+salts agar. This low spore yield did not increase on continued incubation. However, incubation at 1·1° for 10 weeks increased spore production to 50%.

The heat resistance of the spores was relatively low. Spores produced at 1·1° were more heat labile ($D_{80°}$ = 12·8 min.) than those formed at 20° ($D_{80°}$ = 17·5 min.). The numbers of colonies formed on agar were the same whether plates were incubated at 20° or 1·1°. This was true with both heated and unheated suspensions.

Characters

The morphological and general properties of the twelve isolates were the same and are summarized as follows:

Morphology. Vegetative rods of average size 0·5 × 4–6 μ with rounded ends, occurring singly. The rods are motile. Gram-negative at all stages of growth. The sporangium is swollen; spindle-shaped to clavate. Average spore size in Gram-stained preparations 1·0–1·5 × 1·5–2 μ, ellipsoidal, subterminal, with remnants of sporangium usually adhering. Spore wall easily stained.

Nutrient agar. After 4 days at 20°, colonies small, discrete, opaque, smooth with translucent, fimbriate edges, 0·5–1·0 mm. diameter.

Nutrient agar slope. After 4 days at 20°, scant, greyish-white growth, smooth, translucent to slightly opaque.

Glucose agar slope. Growth slightly heavier than on nutrient agar.

Nutrient broth. Growth poor, with sedimentation. Vigorous aeration markedly increases growth.

pH value of glucose broth culture: pH 5·4–5·8 after 7 days at 20°.

NaCl broth. Growth in 2% but not in 5% NaCl.

Potato. Growth spreading; thin, glistening, no pigmentation.

Indole. Not produced.

Crystal violet broth. No growth in 1/1,000,000 crystal violet.

Soybean agar slopes. No growth.

Fermentation tests. Acid but no gas (with ammonium salts as nitrogen source) from glucose, xylose, ribose, galactose, lactose, sucrose, maltose, salicin, mannitol.

Metabolism. Aerobe, facultative anaerobe. Optimal growth 15–20°, no growth at 30°. Growth and sporulation at 0°. Growth in glucose broth under anaerobic conditions pH 5·6–5·8.

Voges–Proskauer reaction. Negative.

Nitrate reduction to nitrite. Negative.

Urease. Negative.

Catalase. Positive.

Starch hydrolysis. Negative.

Gelatin hydrolysis. Negative.

Methylene-blue reduction. Negative.

Nutrition. Will not produce spores in brain + heart infusion medium. Spores formed in nutrient broth and agar, potato infusion + salts agar, potato slopes, and glucose + ammonium salts medium. Yield of organisms in all broth media is greatly increased by vigorous aeration.

Electron microscopy

The Gram-negative property of this organism was exhibited in all phases of growth, even before cell division had occurred after germination. However, electron micrographs of thin sections (Pl. 1, fig. 2) showed the boundary layers of the vegetative form to consist of a relatively thick outer wall and a thin inner membrane. These correspond in their dimensions to the structural cell wall and the cytoplasmic membrane of Gram-positive bacteria, and differ markedly from the configuration considered typical of the Gram-negative bacteria, with its outer limiting unit membrane.

The mature spore is shown in thin section in Pl. 1, fig. 3. Its structures include the normal laminated inner coat and dense outer coat, the latter exhibiting a pronounced ridge structure reminiscent of *Bacillus polymyxa* (Holbert, 1960). The mature spore usually remains within the sporangium.

Chemical analysis of cell walls

In view of the apparent conflict between data obtained from the Gram staining reaction and from electron micrographs, chemical analyses were made on preparations of the cell walls of vegetative forms. The results, given in Table 1, are the means of three determinations on each of three isolates of the organism. The appearance of cell walls prepared for analysis is shown in the electron micrograph of Pl. 1, fig. 4. The amino acids and amino sugars identified in the acid hydrolysate, and their relative molecular proportions, were: alanine (5), lysine (5), proline (4), glutamic acid (4), diaminopimelic acid (4), serine (3), aspartic acid (3), isoleucine/leucine (3), valine (3), tyrosine (2), methionine (2), cysteine (2), glycine (2), threonine (2), glucosamine (1), muramic acid (1).

Table 1. *Analysis of cell-wall preparations of Bacillus macquariensis n.sp.*

	Dry weight (%)
Total nitrogen	13·7
Total phosphorus	0·66
Total amino sugars	4·68
Total lipids	6·23

DISCUSSION

Bergey's Manual (1957) divides the genus *Bacillus* into three morphological groups. The new species *Bacillus macquariensis* produces a terminal ellipsoidal spore which swells the sporangium, and therefore belongs to the morphological group 2.

Comparison with the named species within this group shows that the new isolate most closely resembles *B. pulvifaciens*. However, *B. macquariensis* can be distinguished from *B. pulvifaciens* by a number of characters, of which the following are among the more important.

(i) While the optimum temperature for growth of *Bacillus pulvifaciens* is 37° (with poor growth at 28°), the organism now described has a temperature range of 0–25°, the optimum for growth being 15–20°. Its ability to grow and to produce spores at low temperatures distinguishes it not only from *B. pulvifaciens* but from all named species in the genus.

(ii) The vegetative forms of *Bacillus macquariensis* are Gram-negative at all stages. Growth on nutrient agar is scant, smooth, and greyish white. Addition of glucose to this medium increases growth slightly. Growth in nutrient broth is also very scant, but is greatly increased by vigorous aeration. Availability of oxygen may also be important in sporulation, as the percentage of mature spores formed on agar slope cultures exceeded that produced in liquid cultures, even when the latter were aerated vigorously.

(iii) The new species, unlike *Bacillus pulvifaciens*, can utilize ammonium salts as sole nitrogen source, will not grow on soybean slopes, will not hydrolyse gelatin or casein, nor grow in presence of 5% NaCl.

It is of interest that this organism, Gram-negative under all conditions and very sensitive to crystal violet, possesses a cell wall and membrane structure usually associated with Gram-positive bacteria. Cell-wall preparations, which appeared in electron micrographs to be virtually free from cytoplasmic membrane, had a chemical composition which was not, from the tabulated summaries of Salton (1963), completely typical of either Gram-positive or Gram-negative bacteria. However, the low mucopeptide content (as shown by the amino sugar value) and the wide range of amino acids present, correspond to analyses of bacteria which are Gram-negative.

The name *Bacillus macquariensis* is proposed for this new species, and is derived from the location of soils from which it was isolated. Three isolates have been deposited at the National Collection of Type Cultures, the type strain being NCTC 10419.

We are indebted to Dr F. Jacka, of the Antarctic Division of the Department of External Affairs, for the Macquarie Island soil samples; to Mr P. R. Maguire for the light micrographs; and to Dr W. J. Scott for his interest and advice.

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EXPLANATION OF PLATE

Fig. 1. Photomicrograph of a Gram-stained film of *Bacillus macquariensis* n.sp.; ×2000.

Fig. 2. Electron micrograph of a vegetative form of *Bacillus macquariensis* n.sp.; ×60,000.

Fig. 3. Electron micrograph of a mature spore of *Bacillus macquariensis* n.sp.; ×110,000.

Fig. 4. Electron micrograph of a vegetative cell-wall preparation of vegetative *Bacillus macquariensis* used for chemical analysis; ×30,000.

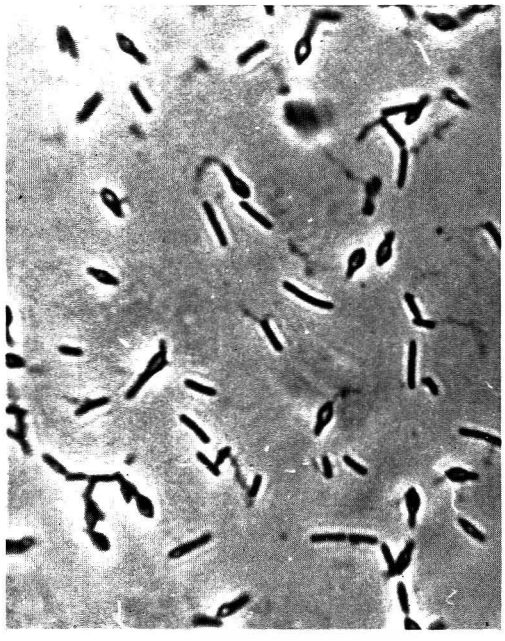

Fig. 1

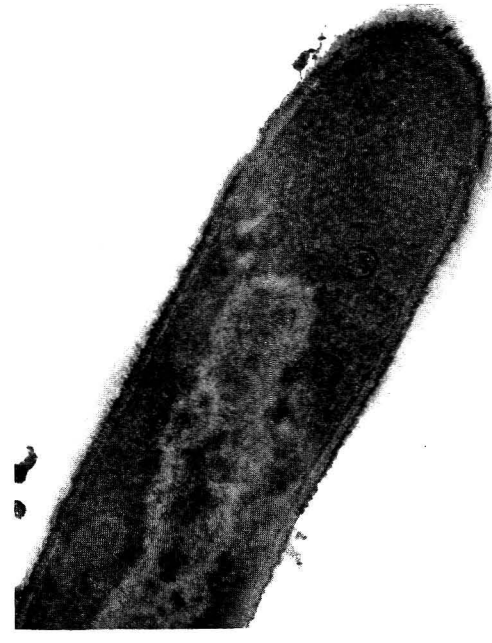

Fig. 2

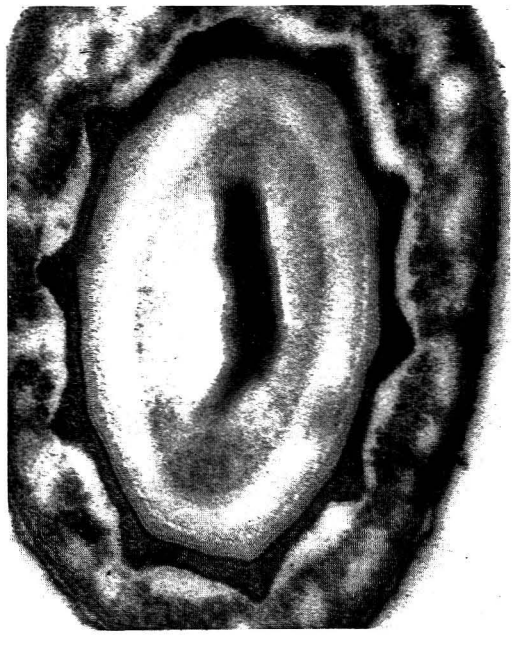

Fig. 3

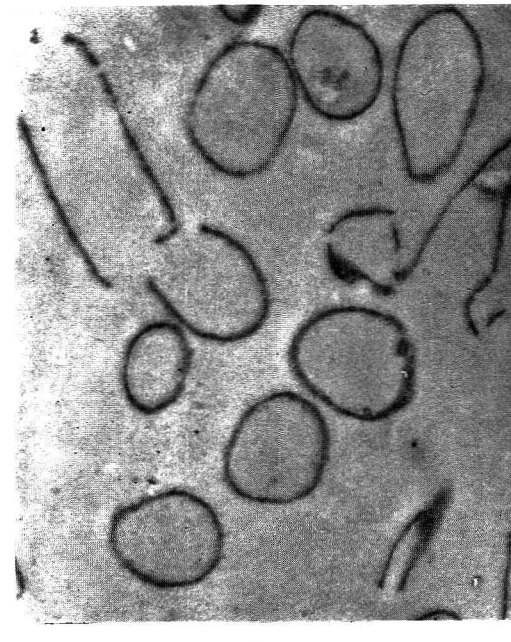

Fig. 4

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Persistent Non-Cytocidal Infection of BHK21 Cells by Human Parainfluenza Type 2 Virus

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SUMMARY

The BHK21–C13 line of hamster fibroblasts was infected with human parainfluenza virus type 2. The infection persisted through 40 subcultures without affecting the plating efficency of the cells and without degeneration of the monolayers. Although all cells contained fluorescent-staining virus antigen and most became specifically haemadsorbing and contained inclusion bodies, the titre of virus in 4-day-old cultures was only equivalent to 0·0001 plaque-forming unit/cell. Infection of cells continued when clones were grown in anti-parainfluenzal serum. There was only very slight interference to infection with other viruses and superinfection with parainfluenza type 2 virus caused degeneration of the culture. Persistent infection of BHK21 cells with human type 2 parainfluenza virus produced no loss of contact inhibition and no ability to grow in semi-solid media, changes which are characteristic of BHK21 cells when transformed by polyoma virus or Rous sarcoma virus.

INTRODUCTION

There are now many descriptions of persistent infection by viruses in continuous cultures of animal cells. In many of these the balance between virus and host is controlled by the presence of specific antibody or of interferon in the system (Ginsberg, 1958; Isaacs, 1963), which limit infection to a minority of the cells in the culture. Continuously infected cultures have recently been described in which antibody is unnecessary and interferon not predominant in maintaining equilibrium, and the infecting viruses are frequently myxoviruses or other RNA-viruses which resemble myxoviruses (Rustigian, 1962; Walker & Hinze, 1962*a*, *b*). The host cells are usually epithelial. Exceptions to this type of host are found in chronic infection of BHK21 cells with rabies virus (Fernandes, Wiktor & Koprowski, 1964) and in a fluctuating balance between polyoma virus, a DNA-virus, and mouse L cells (Henle, Hinze & Henle, 1963; Hare & Morgan, 1964).

The present account of persistent infection of BHK21 cells is of interest because the cells are a line of diploid fibroblasts which, when transformed by polyoma virus (Macpherson & Stoker, 1962) or Rous sarcoma virus (Macpherson, 1965), show a characteristic loss of contact inhibition and a new ability to grow in semi-solid media, but which are largely unaffected in cultural behaviour when all cells in the culture are carrying parainfluenza virus type 2.

METHODS

Media. Growth medium (ETC) is a modified Eagle medium containing 10% (v/v) calf serum and 10% (v/v) tryptose phosphate broth. Occasionally the species or concentration of serum was changed, as mentioned in the text. Overlay medium consisted of 0·9% (w/v) Difco Bacto agar with 5% (v/v) inactivated calf serum in Eagle medium.

Cells. The continuous line of hamster kidney cells BHK21–C13 (Stoker & Macpherson, 1964) was grown in ETC at 37° in 4% (v/v) CO_2 in air. Subcultures of infected and non-infected cells were made every 3 or 4 days by inoculating about 2×10^6 cells into 10 ml. of ETC. When first used, the cells had been subcultured for at least 200 generations and will be referred to as C13 cells. BHK21–C13-404 and C13-405 were cell batches 3 or 4 passages distant from the stock clone C13 and recovered after preservation at −80° in ETC with 5% (v/v) glycerol. They will be designated C13-404 and C13-405 cells.

A line of polyoma-transformed virus-free cells—BHK21/13/PyY (Stoker & Macpherson, 1964)—was also used. Secondary cultures of rhesus-monkey kidney were used for preparing stocks of parainfluenza virus and a line of HEp2 cells was used for titrations of virus infectivity. The cells were grown in ETC and maintained during virus growth by replacing the calf serum with 2% (v/v) horse serum after inoculation of virus. Cells were removed from glass or plastic surfaces with 0·05% (w/v) bovine trypsin in 0·02% (w/v) EDTA.

Virus. Stocks were prepared from a strain of parainfluenza virus type 2, grown in rhesus-monkey kidney cells for 5 successive passages, and were stored at −60°. Titres varied from 10^6 to 10^7 plaque-forming units (p.f.u.)/ml. by the haemadsorption method given below. Viruses other than parainfluenza type 2 were assayed on BHK21 cells as described by Gharpure (1965). Assay of parainfluenza virus-infected C13 cells by immunofluorescence followed previous methods for polyoma virus infection (Fraser & Gharpure, 1962).

Titration. Parainfluenza virus was titrated on C13 monolayers by haemadsorption. Washed monolayers were inoculated with 0·1 ml. of virus dilution which was adsorbed for 30 min. at 37°, when the inoculum was removed and 5 ml. of overlay medium applied. After 3 days of incubation at 37° the agar was peeled off, the monolayer washed and covered with a 1% (v/v) suspension of guinea-pig red cells in saline. These were allowed to adhere for 20 min. at room temperature, then washed off gently and the monolayers examined with a low-power objective. Plaques were easily seen, very variable in size, irregular in shape, often branching to follow the lie of the cells, and gave reproducible counts in proportion to each dilution of inoculum. Overlapping of plaques caused a decrease in the apparent count at numbers above 150 per plate. Titres are given in p.f.u./ml.

Antiserum. Rabbit immune sera were inactivated at 56° for 30 min. and stored at −20°. Serum at 1/25 dilution neutralized 10^4 p.f.u. when equal volumes of serum and virus suspension were allowed to react for 30 min. at room temperature.

Fluorescent staining. The parainfluenza 2 antiserum was used in the indirect method of immunofluorescent staining with anti-rabbit goat globulin conjugated with fluorescein isothiocyanate as second layer. Infected cells, grown on coverslips, were fixed at suitable times in acetone for 10 min. at room temperature. Uninfected

cells and sera immune to influenza and polyoma viruses were used in appropriate controls of the specific staining. Photographs were taken in a Reichert fluorescence microscope on Ektachrome high-speed film.

Growth rates. Infected and uninfected cells were plated on 60 mm. dishes over circular coverslips under ETC. Cell counts were made from a fixed number of randomly chosen fields on coverslips removed from each culture at the 1st, 2nd and 3rd days of incubation and stained with Giemsa's stain.

Plating efficiency of cells. One ml. of cell suspension containing 300 or 100 cells was plated in each of 6 plates over feeder layers of X-irradiated mouse-embryo cells. Two plates were used for haemadsorption counts by the method given below. One week later colonies on the other four plates were fixed in 10% (v/v) formol saline, stained with Leishman's stain and counted.

Agar culture. Suspension culture for the selection of virus-transformed cells was described by Macpherson & Montagnier (1964); this technique was used here.

Examination of monolayers by haemadsorption. The density and firmness of haemadsorption on cultures of parainfluenza-infected cells varied with the cultures, in none of which it was possible to count individual haemadsorbing cells in the confluent monolayer. A modification was therefore made of the original method of Vogel & Shelokov (1957).

Cells from a monolayer were well dispersed in ETC, 5×10^4 cells in 60 mm. Petri dishes, and because freshly trypsinized C13 PF2 cells (see Results), unlike influenza-infected cells, are always non-haemadsorbing they were examined after overnight culture by washing the cells with normal saline, adding 2·0 ml. 0·25% (v/v) guinea-pig red cells and adsorbing these for 20 min. at room temperature. The Petri dish was then filled with normal saline containing 1% (v/v) inactivated calf serum, covered with a 90 mm. lid from a glass Petri dish and inverted. Five ml. of serum saline was then added to the lid. After 1 hr, when red cells had fallen into the lid, the dish was moved aside from the obscuring layer of red cells and the number of haemadsorbing and non-haemadsorbing C13 cells counted with low and high-power objectives. The number of cells counted was 200–400 on each plate, except when most cells seemed to be positive, when fewer sufficed. Cells to which two or more erythrocytes adhered were scored as positive. Cells tended to round up in serum saline, but did not come off the glass for several hours. Data on the validity of the method appear under Results. The specificity of the reaction was tested from time to time with a 1/5 dilution of specific antiserum which neutralized all haemadsorption. A 1/25 dilution of antiserum decreased the count of positive cells and the number of adherent red cells on each positive cell. Antisera to parainfluenza types 1 and 3 and various anti-influenzal sera had no neutralizing effect. Occasional preparations of red cells adhered non-specifically to glass or to plastic, but non-specific adhesion tended to disappear when the tests were left inverted for some hours or the plates tapped firmly from time to time. When non-specific adhesion persisted, tests were made with fresh red cells.

RESULTS

When 0·05 ml. of parainfluenza type 2 virus, titre 2×10^6 p.f.u./ml. was inoculated into about 5×10^6 C13 cells, the monolayer showed no cytopathic changes 2 days after infection, but testing with guinea-pig red cells gave confluent, firm

haemadsorption. Subcultures were made from these monolayers by trypsinizing the cells and inoculating about 2×10^6 washed cells into 10 ml. of ETC. Monolayers again grew that gave confluent haemadsorption at the third day of incubation and showed no cytopathic effect. Such cells were termed C13–PF2 cells and serial passages from the 4th pass onwards were examined in greater detail. They will be referred to as C13–PF2 cells except where exact designation is necessary.

Two further infected cultures were made for comparison, C13-404–PF2, at a virus-to-cell ratio of 1 and C13-405–PF2 at a ratio of 5. There was, this time, visible cytopathic change in both starting cultures, greater with the higher virus dose which delayed the formation of a complete monolayer for 6 days. No experiments were made to establish the factors which initially produced this non-cytopathic haemadsorption.

C13-404–PF2 and C13-405–PF2 thereafter behaved similarly and only the observations made on C13–PF2 and C13-404–PF2 are given in the paper.

Table 1. *Proportion of haemadsorbing cells and the titre of released parainfluenza type 2 virus in successive cultures of C13–PF2 cells*

	Series I, C13–PF2		Series II, C13-404–PF2	
Passage no.	% haem-adsorbing cells	Virus titre p.f.u./ml.	% haem-adsorbing cells	Virus titre p.f.u./ml.
1	—	—	92·0	$7{\cdot}5 \times 10^6$
2	—	—	93·4	$2{\cdot}75 \times 10^5$
3	95·6	$1{\cdot}62 \times 10^5$	—	—
4	84·3	—	18·0	—
5	85·4	—	31·1	—
6	81·6	—	32·4	—
7	66·8	—	—	—
11	13·8	$1{\cdot}68 \times 10^3$	—	—
12	31·6	9×10^2	—	—
13	67·0	—	—	—
15	20·0	—	—	—
17	41·8	—	—	—
18	39·6	—	—	—
19	53·8	—	—	—
21	79·6	1×10^2	—	—
22	79·4	$1{\cdot}2 \times 10^2$	—	—
23	91·3	$0{\cdot}6 \times 10^2$	—	—
31	99·0	$1{\cdot}25 \times 10^2$	—	—
32	99·9	$1{\cdot}75 \times 10^2$	—	—
33	—	—	76·0	—
34	98·5	$0{\cdot}1 \times 10^2$	40·0	—
36	—	—	30·0	$0{\cdot}4 \times 10^2$
37	94·5	—	60·0	—
38	—	—	76·5	$0{\cdot}35 \times 10^2$
39	—	—	81·9	$0{\cdot}5 \times 10^2$

Persistence of infection during continuous propagation of cells

All three infected cultures could be propagated as described in Methods without distinguishable alterations of the macroscopic or microscopic appearances of unfixed cultures from uninfected C13 cells. Infectious parainfluenza virus was recovered at intervals throughout the passages shown in Table 1 and the widespread nature of

the infection is shown by the fact that in all cultures haemadsorbing cells were present and usually predominated.

Virus yields. The titre of infectious virus in supernatant fluids of C13–PF2 cells and C13-404–PF2 cells, harvested at the time of subculture, fell rapidly from about 10^6 p.f.u./ml. in the beginning of the series to less than 100 p.f.u./ml. at the end. Since the number of cells in each culture was $10–16 \times 10^6$ in 10 ml. of fluid, this represented a ratio of about 1·0 p.f.u./cell at the beginning of the chronic infection and 0·0001 p.f.u./cell at the end. Similar titres were found when the virus was titrated on BHK21 cells, HEp2 cells and monkey kidney cells. No higher yields were found at intermediate times between subculturing and harvesting the cells, nor was the amount increased by freezing and thawing the cultures. Yields of haemagglutinin were too low, varying from less than 1 up to 8 agglutinating doses per ml., against 1% guinea-pig red cells, to correlate yield of haemagglutinin with the infectious titre of the virus or with the amount of haemadsorption.

The haemadsorption count and its significance. There was a tendency in both series for the proportion of haemadsorbing cells to diminish towards the middle, and to increase again towards the end of the series. There was no concomitant decrease and increase in the titre of infectious virus. Continued passage resulted in a less intense haemadsorption even when the proportion of haemadsorbing cells remained high. Fewer red cells stuck to each cell and they were more easily washed off, but strongly and weakly adsorbing C13–PF2 cells existed in the same culture. Different degrees of haemadsorption are illustrated in Pl. 1, figs. 1–3.

As explained in Methods, it was necessary to decide whether cells which had been removed from a monolayer and which became haemadsorbing during overnight culture represented cells already producing virus or cells invaded by virus during or following plating. The low p.f.u./cell ratio in the late passages made it virtually impossible for infection during plating to account for the large number of cells showing specific haemadsorption. Secondary infection was disproved by growing cells under an agar overlay. Equal numbers of cells from various passages of C13–PF2 cells were plated in ETC and one half the number of plates overlaid with nutrient agar after 5 hr incubation at 37°, when cells had stuck to the glass. After incubation for a further 18 hr, agar was removed and cells washed and tested by the inversion method of haemadsorption. There was neither significant difference between the proportion of haemadsorbing cells in ETC and the proportion under agar nor any great loss of cells on removing the overlay (Table 2).

Subsidiary tests showed in addition that harvesting and plating the C13–PF2 cells in 1/25 antiserum did not decrease the proportion of haemadsorbing cells. They also became positive after plating even when nutrient agar was replaced by serum saline or when the incubation temperature was decreased to 20°, conditions under which newly infected C13 cells did not produce specific haemadsorption. Haemadsorption as tested by the method used thus indicated a property of the cells that had been removed temporarily during subculture.

Studies were made to see whether haemadsorbing cultures contained any other evidence of virus infection.

Inclusion bodies. Staining of C13–PF2 cells with dilute Giemsa's stain for 20 hr showed many inclusion bodies that were not present in uninfected C13 cells. They varied from sharply defined, densely eosinophilic, oval bodies in the cytoplasm, to

irregular patches of faintly staining material (Pl. 1, figs. 6, 8). The number of cells containing such bodies was greater in the early passages and roughly related to the intensity of haemadsorption. No nuclear inclusions were seen. A small proportion of multinucleate cells was usually present.

Immunofluorescent staining. Plate 1, fig. 4 shows cytoplasmic masses of brightly staining specific antigen which was present as loose aggregates and also as brilliant oval bodies. These corresponded exactly to irregular eosinophilic masses (Pl. 1, figs. 5, 6) or to the oval inclusions (Pl. 1, figs. 7, 8). The amount of specifically stained material was roughly proportional to the amount of haemadsorption, but no cells which were non-haemadsorbing were quite free of virus antigen.

Table 2. *Comparison of the proportion of hcemadsorbing cells in C13–PF2 cells, when plated in growth medium and under agar*

		Growth medium			Agar		
C13–PF2 passage no.	No. cells plated	No. cells counted	% haem-adsorbing cells	Average no. cells per field	No. cells counted	% haem-adsorbing cells	Average no. cells per field
7	10^5	212	67·4	—	230	65·6	—
9	10^5	169	19·8	—	590	22·0	—
18	10^4	245	35·1	9·07	204	41·6	7·9
	10^5	574	37·4	114·3	496	39·6	99·2

— = not done.

Infectious centres. The number of virus-yielding cells in a culture can normally be estimated from the proportion of infectious centres formed on plating a known number of cells. Four different subcultures of C13–PF2 cells and two of C13 cells, freshly infected at a ratio of about 3 p.f.u./cell, were tested by plating up to 10^4 cells on monolayers of BHK21 or of monkey kidney cells. The proportion of infectious centres was always less than 1% of the cells plated. It was not therefore possible to decide how many C13–PF2 cells produced infectious virus.

Culture in antiserum. Cultures were also made in specific antiserum. Six successive passages, each of 2×10^6 cells, made in 1/5 and 1/10 rabbit immune antiserum did not reduce the proportion of haemadsorbing cells present in the final culture when the serum was removed.

The effect of cloning C13–PF2 cells

Continuous cultivation of mass cultures of C13–PF2 cells was similar to the passage of uninfected cells. Since each cell was infected, in that all contained virus antigen and most displayed specific haemadsorption when tested, it was possible to study persistent infection arising from single cells.

Plating efficiency. The proportion of viable cells in C13 and C13–PF2 cultures was studied by comparing the number of colonies appearing from a given inoculum of each type of cell (see Methods). The plating efficiency of the parainfluenza-infected cells is equal to that of uninfected cells; and although virus antigen must be present at the surface of haemadsorbing cells, plating efficiency was not diminished in virus-specific antiserum (Table 3).

Haemadsorption on single colonies. Cells were plated as in the plating efficiency

test, but no feeder layers were used. An inoculum sufficient to provide 3–30 colonies/plate was used, and at the end of the incubation period these were not fixed but examined by haemadsorption. They could be divided into haemadsorbing and non-haemadsorbing colonies. Some colonies showed only slight haemadsorption, a few hundred red cells being scattered over the surface of the colony, others showed dense, confluent haemadsorption through which the C13 cells could not be seen. The presence of non-haemadsorbing colonies at the end of incubation for 7 days and the fact that the proportion of haemadsorbing colonies roughly equalled the proportion of haemadsorbing cells at the start of the experiment (Table 4), suggests that spread of virus from colony to colony was infrequent.

Table 3. *Plating efficiency of C13–PF2 cells*

A. In ETC, C13–PF2 cells compared with C13 cells

	Number of colonies from 300 cells/plate				
	Plate				
	1	2	3	4	Average
C13 cells	134	165	133	129	140
C13–PF2 cells	127	139	141	134	135

B. C13-404-PF2 and C13–PF2 cells in ETC and in ETC plus anti-parainfluenza serum 1:10

		Number of colonies from 300 cells/plate			
		Plate			
Series and passage no.	Medium	1	2	3	Average
C13-404–PF2, 4th pass	ETC	174	163	158	165
	ETC + antiserum	181	178	159	173
C13–PF2, 30th pass	ETC	191	174	190	185
	ETC + antiserum	185	205	193	194

Table 4. *Comparison of the proportion of haemadsorbing C13–PF2 cells, after 24 hr incubation, with the proportion of haemadsorbing colonies, from the same culture, after 7 days incubation*

C13–PF2 passage no.	% haem-adsorbing cells at 24 hr	No. cells/plate	No. colonies counted on all plates	% haem-adsorbing
22	78	300	90	64·4
		100	44	65·9
34	98·5	300	54	100
		100	16	100

Subculture of single colonies. Cells negative to haemadsorption have been shown above to contain specific virus antigen. It is probably for this reason, and not because of spread of infectious virus from colony to colony, that non-haemadsorbing colonies have always given haemadsorption-positive cells when subcultured to make monolayers. Table 5 gives results of one series in which the progeny of all colonies was haemadsorbing when tested in the usual way for the proportion of haemad-

sorbing cells. It will be seen from Table 5 that the intensity of haemadsorption was less in subcultures derived from non-haemadsorbing colonies. No attempt was made to select completely uninfected cells by this method.

Single-cell cultures. The technique of picking single colonies could not exclude the chance of secondary infection of the colony during sampling or in the haemadsorption test. The passage of infection from parent cell to progeny was confirmed by culturing single C13–PF2 cells in anti-parainfluenza serum. Of 12 clones grown in droplets, 6 were cultured in ETC and 6 in 1/25 antiserum. All droplet cultures were grown into monolayers in antiserum and all gave haemadsorbing cells when the serum was removed and the cells tested by overnight culture. Six clones from the single-cell cultures in antibody were recloned in antiserum with the same haemadsorption result as before and two of these were passed a third time in single cell culture. These were haemadsorption-negative at the first pass, but became positive on further culture. Thus it was certain that infection could be passed intracellularly to daughter cells, but the question of eventual cure of the culture from parainfluenzal infection was not settled.

Table 5. *Haemadsorption in monolayers derived from single colony cultures of C13–PF2 cells*

Origin	1st passage, single colonies	2nd passage, monolayer from each colony
A. A colony with confluent haemadsorption	5 haemadsorbing	→ 5 haemadsorbing*
	6 non-haemadsorbing	→ 6 haemadsorbing†
B. A colony with moderate haemadsorption	4 haemadsorbing	→ 4 haemadsorbing*
	2 non-haemadsorbing	→ 2 haemadsorbing†

* Haemadsorption equal in density to colony A or colony B.
† Haemadsorption 80% less dense than colony A or colony B.

Serial single cell cultures were also made in stronger antiserum, 1/9 in ETC, but cultures died out at or before the 4th single-cell subculture. Whether this was an effect of the anti-parainfluenzal globulin on the surface of the cell or whether the cultural conditions favoured the eventual lytic cycle of the virus is not known. No attempts were made to recover infectious virus from single cells because of the low yield of virus (0·0001 p.f.u./cell) in monolayer cultures.

Cultural characteristics of C13–PF2 cells

Since no obvious cytopathic effect had been seen during passage of mass cultures of C13–PF2 cells, the growth rate and plating efficiency in fluid medium and agar were tested, as described under methods.

Growth rate. No significant difference was detected between C13 cells and C13–PF2 cells (Table 6).

Growth in agar. In 3 out of 4 experiments using the agar technique, it was found that C13–PF2 cells, like C13 cells, did not proliferate when colony formation in agar was attempted (Table 7). In the same tests polyoma-transformed C13 cells produced too many colonies to be counted.

Interference and superinfection. Interference with plaque formation by other viruses is shown in Table 8, which compares the number of foci on C13–PF2 monolayers with the number on monolayers of C13 cells. There was a significant, but slight, interference with encephalomyocarditis virus, with the NWS strain of influenza A and with polyoma virus but not with vaccinia. Decrease of plaque diameter on C13–PF2 cells was also a sign of interference. The mean diameter of 20 plaques of EMC virus on C13 cells was 9·6 mm. (range 9·0–11·0) and on C13–PF2 cells the mean was 5·5 mm. (range 4·0–6·5).

Table 6. *Growth rate of C13–PF2 cells compared with that of C13 cells*

		Average no. of cells in 10 fields at:			Multiplication factor at
		24 hr	48 hr	72 hr	72 hr
C13–PF2	1st slip	43·8	169·6	331·5	—
	2nd slip	37·6	161·2	346·5	—
	Average	40·07	165·4	339	×8
C13	1st slip	40·2	154	414	—
	2nd slip	54·6	139·4	364	—
	Average	47·4	147	389	×8

Table 7. *Comparative growth in agar of C13–PF2 cells, C13 cells and polyoma-transformed BHK21 cells (Py/Y)*

	C13–PF2		C13-404–PF2	
Expt. no.	1	2	3	4
Inoculum No. cells/plate	2×10^4	5×10^4	5×10^4	5×10^4
	No. of colonies/plate (5 plates)			
C13–PF2 or C13-404–PF2 cells	19	0	0·2	0·2
C13 cells	0	0	0·2	0·2
Py/Y cells	570	Very numerous	Very numerous	Very numerous

Table 8. *Number of foci produced by four viruses on monolayers of C13–PF2 cells compared with the number produced by an equal inoculum on C13 cells*

Virus	C13–PF2, monolayer	C13, monolayer	Ratio, % C13-PF2/C13
Encephalomyocarditis	13*	23·5	55 %
NWS, influenza A	134*	325	41 %
Vaccinia	47·5*	57·5	83 %
Polyoma	0·18†	0·45	41 %

* Average number of plaques on 4 plates.
† Percentage of fluorescent-stained nuclei in 40,000 cells.

Plaque titration of parainfluenza virus on C13–PF2 cells was not possible but, if there were any interference to superinfection with the same virus, it was not absolute. The inoculation of 0·2 ml. of undiluted stock parainfluenza type 2 virus caused confluent haemadsorption and destruction of the monolayer 4 days later.

Polyoma-transformed BHK21 cells, free of polyoma virus, were readily infected by parainfluenza type 2 virus and persistent infection followed.

Reaction to altered cultural conditions. Several attempts were made to upset the balance of synthesis between virus and cells. Replacement of ETC with a medium containing only 1 % calf serum resulted in destruction of the cells in 3 or 4 days, but the titre of infectious virus was not increased and the concentration of haemagglutinin was only occasionally increased. Temperatures of incubation were varied from 37° to 32°, 35° and 39·5°. These did not alter the state of C13–PF2 cells or alter the titre of cell-free virus. Monolayers of C13–PF2 cells degenerated before similar monolayers of C13 cells when incubation was prolonged to 6 or 7 days in ETC. There was no increase in the yield of infectious virus but the yield of haemagglutinin rose as high as 16 agglutinating doses per ml.

DISCUSSION

The principal features of this persistent association between parainfluenza type 2 virus and BHK21 cells were the continuous production of antigen, shown by haemadsorption and by fluorescent antibody, the low yield of infectious virus and the unaltered cultural characteristics of the infected C13–PF2 cells. The fall and rise in the proportion of haemadsorbing cells may have been caused by extraneous factors which were not tested, such as the presence of antibody or inhibitors to parainfluenza virus in different batches of calf serum. It may also be a rhythmical alteration similar to changes in the yield of infectious influenza virus that occur on passage of infected cultures (Tyrrell, 1959). This will only be apparent when the infected cell line is propagated further.

The large amount of antigen indicated by haemadsorption and also by immunofluorescent staining is, surprisingly, not accompanied by large yields of virus haemagglutinin, but the rough correlation between haemadsorption and specific immune staining does suggest that haemadsorption is dependent on the amount of antigen being extruded from the cell. When cytoplasmic stores diminished haemadsorption was absent. There was no concentration of virus antigen at the cell margin of C13–PF2 cells as there is with influenza A antigen in the same species of cell (K. B. Fraser, personal observation). The loss of haemadsorbing properties in newly trypsinized cells and their recovery under conditions which did not permit virus synthesis also suggests that surface antigen was replaced from the cytoplasmic virus.

The small quantity of infectious virus in C13–PF2 cultures, which was not due to an attenuated infectivity for C13 cells alone, may well come from a minority of cells which produce large individual yields of virus and fail to divide. The higher yield of virus in the early passages of the series, in conjunction with undiminished plating efficiency of cells, suggests that, initially at least, most cells produced some infectious virus. It is clear that parainfluenza virus-infected cells divided, as was already known to Gresser & Enders (1961), who used non-continuous cell lines; it is not known whether dividing cells synthesized virus. The very poor plating efficiency of infectious centres and the very low yield of released virus per single cell make these questions difficult to decide.

There are many similarities between C13–PF2 cells and other systems in which

persistent infection of the majority of cells in culture has been described. Continuing production of measles antigen by infected HeLa cells with little production of infectious virus (Rustigian, 1962) is very similar to the C13–PF2 system. In chronic infection of human conjunctival cells with mumps virus (Walker & Hinze, 1962*a*) auto-interference was striking, in contrast to C13–PF2 cells; interference with unrelated viruses was negligible. In the mumps system, anti-virus serum decreased the amount of antigen in each cell, but all cells became haemadsorbing when antiserum was removed. (Walker & Hinze, 1962*b*). This again is similar to C13–PF2 cells.

The pathogenicity of mumps virus decreased in chronically infected cells, and rabies virus also has been reported to lose virulence in persistently infected rabbit and hamster cells (Fernandes *et al.* 1964). On the other hand, interference in rabies-infected cultures was not confined to auto-interference or even to viruses of the same presumed group.

Persistent infections by mumps, rabies and parainfluenza viruses show certain common features: no great effect on the cultural characteristics of the cells; a large percentage of infected cells present in the culture; diminished infectivity or virulence of the virus; inaccessibility of the virus to immune serum; the presence of cytoplasmic inclusion bodies and aggregates of virus antigen; rather poor, but variable, resistance to infection by other viruses. The type of association is distinct from steady-state cultures which are maintained by cytolytic infection of a minority population of cells amongst a resistant or protected majority.

The use of diploid BHK21–C13 cells in this study has a bearing on virus transformation not illustrated in previous studies. Alteration of the morphology of the colonies, and acquisition of ability to grow in agar, are manifestations of virus transformation by either polyoma virus (Macpherson & Stoker, 1962) or Rous sarcoma virus (Macpherson, 1965). They are readily demonstrable in BHK21 cells and are usually regarded as a result of close integration of the virus genome with the cell. It is thus of some interest that persistent infection of BHK21 cells with parainfluenza virus is not accompanied by any of the cellular changes that are characteristic of virus transformation. This shows that persistent infection alone is not the cause of virus transformation.

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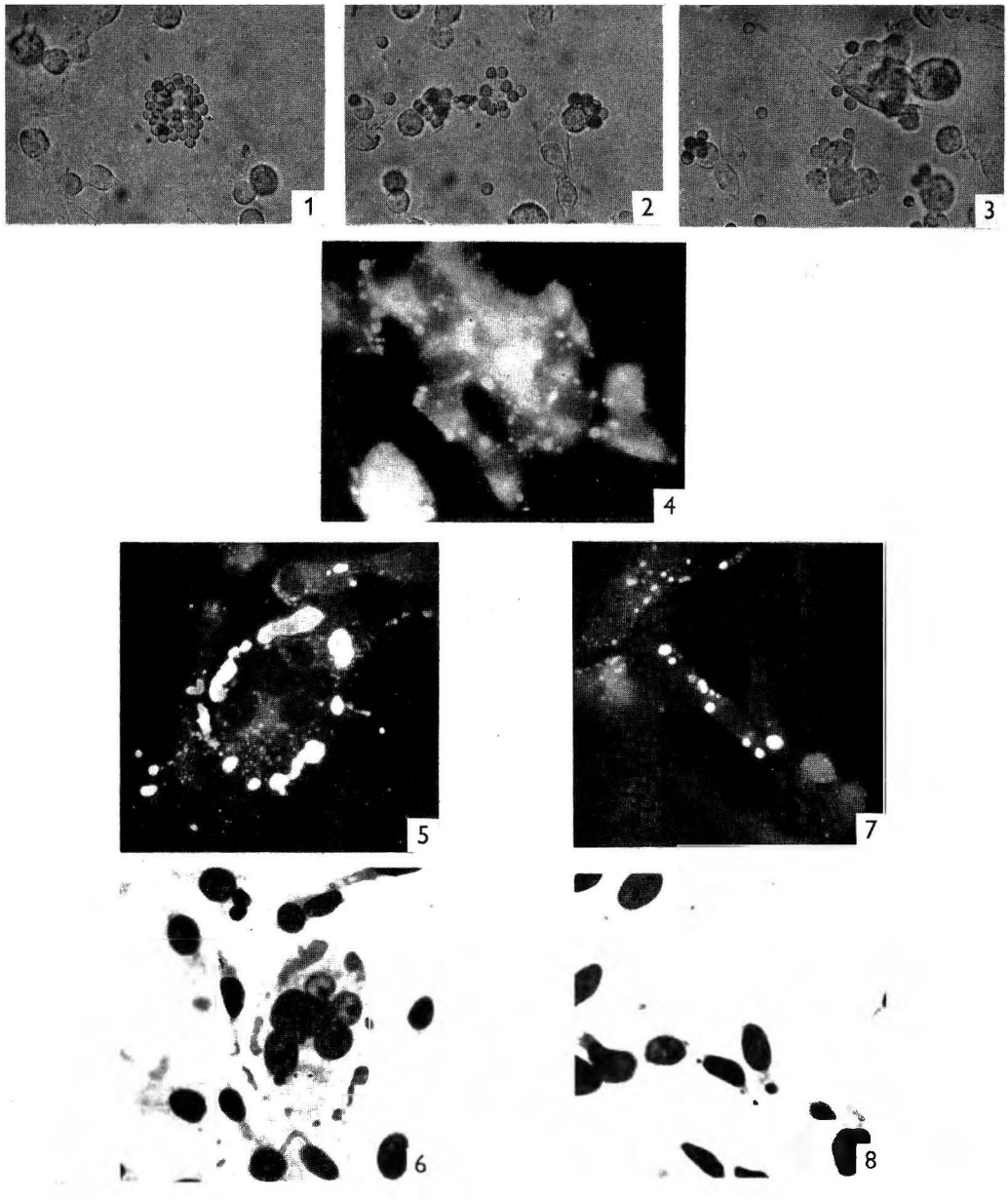
EXPLANATION OF PLATE

Figs. 1–8. Different intensities of specific haemadsorption of guinea-pig erythrocytes in the same culture of C13–PF2 cells. Culture cells rounded by the action of non-buffered serum-saline. Fig. 1. Dense haemadsorption to a single cell; ×480. Fig. 2. Moderate haemadsorption to 3 cells; ×480. Fig. 3. Scanty haemadsorption to 4 cells, one on a lower plane; ×480.

Fig. 4. Immunofluorescent staining of C13–PF2 cells, 85% of which were haemadsorbing. Virus antigen (white) present in all cells as inclusion bodies and as diffuse antigen. ×480.

Figs. 5–8. A comparison of fluorescent-stained virus antigen with inclusions in C13–PF2 cells. Fig. 5. A multinucleate cell showing irregular aggregates and discrete flecks of virus antigen; ×480. Fig. 6. The same cell stained with dilute Giemsa's stain. Note the correspondence of cytoplasmic inclusions with fluorescent-staining of virus antigen. ×480. Fig. 7. Three cells showing circular and oval cytoplasmic aggregates of virus antigen; ×480. Fig. 8. The same cells, stained with dilute Giemsa's stain, to show correspondence of inclusions with fluorescent-staining; ×480.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Immunoelectrophoretic Analysis of Cytoplasmic Proteins of *Neurospora crassa*

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SUMMARY

Immunoelectrophoretic analysis (IEA) of cytoplasmic proteins of *Neurospora crassa* revealed more than 30 antigenic components in detectable concentrations in crude extracts. The IEA patterns varied somewhat with culture conditions and age, and with strain. A one-gene polymorphism was recognized but was not associated with any known biochemical or morphological mutant character. Comparison of the IEA patterns given by proteins of several species of fungi with antisera against *N. crassa* revealed the prominent phyletic affinities among the protein antigens within the class Ascomycetes. The results are discussed with emphasis on the methods and the interpretation of immunoelectrophoretic variability.

INTRODUCTION

There are indications that immunochemical techniques when judiciously applied will prove as useful in neurospora research as they have in the study of other micro-organisms. The object of this report is to illustrate the application of immunoelectrophoretic analysis (IEA) to a variety of problems which may arise during an investigation of the proteins of any micro-organism. The experiments are presented as procedures, rather than with intent to report completed investigations. Nearly all of them have been made in conjunction with other techniques during the conduct of continuing research projects.

The main current uses of IEA in neurospora research are: (1) to enumerate the minimal number of antigenic protein components with which one has to contend; (2) to monitor the purification of protein components; (3) to detect qualitative and gross quantitative variations due to known genetic differences; (4) to evaluate effects of different culture conditions; (5) to detect unsuspected genetic polymorphisms among otherwise comparable strains. The application of IEA (or any qualitative immunochemical technique) to one of the last three mentioned purposes must obviously consider the other two as possible causes of any observed differences. Additional considerations of importance to the interpretation of IEA patterns of intracellular antigens are the relative effectiveness of extraction procedures for different strains, different cells and different proteins, and the relative stability of extracted components under subsequent experimental or storage conditions. Solutions of cytoplasmic proteins, and especially crude extracts, are not as stable as blood serum for example, where IEA has been applied with notable success for many of the objectives listed above.

We have reported several preliminary observations on the variability of IEA patterns among morphological mutants of *Neurospora crassa*, and a comparative study with certain other species of ascomycetes (Williams & Tatum, 1961). More species have been examined in an effort to determine some of the phyletic affinities of neurospora. Selected results are shown in the present survey. Other demonstrations are: (1) examples of specific identification procedures applicable to protein components; (2) the effect on the IEA pattern of curtailing a nutritional requirement in the medium; (3) the detection and verification of a one-gene polymorphism not associated with any known biochemical or morphological mutant character.

METHODS

Organisms. Wild-type *Neurospora crassa*, SYR 17–3 *A*, and the various biochemical and morphological mutants used are maintained in the collection of this laboratory. Mutants B28 and B132 were originally obtained from Dr V. W. Woodward, then of Brookhaven National Laboratory. The *slime* mutant was kindly supplied by Dr S. Emerson of the California Institute of Technology.

Triangularia, pleurage, and neocosmospora species were obtained from Dr A. Hervey of the New York Botanical Garden. Sordaria and gelasinospora species were furnished by Dr L. S. Olive of Columbia University. *Neurospora tetrasperma* and *N. sitophila*, *Penicillium uticae* and *Saccharomyces cerevisae* were from the collection in this laboratory.

Culture methods. Unless otherwise stated, mycelial growth was obtained in liquid medium at 30° in 15 l. aerated carboys. Conidial suspensions from 5- to 7-day old slopes were used for inoculation. Glycerol sucrose complete (GSC) medium of Vogel (1956) was generally used except for special purposes mentioned in text. Mycelium mass was collected on a 25 cm. Büchner funnel, washed with distilled water, lyophilized, pulverized, weighed and stored at 5°.

Crosses were made by standard procedures on the chemically defined crossing medium of Westergaard & Mitchell (1947). Progeny are designated by the strain number of parents (e.g. 37041), the cross-number in a series e.g. (1, 2, 3, etc.), and the ascus number and ascospore number in parentheses. A sample designation would be 37401–2(1–8).

Extraction procedures. Dry pulverized mycelium was ground to a fine powder in a cold mortar. Extractant, 5 parts (w/w) for IEA, was added gradually with continued grinding until the homogeneous paste could be transferred to centrifuge tubes, one part of extractant being retained to flush the mortar into the tube. Extraction mixtures were centrifuged for 90 min. at about 90,000*g* in the Spinco SW39 swinging-bucket rotor. The clear supernatant fluid was pipetted off for analysis. The protein content of such extracts ranged from 30 to 40 mg./ml. by biuret assay with a serum albumin standard. The extractant used was generally the same solution as that used in electrophoresis, although this was not essential.

To obtain immunizing antigens the same general procedure was followed, using 10 parts (w/w) of 0·1 M-NaCl in 0·05 M-sodium phosphate buffer (pH 7·2) as the extractant. The extraction mixture was centrifuged at 15,000 to 18,000*g* and the uncleared supernatant fluid was used as such in immunization procedures.

Immunization and antisera. Strains SYR 17–3 *A* (wild) and 37401–1(6–3) *a*, *inos*

were the *Neurospora crassa* strains used for the preparation of antigens for the immunization of two separate groups of rabbits. The antigen suspension was emulsified into an equal volume of Freund's complete adjuvant (Difco) and 2 ml. were divided among several injection sites. Subsequent injections were given intravenously in series of three increasing doses with intervals of one day. Antisera were tested one week after the end of each series, and bulk bleedings (25 ml.) began when IEA patterns from individual animals were sufficiently complex. Pools were made of bleedings which gave similar patterns. Special pools were blended from these for specific purposes.

Immunoelectrophoretic analysis (IEA). The microtechnique on 25 × 75 mm. glass slides was used. Other conditions were as originally described by Grabar & Williams (1955): electrophoresis at room temperature for 90 min. at 5 V/cm. in veronal buffer, ionic strength 0·038, pH 8·2. Development of patterns by diffusion of antiserum continued up to 40 hr at 5°. Slides were washed, dried, and stained with bromphenol blue. Patterns were analysed on photographic enlargements obtained by using the slides as negative plates.

Identification of specific proteins. Catalase and the 'non-specific' carboxylic esterases were identified according to the methods of Uriel (1960), catalase by immersion of the dry slides for a few seconds in 3 % H_2O_2, esterase by β-naphthyl-acetate solution followed by diazo blue B as coupling agent. Alkaline phosphatase was detected by immersion in a solution of naphthol AS MX phosphate followed by diazo blue B. Glucose-6-phosphate dehydrogenase detection was done by an adaptation of a histochemical method which used the substrate, TPN, KCN, $MgCl_2$, and 2(*p*-iodophenyl)-3(*p*-nitrophenyl)-5-phenyl tetrazolium chloride (Nutritional Biochemicals Corp.).

RESULTS

Enumeration of antigenic components

Every antiserum develops an IEA pattern which is in some way unique. It is not possible, therefore, to establish a single basic pattern. The most to expect is that a pattern may be recognized as more or less typical and complete with respect to the components of interest and to components which serve as mobility standards. Plate 1, fig. 1A, illustrates a pattern of *Neurospora crassa* strain 37401-1(6-3) *a, inos.* Electrophoresis was done with 60 μg. extract protein, and the pattern was developed by antiserum pool N-II concentrated 10 times. On the original photograph 22 reaction lines were clearly distinguishable. In addition, there are several other components which are known to be precipitated, most likely in extreme antibody excess. The antibody excess reaction is one of diffuse, often faint precipitation. It is likely that several such reactions comprise the hazy background in the region to the left of the starting well. Two arcs, SS and RS are rather constant features of IEA patterns of *Neurospora crassa* extracts, and are used as mobility standards. The slow standard component (SS) is isoelectric around pH 8·2, and the rapid standard component (RS) migrates approximately like serum albumin.

Identification of precipitates

Before staining with bromphenol blue, the slide shown in Pl. 1, fig. 1, A was immersed in 3 % H_2O_2 for 5 sec., then washed thoroughly in water. Catalase, which is

active even when precipitated by specific antibody, breaks down H_2O_2 and produces bubbles of O_2 in the pattern. The short line locates the catalase precipitate. Without the H_2O_2 treatment it was sometimes impossible to distinguish this precipitate from the hazy background.

Many enzymes can be detected in this manner; as yet none of the more prominent arcs in the pattern has been identified as that of a specific enzyme. The enzyme precipitates detected thus far (such as the alkaline phosphatase, Pl. 1, fig. 1 B) or the various non-specific esterases (Pl. 1, fig. 1 D, E) are typical of an antibody excess reaction suggesting very small quantities of the antigenic protein. The glucose-6-phosphate dehydrogenase precipitate (Pl. 1, fig. 1 C) was not in extreme antibody excess but there was not sufficient protein in the antigen-antibody complex to give visible staining with bromophenol blue.

Except for D, in Pl. 1, fig. 1, the total IEA protein patterns would be the same as Pl. 1, fig. 1 A, since the same extract was used for all. Pl. 1, fig. 1 D was produced with an extract of the *slime* mutant of *Neurospora crassa*, a strain devoid of organized cell-wall. In addition to having several other mutant genes (*arg*, *ad-2*, *nic-1*, *lys-3*, *aur*, *cr*, *os*), *slime* seemed deficient for 2 of 4 esterases. The *slime* pattern may be compared with a wild-type pattern in Pl. 2, fig. 4. These patterns were obtained by shorter development times, hence are not as complex as that of Pl. 1, fig. 1 A.

Pattern variation

We reported previously (1961) that the IEA pattern and the total protein distribution by moving boundary electrophoresis vary with the age of the culture. Between 1 and 5 days of growth in liquid medium there is a marked increase in the relative amounts of certain of the more rapidly migrating components in the region of the rapid standard-mobility component RS. This observation was confirmed with strains other than the wild type, particularly with two morphological mutants of *Neurospora crassa*. One of these (R 1006) is a temperature-sensitive strain, growing as a colonial at 30° but with almost normal morphology at 25°. In addition to showing variability with respect to age, there were also differences in the IEA patterns of cultures of similar age grown at the two different temperatures. Since the change of temperature also produced a change of morphology, it was of interest to compare the patterns obtained with a non-facultative morphological mutant and with the the wild strain. There appeared to be no correlation of the pattern modifications with colonial morphology as such. Since some mutant strains of *N. crassa* grow more rapidly than others, and since any one strain may grow at different rates according to the temperature, the chronological age of a culture was not thought to be a suitable basis for comparison of strains with one another. Comparable dry weight of mycelium'given volume of medium during rapid growth was used as a better approximation of physiological age. Patterns also vary depending upon the method of preparing 'comparable' extracts for analysis. Extractable protein/unit dry weight mycelium varies with age and also from strain to strain. Extracts may be adjusted to equal total protein or compared on the basis of equivalent solvent: mycelium=dry-weight ratio. Any approach must find its rationale in experimental results, since there is no way of being certain what proportion of the total extractable protein contributes to the readily observed IEA pattern, or how this proportion may vary with strain or with age of a culture. There is at least one

report that protein synthesis in Neurospora continues even after the increase of mycelial mass ceases (Ballantine & Stephens, 1951).

IEA patterns do not vary markedly with different extracting solutions. Veronal buffer (0·038 M, pH 8·2), phosphate buffer (0·05 M, pH 7·6) or distilled water give similar patterns when the extract is fresh. On standing at 5°, or after freezing and thawing, however, there are differences apparently related to differential stability of some components in the different solvents. With some strains or cultures there is copious spontaneous precipitation of extracted material on standing or after freezing and thawing, irrespective of the extracting solvent. This material does not seem to comprise much of the protein which contributes the major components of the IEA pattern. Reconstituted ammonium sulphate precipitates are somewhat more stable in solution, but at 70% saturation a considerable number of the proteins which constitute the total pattern remain in the supernatant fluid. Dialysis against 4·8 or 5 M-$(NH_4)_2SO_4$ yields a precipitate which when reconstituted gives a pattern similar to that of the fresh crude extracts.

Variability with nutritional deprivation

Biochemical mutants of *Neurospora crassa* which require methionine (4984, *me-7*), arginine (27497, *arg-5*), or inositol (17401, *inos*), respectively, were grown in media containing decreasing amounts of the nutritional requirement, from the optimal concentration to that at which growth was possible but severely limited. Cultures were incubated for 3 days in 500 ml. medium on a rotary shaker. All inocula consisted of 30×10^6 washed conidia in minimal medium. Mycelia were harvested, lyophilized, and weighed in the usual fashion. Extracts were prepared with 10 ml. veronal buffer/g. mycelium. Extractable protein/unit dry wt. mycelium was about the same for all extracts of the *arg* and *me* mutants, but decreased progressively with lower concentrations of inositol in the medium in the case of the *inos* mutant. When the extracts were compared by IEA at equal protein concentrations, modifications in the patterns were most marked for the *me* mutant and slight for the *arg* and *inos* mutants. The comparative patterns of extracts of the *me* mutant developed with antiserum pool N-VI-A are shown in Pl. 1, fig. 2. They represent cultures grown: (A) with optimal methionine concentration (40 μg./ml.); (B) with suboptimal concentration (20 μg./ml.) which did not impair growth in 3 days; (C) with minimal concentration (5 μg./ml.) at which growth was severely limited. Those antigenic components which are modified noticeably in relative concentration are marked with short vertical lines.

Genetic polymorphism

The component labelled VMP (variable mobility protein) in Pl. 1, fig. 1 A first attracted attention because in the patterns of most of strains examined, whether wild or mutant, this are indicated that the component migrated more slowly than the rapid standard mobility component (RS). The F (fast) character appeared in two of eight strains derived from a single cross made for other purposes. A fast variant of mating type *a* was crossed with a slow strain of mating type *A* to determine the mode of inheritance of the mobility variation of the protein. Ten asci were isolated and the 8 ascospores numbered sequentially. Each ascospore was cultured and tested for inositol requirement and mating type. Two-day cultures in 300 ml. complete medium were

harvested in the usual way, giving yields of 0·7 to 0·9 g. dry wt. mycelium. Extracts were adjusted to equal protein concentration and examined by IEA. A special antiserum pool (N-V) was blended from three individual rabbit antisera and concentrated ten-fold. It contained antibody against VMP but did not develop prominent arcs for other components with which it might be confused. Plate 2, fig. 3, shows the patterns of the parents, *Neurospora crassa* 37401–1(6–3) *a* and 1(5–3) *A*, along with those of the even numbered isolates (one member of each pair) from the first ascus. VMP is marked on each pattern and mating types are indicated. It is seen that F VMP occurs in 2(1–2)*a* and 2(1–8)*A* in a segration pattern typical of a single-gene character.

Table 1. *Neurospora crassa: segregation of VMP characters in backcross progenies*

Back-cross No.	Cross*	Analysis of progeny: Ascospore no.	Mating type	VMP type†
1	2(1–1)a × 1(5–3)A	1	a	F
		3	a	f
	F f	5	A	f
		7	A	F
2	2(1–2)a × 1(5–3)A	1	A	f
		3	A	f
	F f	5	a	F
		7	a	–
3	2(1–3)A × 1(6–3)a	1	A	F
		3	A	F
	f F	5	a	f
		7	a	f
4	2(1–4)A × 1(6–3)a	1	a	(f)
		3	a	(f)
	f F	5	A	F
		7	A	F
5	2(1–5)a × 1(5–3)A	1	a	(f)
		3	a	(f)
	f f	5	A	(f)
		7	A	(f)
6	2(1–6)a × 1(5–3)A	1	A	(–)
		3	A	(f)
	f f	5	a	(f)
		7	a	(f)
7	2(1–7)A × 1(6–3)a	1	a	F
		3	a	F
	F F	5	A	F
		7	A	F
8	2(1–8)A × 1(6–3)a	1	a	F
		3	a	F
	F F	5	A	F
		7	A	F

* x(y–z): *x*, cross no. in a series; *y*, ascus no. from cross; *z*, ascospore no. in ascus.
† F, fast VMP; f, slow VMP; ( ), one extract only; –, are unidentifiable.

The homology of the fast and slow VMP was established by immunochemical identity reactions in pattern-interaction slides described by Williams & Wemyss (1961). The 8 ascospore cultures from ascus 1 were back-crossed to the parent of the

opposite mating type and an ascus from the progeny of each cross was isolated, tested, cultured and harvested for IEA as before. IEA results with extracts from cultures of odd-numbered ascospores from each backcross, shown in Table 1, are consistent with a single gene difference determining a higher or lower mobility of the VMP.

There was some indication that, in addition to the genetic factor, cultural conditions played a role in the mobility of this protein. The VMP of temperature-sensitive strain *Neurospora crassa* R 100 is fast when the culture is grown at 25° and slow at 30° when the strain shows colonial morphology. However, B 28 and B 132, other colonials, have a fast and a slow VMP, respectively, at 30°, so it is not a characteristic which is associated with colonial growth or temperature *per se*. In the 37401 *inos* strains used for the genetic analysis of VMP there was no noticeable impairment of growth on minimal medium supplemented with 50 mg. inositol/l.

Species differences and phyletic affinity

Several species of ascomycetes were cultivated on complete medium; growth proceeded at widely different rates. Most of these cultures were harvested 2 days after inoculation, but some required up to 7 days to yield sufficient mycelial growth for harvest. Neurospora, gelasinospora neocosmospora and most sordaria species grew quite well, producing between 11 and 21 g. dry wt. /15 l. medium in 48 hr. *Triangularia bambusae*, *Pleurage anserina*, *Sordaria fimicola* and *Penicillium uticae* grew poorly, and produced less than 5 g./15 l. in 5 days.

Extracts were compared on the basis of equal protein concentration with two different antiserum pools. Pool N–II prepared with antiserum from five rabbits immunized with Neurospora proteins over a period of 5 months revealed striking differences in the IEA patterns of the various species. Antigenic homologues were detectable, however, in the extracts of such distantly related species as the common yeast *Saccharomyces cerevisiae*. Pool N–VI–A was one vol. pool N–II and 2 vol. serum from two rabbits which had been immunized for more than 7 months.

Plate 2, fig. 4, illustrates the patterns of three species considered to be fairly near relatives of *Neurospora crassa* and which grew well on the medium used. The patterns were developed for 24 hr with pool N–II antiserum. It is evident from the number and intensity of the arcs of specific precipitate that gelasinospora and sordaria species are more distant relatives of *N. crassa* than is *N. sitophila*. When the same extracts were compared by development with pool N–VI–A antiserum, however, the striking differences in number and intensity of the reactions disappeared; such patterns are shown in Pl. 2, fig. 5. It is important for studies of this nature to show that the actual antigenic composition of the extracts from different species are similar, a fact which can be revealed by the less specific antibodies present in pool N–VI–A antiserum. The antibodies of pool N–II, which are more specific for *N. crassa* protein antigens may then be confidently used to assess phylogenetic distance among species.

DISCUSSION

In spite of the sensitivity, specificity and resolving power of immunoelectrophoretic analysis (IEA), there are the obvious limitations inherent in all immunochemical techniques. First, one is at the mercy of an animal, in our case the rabbit,

which is unconcerned with the research aims of the investigator. The antibodies produced may be highly specific or rather non-specific, as illustrated by the relative activities of antiserum pools N–II and N–II–A reacted with antigens from different species of fungi (see Pl. 2, figs. 4, 5). Also, the amounts of antibodies produced may have no relationship to the relative concentrations of the antigens in the extract to be analysed, as indicated by the significant number of antibody-excess reactions in the IEA patterns. Further, every animal immunized with a mixture of antigens as complex as a protoplasmic extract will produce a different spectrum of specific antibodies in different relative amounts.

All of these variables, however, may prove useful in a given research problem. Individual antisera may be used for their particular qualities or they may be selectively pooled for special applications, as was done for the analysis of the VMP protein and the comparison of the several species of ascomycetes. The fact that significantly more antibody is produced to minor components, such as certain enzymes, relative to their concentration in the extract, makes broad spectrum antisera valuable tools to monitor the purification of these antigens. As their relative concentrations in fractions are increased, their corresponding arcs of precipitate will become sharper, permitting their clear distinction from antigenic impurities. Specific identification tests for enzymes in immune precipitates have been devised for many enzymes (Uriel, 1966). Roberts & Pateman (1964) reported the use of such techniques in studies of glutamate dehydrogenase of neurospora.

It is now a commonplace that the protein composition, both in quality and quantity, is as characteristic of a species as the gross morphology or the metabolic and biosynthetic pathways. IEA and other immunochemical methods offer the possibility of examining this degree of phenotypic expression in broad but sensitive parameters. In organisms with which environmental as well as genetic manipulation is possible, investigations by IEA might well uncover important new directions for the study of heredity/environment interaction in adaptive processes. No special claim is made in this regard for the VMP component described in these experiments, but proteins with such properties might have special interest. Its identification with some structure or function might provide a means to assess the importance of minor changes in protein structure. The VMP component might also serve as a strain marker among otherwise wild-type cultures.

The analysis of different species has theoretical as well as practical implications. In higher organisms, such as the mammals and specifically the Primates, immunochemical correspondence has been shown to be consistent with accepted systematics and phylogeny (Goodman, 1963; Williams, 1964; Hafleigh & Williams, 1966). The systematics of micro-organisms are in many cases based on characters that have not been considered in the classification of higher organisms. In addition, phylogenetic relationships, and thus evolutionary processes, can only be surmised because of the lack of any extensive palaeontological evidence. Among systematic or taxonomic relatives of micro-organisms, however, the relative correspondence of macromolecular structures such as the nucleic acids (McCarthy & Bolton, 1963; Drs S. K. Dutta, W. P. McWhorter and V. W. Woodward, personal communications) and the proteins should reveal phylogenetic relationships. Studies of this sort may distinguish between systematic schemes which reflect true biological affinities and those which are merely convenient. At present only the proteins provide

material for assessing structural similarities in defined portions of the genomes of different species. Quantitative and qualitative analysis of these similarities, in providing a measure of genetic relatedness, provides also the starting-point for a rational evaluation of evolutionary trends among micro-organisms. Immunochemical analysis of structural correspondence, while not ideal theoretically, does offer the possibility for extensive collection of data which are satisfactory for first approximations of phylogenetic relationships.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. IEA pattern of *Neurospora crassa* extracts. A. Total pattern of 37401–1(6–3)*a*, *inos*. Slide stained with bromphenol blue after immersion in 3 % H_2O_2 to reveal the position of catalase (indicated by short vertical line). The variable mobility protein (VMP) is shown in Fig. 3. RS and SS refer to the rapid and slow standard mobility components, respectively. B. IEA procedure identical to that of A with staining for alkaline phosphatase (AP) activity. Picture taken before counterstaining with bromphenol blue. C. IEA procedure identical to that of B with staining for glucose-6-phosphate dehydrogenase (G-6-PDH) activity. D. and E. Extract of *slime* mutant

(D) and of *N. crassa* 37401–1(6–3)*a*, *inos* (E) compared on same slide for non-specific carboxylic esterases.

Fig. 2. IEA pattern of extracts of methionine-deficient *N. crassa* mutant (4894, *me*-7) grown on minimal media augmented with methionine 40 μg., 20 μg. or 5 μg./ml. Antigenic components which were affected by decreasing the amount of the nutrient are indicated by short vertical lines.

PLATE 2

Fig. 3. IEA patterns showing variable mobility protein (VMP) positions in extracts of cultures of even-numbered ascospores from a single ascus derived from the cross of *N. crassa* strains 1(5–3)A and 1(6–3)a.

Fig. 4. IEA patterns of *Neurospora crassa*, *slime* mutant (see Fig. 1D), *N. sitophila*, a gelasinospora species, and a sordaria species developed with antiserum to *N. crassa* antigens. Note greater similarity of antigens of *N. sitophila* to those of *N. crassa* than to antigens of other genera.

Fig. 5. IEA patterns of same species as shown in Fig. 4 developed with an antiserum pool containing a wide range of specific antibodies. Many more antigenic homologues are revealed in the non-neurospora species by this antiserum than the more specific pool used to obtain the patterns in fig. 4.

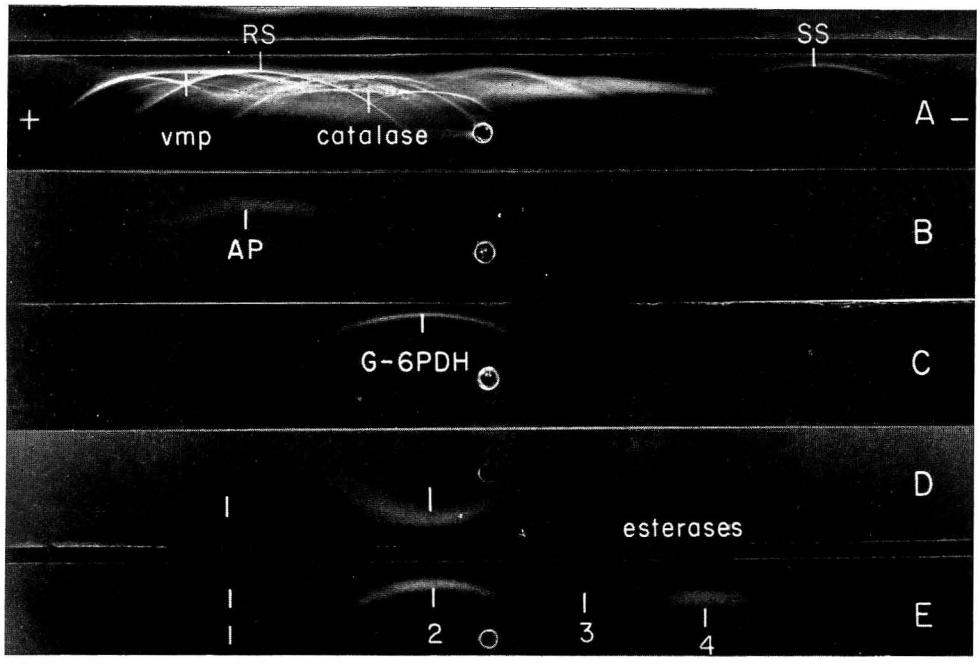
RS
SS
+
vmp
catalase
A
–
AP
B
G-6PDH
C
D
esterases
E
1
1
2
3
4

Fig. 1

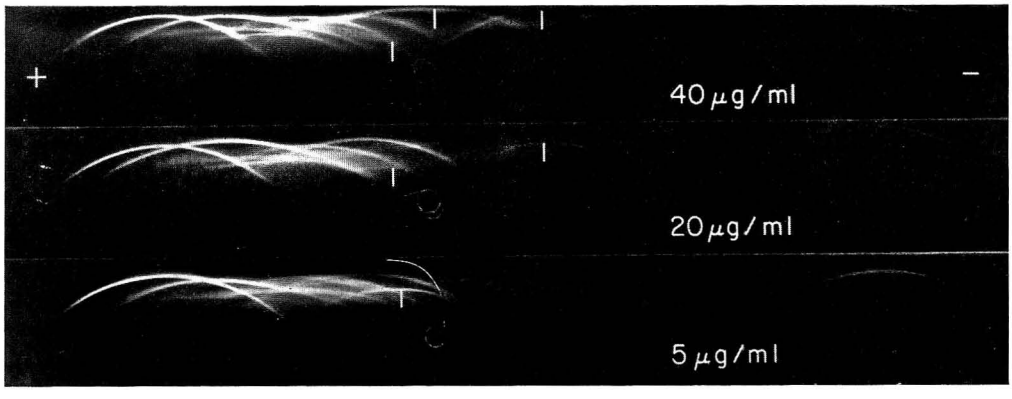
+
40 μg/ml
–
20 μg/ml
5 μg/ml

Fig. 2

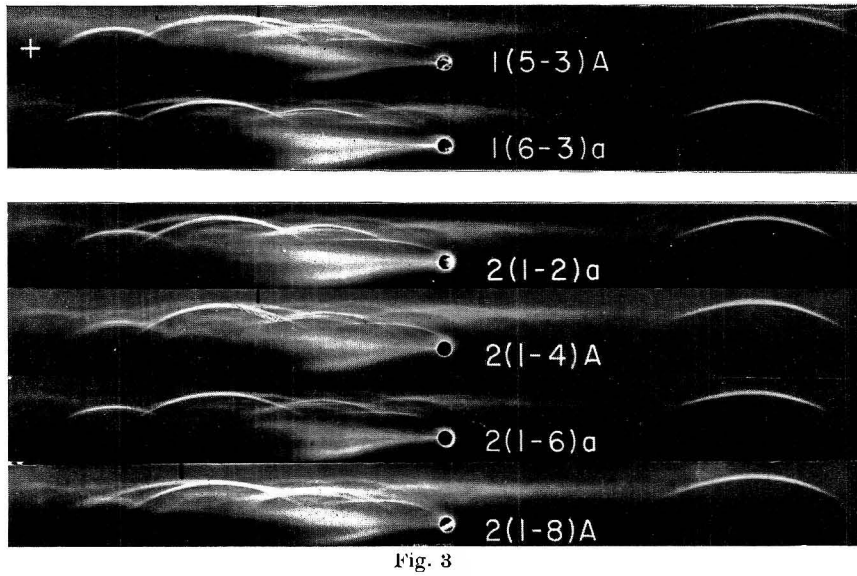

Fig. 3

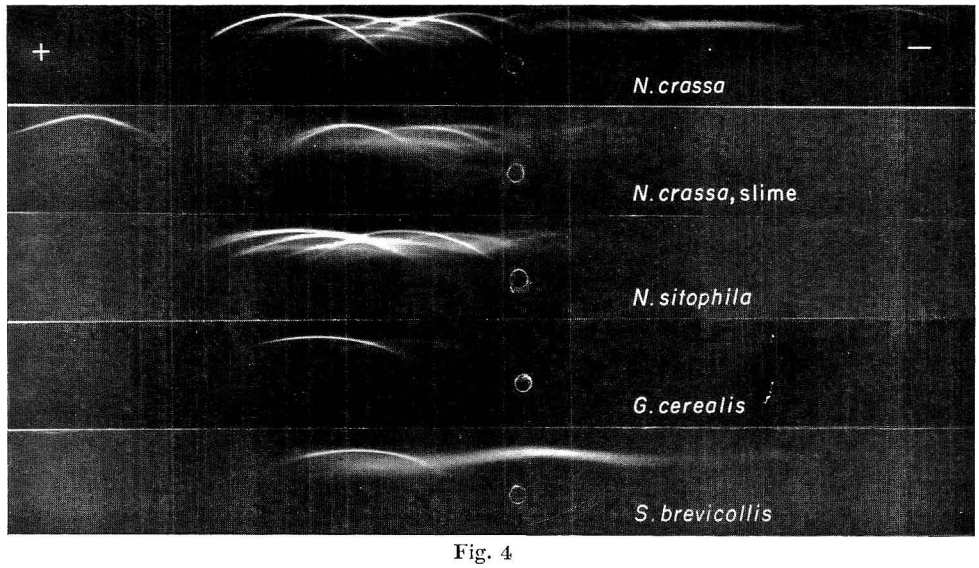

Fig. 4

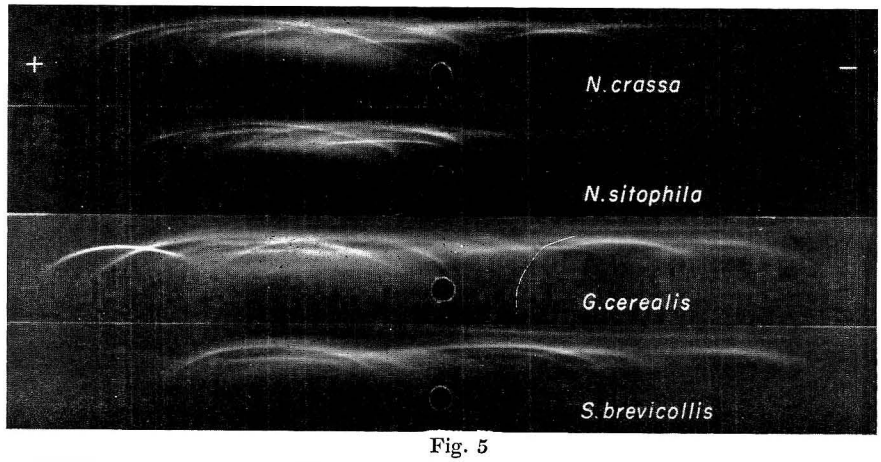

Fig. 5

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A Conidial Actinoplanes Isolate from Blelham Tarn

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(*Received* 9 *November* 1965)

SUMMARY

An *Actinoplanes* sp. (Actinomycetales) isolated from Blelham Tarn is described and illustrated. It is characterized by a filed arrangement of spores both in sporangia and conidiophore systems, suggesting that these two types of reproductive structure may develop in an essentially similar manner.

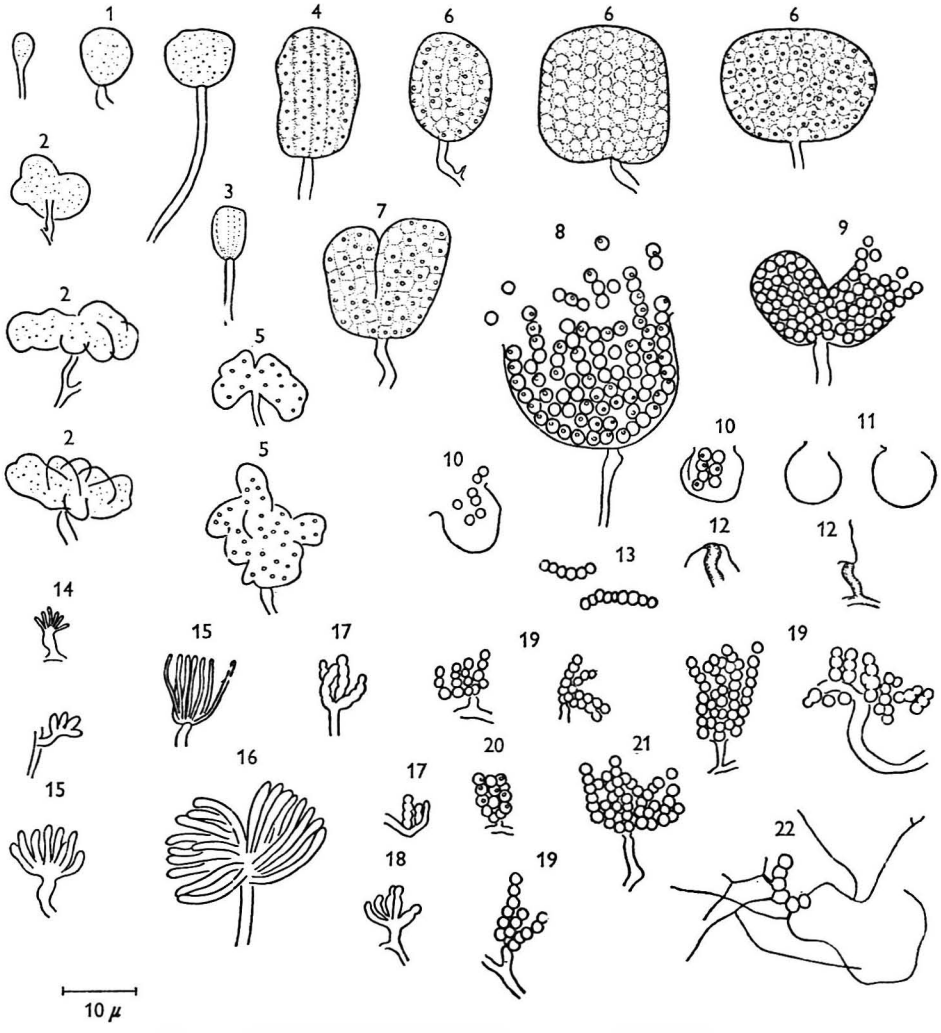
INTRODUCTION

Actinomycetales bearing sporangia were first reported by Couch in 1949 and placed in a special family, the Actinoplanaceae. Subsequently Couch (1955, 1963) distinguished several constituent genera of which *Actinoplanes* has been represented the most frequently in his isolations, which have largely been from soil. The purpose of this contribution is to describe an actinoplanes isolate which has several interesting morphological features; in particular it bears striking conidiophore systems in addition to sporangia.

METHODS AND RESULTS

The isolate was obtained by plating and incubating a freshly collected surface water sample from Blelham Tarn (English Lake District) on starch casein agar (Küster & Williams, 1964). Subcultures on the latter medium were creamy yellow in colour, and with a rather diffuse colony margin. Subcultures were also set up on pinus pollen suspended in sterile lake water. Under these conditions growth of the organism was particularly prolific and clearly displayed and gave most of the observations reported. Sporangial initials occurred at hyphal tips (Fig. 1; Pl. 1, fig. 1) and it was sometimes possible to see some indication of a longitudinal arrangement of contents very early in development (Fig. 3). At maturity the sporangium was normally oblong (Fig. 6) or suburceolate (Pl. 1, figs. 2, 3); a double structure was not unusual (Figs. 7, 9; Pl. 1, fig. 7). As with other actinoplanes isolates which have been studied in this laboratory, dehiscence could usually be induced by mounting ripe material under a coverglass. However, the very rapid sequence of visible sporangiospore differentiation and dehiscence often observed in these cases, particularly well exemplified in an isolate from soil from Kynance Cove, Cornwall (Pl. 2), was not a feature of the Blelham Tarn isolate. In the latter sporangiospore outlines, arranged in a characteristically filed fashion, were distinguished when the material was first mounted (e.g. as in Fig. 4; Pl. 1, fig. 3). There was a more leisurely accentuation and rounding off of these outlines (Fig. 6; Pl. 1, fig. 4) followed by a correspondingly protracted deliquescence of sporangial walls, either partial (Figs. 10, 11) or more or less complete (Fig. 12). Sporangiospores were

motile, but there was a considerable time lag (up to several hours) before they became highly active. The usual pattern was to observe a background of highly active sporangiospores traversing the microscope field with a foreground of sporangia



Camera-lucida drawings of *Actinoplanes* sp. from Blelham Tarn.

Fig. 1, young sporangia. Fig. 2, irregularly shaped specimens occasionally observed. Fig. 3, young sporangium with indication of longitudinal internal arrangement. Fig. 4, sporangium with refractive globules and outlines of sporangiospores first apparent. Fig. 5, a corresponding stage in irregularly shaped sporangia. Fig. 6, sporangiospore outlines more clearly defined. Fig. 7, a double sporangium structure. Figs. 8–10, dehiscence and sporangiospore liberation. Fig. 11, dehisced sporangia. Fig. 12, dehisced sporangia with very little residual wall material apparent. Fig. 13, liberated sporangiospore chains. Fig. 14, young conidiophore systems. Fig. 15, conidiophores following further growth. Fig. 16, conidiophore system at mature size. Fig. 17, conidiospore constriction beginning. Fig. 18, conidiophores with apical constriction only. Fig. 19, mature conidiophore systems. Fig. 20, conidiospores showing refractive globule. Fig. 21, orderly arrangement of conidiospores breaking down. Fig. 22, germination of a chain of sporangiospore on starch casein agar.

which were dehisced, but the spores in which remained quiescent. These sporangia were difficult to distinguish from conidiophore systems, as described below. The consistent observation of the liberation of pairs or chains of sporangiospores rather than single units (Figs. 8, 10, 13; Pl. 1, figs. 15, 16) gave further confirmation, if any were needed, of the filed arrangement of sporangiospores in the sporangia. These spore chains only exhibited extremely limited motility; the question arose whether they had been squashed out of sporangia prematurely rather than liberated in a normal fashion. The fact that spore chains are viable (Fig. 22; Pl. 1, fig. 17) is deemed to imply that they are in fact a normal phenomenon in this isolate.

Conidiophore systems were distinguished in their earliest stages as clusters of finger-like hyphae radiating from a common stalk (Fig. 14; Pl. 1, figs. 9, 10). The hyphae expanded both in length and girth and eventually superficial constrictions were apparent (Fig. 17). Although the constriction and subsequent separation of conidiospores in a fertile hypha normally proceeded simultaneously along its length, there was evidence that successive separations from the apex were also possible (Fig. 18). At maturity the conidiophore systems consisted of loosely-borne divergent files of conidiospores (Fig. 19; Pl. 1, fig. 14) the orderly arrangement of which tended to break down in the larger examples (Fig. 21).

When subcultures grown on pollen were mounted for microscopic examination mature sporangia and mature conidiophore systems could usually be distinguished initially. However, as the sporangial walls became indistinct at the onset of dehiscence, and this was often accompanied by a loosening of the compact arrangement of the sporangiospores (Pl. 1, fig. 5), it became increasingly difficult to make the distinction. Eventually sporangiospores were distinguishable from conidiospores by their motility, on occasions when this was pronounced. The limits of the size-range of sporangiospores (1·3–1·8 μ) were overlapped by those of conidiospores (1·1–1·8 μ). The refractive globule which was usually a conspicuous feature of sporangiospores was rarely to be distinguished in conidiospores (Fig. 20). Despite these various points of difference between the two types of sporulation unit and spore the difficulty experienced in making a visual segregation gave acute awareness of their essential similarity.

DISCUSSION

Although there is a previous report and description of the occurrence of conidia in the genus *Actinoplanes* (Kalakutskii & Kuznetsov, 1964) and remarks by other authors imply that this is not unusual, the Blelham Tarn isolate would seem to be the only documented instance so far where a definite conidial system, at maturity outwardly similar to the sporangium, has been reported. In this isolate the liberation of spore chains from the sporangia tends to suggest that the filed arrangement of sporangiospores occurs through the septation of parallel lengths of hyphae. Lechevalier & Holbert (1965) produced evidence from electron microscope studies that sporangiospores in an actinoplanes isolate made by them were so derived. In comparing the sporangial and conidial systems in the Blelham Tarn isolate it may be deduced that the main essential difference lies in the enclosure of the whole sporing system in a common membrane in the sporangial system only. Clearly such an ontogeny for the sporangium is quite different from that in the lower aquatic fungi (Chambers & Willoughby, 1964), with which the Actinoplanaceae show such outwardly close affinity.

My thanks are due to Mrs P. J. McDougall for valuable assistance and to Mr T. Cross of the Bradford Institute of Technology for help with the literature.

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EXPLANATION OF PLATES

PLATE 1

Photomicrographs of *Actinoplanes* sp. from Blelham Tarn. All × 1000 except Figs. 17 and 18.

Fig. 1. Young stalked sporangium.

Fig. 2. Field of young sporangia.

Fig. 3. Sporangia with sporangiospores first distinguished.

Fig. 4. Sporangiospores now fully apparent.

Figs. 5, 6. Sporangia with walls deliquesced but zoospores quiescent.

Fig. 7. A double sporangium with wall deliquesced at top right only.

Fig. 8. An irregularly shaped sporangium.

Figs. 9, 10. Conidiophore initials.

Fig. 11. Conidiophore system with oblong conidiospores distinguished.

Fig. 12. Young conidiophore system and sporangium showing close similarity in gross external morphology.

Fig. 13. Conidiophores at full size but conidiospores not constricted.

Fig. 14. Mature conidiospores.

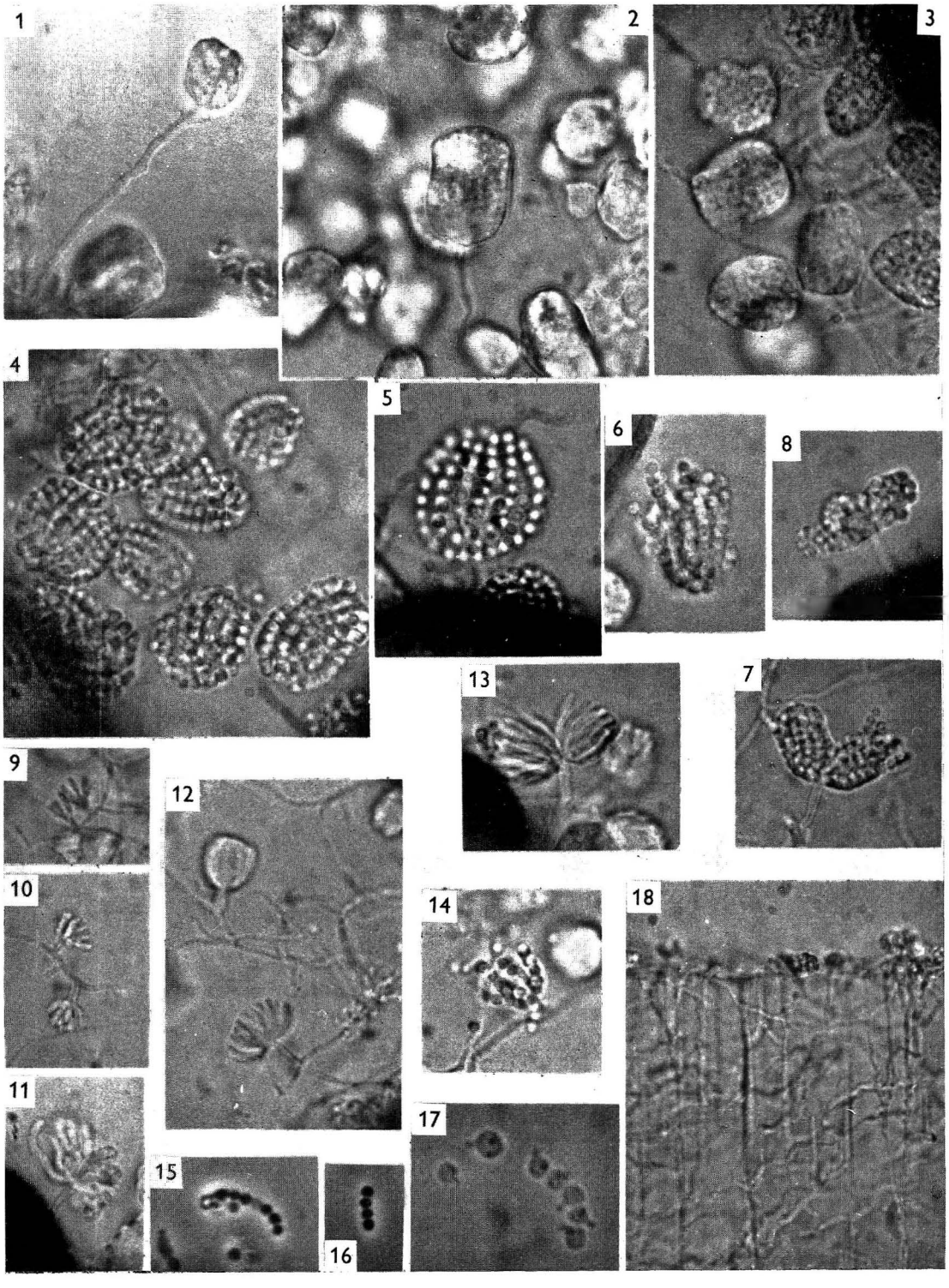
Figs. 15, 16. Chains of sporangiospores.

Fig. 17. Single sporangiospores and a chain (right) germinating on starch casein agar (× 2000).

Fig. 18. Vertical section of growth on starch casein agar showing strongly marked palisade (× 630).

PLATE 2

Photomicrographs of *Actinoplanes* sp. from soil from Kynance Cove, Cornwall showing sequences of sporangiospore differentiation and dehiscence in several sporangia on *Pinus* pollen. The series runs from left to right, top to bottom, and was made over a period of approximately 20 min. All × 630.



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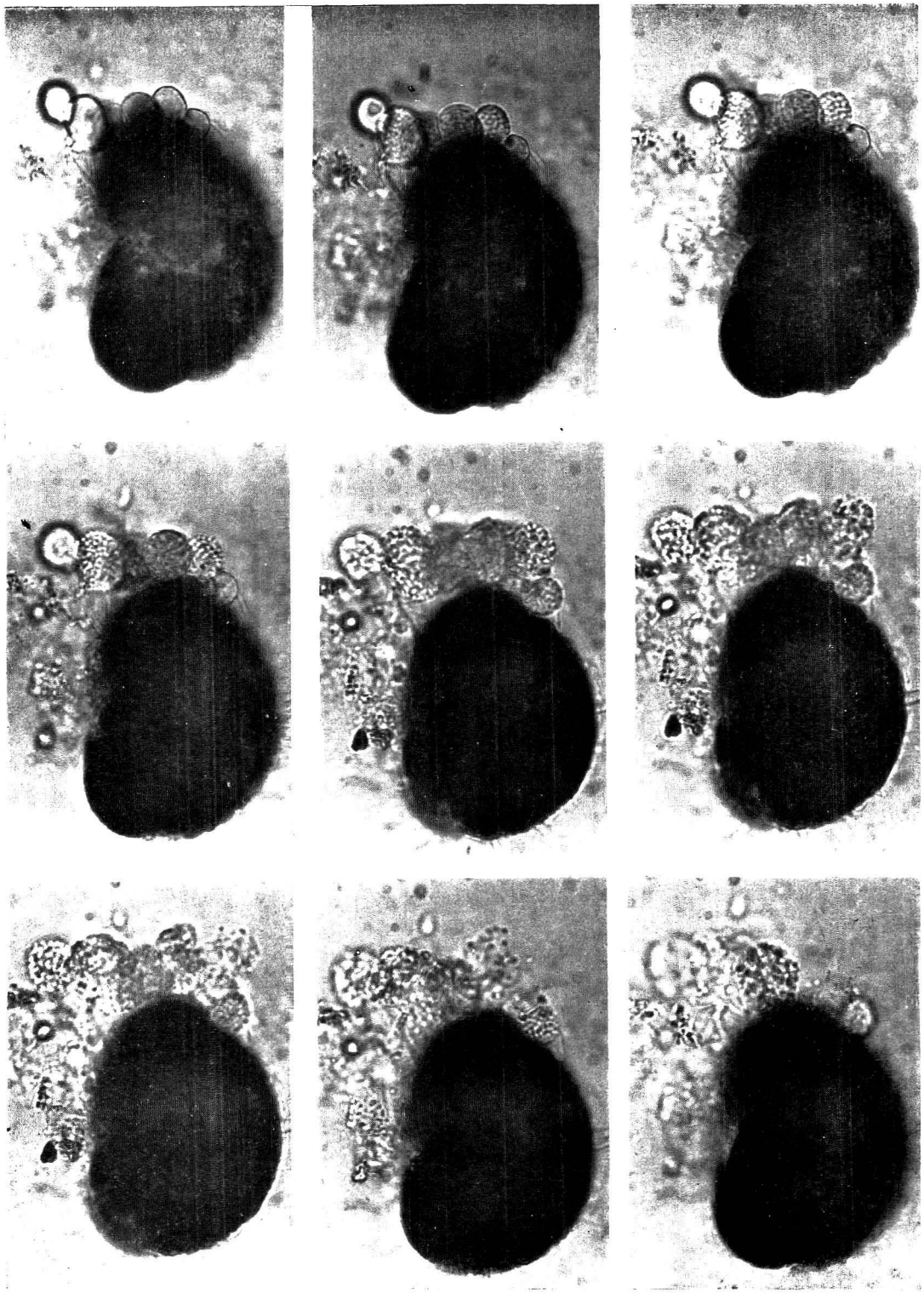

L. G. WILLOUGHBY

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Aconitase and Isocitric Dehydrogenases of *Aspergillus niger* in Relation to Citric Acid Production

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(*Received* 16 *November* 1965)

SUMMARY

Aconitase and NAD- and NADP-linked isocitric dehydrogenases were examined in two strains of *Aspergillus niger*. The mutant strain 72–44 produced higher yields of citric acid on the culture medium used than did the parent strain 72–4. After growth for 22 hr on a medium in which citric acid did not accumulate, the amounts of each enzyme in the parent strain were approximately the same as those in the mutant strain. The enzymes were also found, though in lower amounts, throughout the incubation period of 8 days in both strains grown on a medium in which citric acid accumulated. A 20-fold increase in the concentration of iron in this medium doubled the activity of aconitase in extracts from the mutant strain, though citric acid accumulation was only decreased by 25%. The addition of monofluoroacetate to the culture medium was toxic to the mould and did not stimulate citric acid yields. It is suggested that during the incubation, some recycling of citric acid may take place. The difference in citric acid yields from the parent and mutant strains was not accounted for on the basis of the degrees of aconitase or isocitric dehydrogenase activity.

INTRODUCTION

Under certain conditions of nutritional deficiency strains of *Aspergillus niger* are capable of excreting large quantities of citric acid (Perlman & Sih, 1960). Cleland & Johnson (1954), by using glucose-3,4-^{14}C obtained results which suggested a scheme for the formation of citrate from glucose The distribution of radioactivity in the citrate indicated that there was little recycling of the acid. Ramakrishnan, Steel & Lentz (1955) implied that accumulation of citric acid resulted from a deficiency in the tricarboxylic acid cycle enzymes aconitase and isocitric dehydrogenase. In a culture which yielded 7·1 g. anhydrous citric acid/100 ml. culture fluid, they were unable to detect the two enzymes in extracts prepared after citric acid accumulation had begun. Since strains of *A. niger* differ in their ability to accumulate citric acid they might, on the above hypothesis, be expected to contain different quantities of some enzymes of the tricarboxylic acid cycle. In the present study, two strains of *A. niger* were used. The mutant strain 72–44, which is characterized by restricted growth and delayed sporulation, was derived from the parent strain 72–4 after ultraviolet irradiation; it produces higher yields of citric acid than does the parent on the particular medium used. This paper describes an investigation of aconitase and isocitric dehydrogenase in the two strains of *A. niger* when grown,

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first, on a medium which favoured growth rather than citric acid production, and secondly on a metal-deficient medium which allowed citric acid to accumulate.

METHODS

Organisms. Aspergillus niger Wisconsin strain 72–4, ATCC 11414 (Perlman, Kita & Peterson, 1946), will be referred to as the parent strain. The mutant strain 72–44 was derived by Millis, Trumpy & Palmer (1963) from the parent after ultraviolet irradiation. Stock cultures were maintained as lyophilized spores and working cultures were grown on beer-wort agar slopes. The parent organism spored readily on beer-wort slopes in 3 days, whereas the mutant required 10 days to produce an adequate degree of sporulation.

Cultures on fluid media

Glucose malt-extract yeast medium (GMY). This medium was used for the production of mycelium for enzyme extracts under conditions where citric acid did not accumulate. It was essentially that used by Martin (1954) and consisted of 1% glucose, 3% malt extract, and 0·5% yeast extract dispensed in 100 ml. volumes in 500 ml. Erlenmeyer flasks which were autoclaved at 116° for 10 min.

Fermentation medium (Medium B). This medium developed by Millis, Trumpy & Palmer (1963) was used for studies under conditions for citric acid accumulation. It consisted of a 14% (w/v) solution of sucrose (A1 grade commercial cane sugar, Colonial Sugar Refineries, Australia) which was passed through a cation exchange resin Zeocarb 215 (Permutit Co.) in the H^+ form. To this were added (g./l.): $MgSO_4.7H_2O$, 0·15; KH_2PO_4, 0·30; NH_4NO_3, 1·4; (mg./l.): Cu^{2+}, 0·3; Fe^{2+}, 2·0; and Zn^{2+}, 0·1. The medium was dispensed in 50 ml. volumes in 500 ml. acid-washed flasks and autoclaved at 116° for 10 min.

Inoculation of flasks. Spores from a beer-wort agar slope were suspended in 1/10,000 (w/v) sterile 'Soaxit' (sodium dioctyl-sulphosuccinate; W. Hermon Slade Co., Homebush, N.S.W.). The suspension was shaken vigorously to disperse clumps and the spores counted in a haemocytometer. These were then added to the flasks at the rate of 1×10^4 spores/50 ml. medium.

Conditions of incubation. The flasks were incubated at 30° on a shaker which was rotated at 210 rev./min. with a 2 in. amplitude throw. The flasks were placed at an angle of 15° to the horizontal to improve aeration. Cultures on GMY medium were incubated for 22 hr and those on medium B for various times up to 8 days.

Analysis of cultures

Mould growth. The wet weight of mycelium was used as a rough index of growth. The contents of each flask were filtered through a Buchner funnel with Whatman paper no. 541, washed with ice-cold water, sucked until damp dry, and weighed to the nearest 0·1 g. This mycelium was used for the preparation of enzyme extracts.

Citric acid. The fermentation liquor was separated from the mycelium by filtration through a Buchner funnel with Whatman paper no. 541. The filtrate + washings were made up to 100 ml. and the yield of citric acid was measured by titrating a 4 ml. sample against 0·25 M-NaOH (Millis, Trumpy & Palmer, 1963). The results were expressed as g. anhydrous citric acid/100 ml. original culture fluid.

Sugar utilized. The colorimetric method developed by Somogyi (1952) was used for estimating residual sugar. Samples were diluted to contain between 25 and 600 sugar reducing μg./ml. and sucrose was hydrolysed by heating 1 ml. at 100° for 10 min. with 0·1 ml. 0·5 M-H_2SO_4. On cooling, 0·1 ml. M-NaOH was added to neutralize the sulphuric acid before proceeding with the estimation.

Preparation of enzyme extracts

Mycelium from GMY medium was washed with ice-cold water, filtered through four layers of gauze and pressed dry. It was then weighed to the nearest 0·1 g., and placed in a Petri dish in ice until used.

Mycelium from medium B was filtered on a Buchner funnel with Whatman paper no. 541, and washed with ice-cold water. The mycelial pad was weighed to the nearest 0·1 g. and resuspended in ice-cold water. To neutralize the citric acid, M-KOH was added dropwise to the mixture, with use of a magnetic stirrer, until it remained at pH 7·2 to 7·4. The mycelium was then refiltered and kept in ice until used.

Water was used for the preparation of extracts containing aconitase from mycelium grown on GMY medium, and 0·1 M-borate buffer (pH 7·5) was used for mycelium grown on medium B. For NAD- and NADP-linked isocitric dehydrogenases, 0·1 M-potassium phosphate buffer (pH 7·0), containing 10^{-3} M glutathione (freshly prepared) was used in both cases. The washed mycelium was placed in a 7-inch porcelain mortar cooled in an ice bath and ground for 5 min. with an equal weight of cold washed 600 crystal Corundum. Three times the volume of cold extraction fluid was then added and the mycelium ground for a further 5 min. The temperature was kept below 4° throughout the operation. The extract was centrifuged at 500*g* for 10 min. at 0° to remove the bulk of the Corundum and cellular debris, and it was then centrifuged at 21,600*g* for 15 min. at 0° and the supernatant fluid assayed for enzyme activity.

Extracts of mycelium grown on medium B were treated with protamine sulphate to remove material which absorbed at 260 mμ, and thus interfered with the assay of aconitase. After adding a fifth of its volume of a 1% solution of protamine sulphate the extract was stirred for 15 min. with a magnetic stirrer, then centrifuged at 4600*g* for 10 min. The precipitate was discarded and the supernatant fluid assayed for enzyme activity.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) and expressed as mg./ml.

Enzyme assays

Spectrophotometric measurements were made with a Unicam Spectrophotometer (S.P. 500) with a temperature control unit (S.P. 570) holding two 1-cm. cuvettes of 3 ml. capacity. Tests were made at 30°, a period of 5 min. being allowed before the addition of substrate, to enable the temperature of the test system in the cuvettes to reach that of the water bath.

The unit of activity was defined as an initial rate of increase in extinction of 0·001/min./ml. extract. Specific activity was expressed as units/mg. protein.

Aconitase. The activity of this enzyme was determined by measuring the rate of appearance of *cis*-aconitate at 240 mμ from either citrate or isocitrate according to the method of Racker (1950). To a Unicam cuvette were added: 0·2 M-sodium

citrate, 0·3 ml., or 0·1 M-sodium isocitrate, 0·4 ml., each in 0·05 M-potassium phosphate buffer (pH 7·4); enzyme and water to 3 ml. The blank contained all components other than substrate. The increase in extinction was measured at 1 min. intervals for 5 min.

NAD-linked isocitric dehydrogenase. This enzyme was assayed by measuring the increase in extinction at 340 mμ resulting from the reduction of NAD, according to the method of Ochoa (1948). To a Unicam cuvette were added: 0·5 M-potassium phosphate buffer (pH 7·0), 0·2 ml.; 0·005 M-NAD, 0·3 ml.; 0·0025 M-AMP, 0·3 ml.; 0·1 M-$MgCl_2$, 0·1 ml.; 0·3 M (KCN-freshly neutralized pH 7·2), 0·1 ml.; 0·1 M-sodium isocitrate, 0·1 ml.; enzyme and water to 3 ml. A blank containing all components other than substrate was used in each assay. The increase in extinction at 340 mμ was measured at 30 sec. intervals for 3 min. KCN was included in the assay system because the enzyme preparations contained substances capable of oxidizing the reduced NAD.

NADP-linked isocitric dehydrogenase. The activity of this isocitric dehydrogenase was also determined at 340 mμ. To a Unicam cuvette were added: 0·5 M-potassium phosphate buffer (pH 7·4), 0·2 ml.; 0·0025 M-NADP, 0·3 ml.; 0·1 M-$MgCl_2$, 0·1 ml.; 0·3 M-KCN (freshly neutralized pH 7·2), 0·1 ml.; 0·1 M-sodium isocitrate, 0·1 ml.; enzyme and water to 3 ml. A blank containing all components other than substrate was used in each assay. The increase in extinction at 340 mμ was measured at 30-second intervals for 3 min.

RESULTS

Cultural characteristics

The mutant strain *Aspergillus niger* 72–44 was easily distinguished from the parent strain by its restricted colony development and delayed sporulation on agar media. In GMY liquid medium, it grew at a slightly slower rate than the parent and usually formed discrete pellets about 1 mm. in diameter, whereas the parent formed a mass of loose filamentous growth throughout the medium.

On medium B, the mutant produced a higher yield of citric acid than the parent, generally in the range of 8–10 g./100 ml. (see Trumpy & Millis, 1963) while the yield with the parent was 2–3 g. citric acid/100 ml. Cultures were analysed for the amount of sugar utilized and for weight of mycelium formed. The course of a typical fermentation is shown in Fig. 1.

Enzymic studies under conditions when citric acid did not accumulate

The activity of aconitase in each organism was determined on two occasions after growth for 22 hr in GMY medium. The rate of the reaction citrate → *cis*-aconitate was about half that of the reaction isocitrate → *cis*-aconitate. Although the amounts of enzyme differed in the two experiments the same ratio of reaction rates was obtained with both strains of Aspergillus. This suggests that the strains do not differ greatly in the activity of aconitase or aconitic hydrase. The latter enzyme, demonstrated in extracts of *Aspergillus niger* by Neilson (1956), catalyses the reaction citrate → *cis*-aconitate only. The results are shown in Table 1.

The amount of both isocitric dehydrogenases in the two organisms was determined under the same conditions in three separate experiments (see Table 2). Since

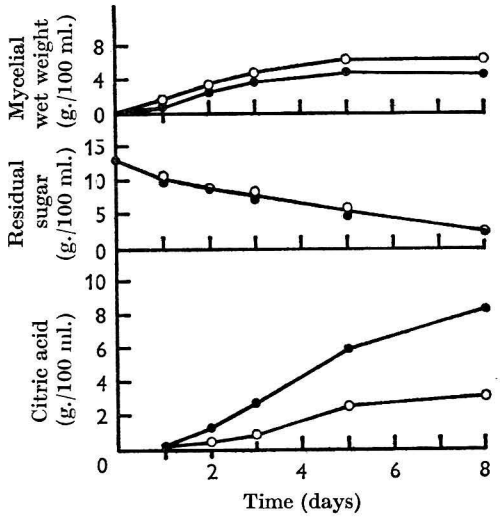

Fig. 1

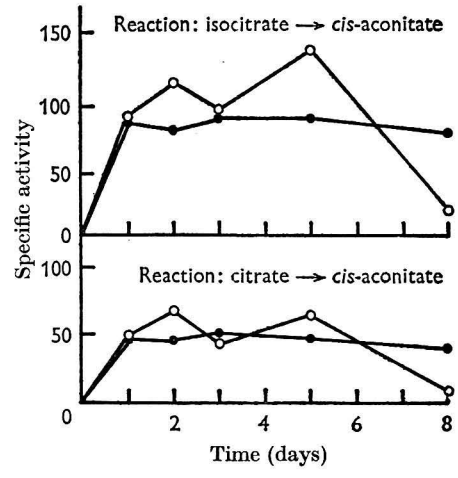

Fig. 2

Fig. 1. Rates of growth, sugar utilization and citric acid production on medium B by *Aspergillus niger* strain 72–4 (○—○) and strain 72–44 (●—●).

Fig. 2. Specific activity of aconitase in *Aspergillus niger* strains during growth on medium B; ○—○ *Aspergillus niger* strain 72–4; ●—● *A. niger* strain 72–44. Specific activity is expressed as enzyme units/mg. protein, one unit being defined as an initial rate of increase in extinction at 240 mμ of 0·001/min./ml. enzyme extract.

Table 1. *Specific activity of aconitase in Aspergillus niger strains grown on GMY medium*

Organism	Specific activity* Reaction citrate → *cis*-aconitate	Reaction isocitrate → *cis*-aconitate	Ratio isocitrate citrate
Exp. *a* *A. niger* 72–4	150	326	2·2
A. niger 72–44	154	366	2·4
Exp. *b* *A. niger* 72–4	106	207	2·0
A. niger 72–44	117	238	2·1

* Specific activity is expressed as enzyme units/mg. protein, one unit being defined as an initial rate of increase in extinction at 240 mμ of 0·001/min./ml. enzyme extract.

there was variation in the specific activities obtained, it is not possible to conclude whether or not there is any significant difference between the two organisms in this respect. Experiment (*c*) showed similar values in the two organisms, suggesting that the differences found in experiments (*a*) and (*b*) were not significant.

Enzymic studies under conditions of citric acid accumulation

Twenty flasks of medium B were inoculated with the parent organism and a similar number with the mutant strain 72–44. Sufficient amounts of material to yield 2 to 4 g. wet wt. mycelium were harvested 1, 2, 3, 5 and 8 days after inoculation and the corresponding enzyme extracts were prepared.

Mycelium which has accumulated citric acid contains some intracellular acid. Initially difficulty was experienced in obtaining active enzyme preparations through failure to keep the pH value of the extracts near to neutral. Active preparations were obtained by washing the mycelium with ice-cold water followed by the dropwise addition of M-KOH to maintain at pH 7·2 to 7·4; an extract was then prepared in the usual manner and assayed.

Table 2. *Specific activity of isocitric dehydrogenases in Aspergillus niger strains grown on GMY medium*

Organism	Specific activity*	
	NAD-linked isocitric dehydrogenase	NADP-linked isocitric dehydrogenase
Exp *a* *A. niger* 72–4	90	35
A. niger 72–44	51	22
Exp *b* *A. niger* 72–4	37	26
A. niger 72–44	22	12
Exp *c* *A. niger* 72–4	42	21
A. niger 72–44	35	19

* Specific activity is expressed as enzyme units/mg. protein, one unit being defined as an initial rate of increase in extinction at 340 mμ of 0·001/min./ml. enzyme extract.

Aconitase and both isocitric dehydrogenases were present during the entire incubation period. The activity of aconitase was about the same in the parent organism as in the mutant (see Fig. 2). The reactions citrate → *cis*-aconitate and isocitrate → *cis*-aconitate were again measured and there was little change in their ratio. The specific activity of aconitase during growth on medium B was about one-third of that found after 22 hr incubation on GMY medium.

The mutant appeared to have slightly higher values of both isocitric dehydrogenases than had the parent (see Fig. 3), but this difference may not be significant. The decrease in activity on the 5th day was unexplained, possibly some denaturation of enzymes took place during preparation of the extracts.

The influence of iron on aconitase activity

Some workers have reported that ferrous ions activate purified preparations of aconitase from mammalian tissues (Dickman & Cloutier, 1950, 1951; Morrison, 1954). By using Morrison's methods in the present work it was found that, when precipitated with ammonium sulphate and dialysed, the partially purified aconitase of *Aspergillus niger* could be activated 4-fold by adding iron and cysteine. Since iron is one of the metals which must be restricted to very low concentrations in the medium in order to obtain high citric acid yields, the relation between iron, aconitase activity and citric acid yield was investigated.

Aspergillus niger 72–44 was inoculated into medium B (Fe^{2+}, 2·0 mg./l.) and into the same medium supplemented with iron (Fe^{2+}, 40 mg./l.) Samples of mycelium were harvested 1, 2, 3, 5 and 8 days after inoculation. Each sample was analysed for the amount of organism, sugar utilized and citric acid yield (see Fig. 4). The presence of additional iron enhanced growth, causing the mould to form larger pellets. The

yield of citric acid decreased by about 25%, but there was no significant difference in the rates of sugar utilization. Aconitase values were about doubled (see Fig. 5), but the yield of citric acid was still high. Again, there was little change in the ratios of the two reactions catalysed by aconitase.

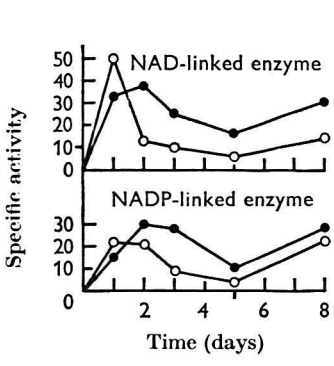

Fig. 3

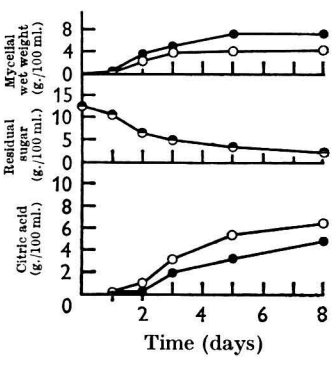

Fig. 4

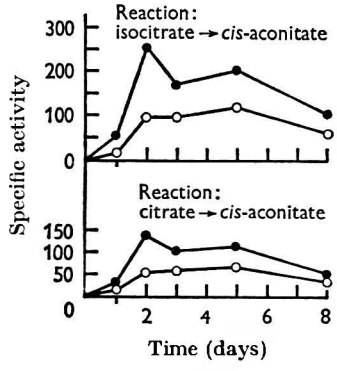

Fig. 5

Fig. 3. Specific activity of NAD-linked and NADP-linked isocitric dehydrogenases in *Aspergillus niger* strains during citric acid accumulation on medium B; ○—○ *A. niger* strain 72–4; ●—● *A. niger* strain 72–44. Specific activity is expressed as units/mg. protein, one unit being defined as an initial rate of increase in extinction at 340 mμ of 0·001/min./ml. enzyme extract.

Fig. 4. Rates of growth, sugar utilization and citric acid production by *Aspergillus niger* strain 72–44 on medium B containing different concentrations of iron; ○—○ Fe^{2+} 2·0 mg./l. ●—● Fe^{2+} 40 mg./l.

Fig. 5. Specific activity of aconitase in *Aspergillus niger* strain 72–44 during growth in medium B containing different concentrations of iron; ○—○ Fe^{2+} 2·0 mg./l.; ●—● Fe^{2+} 40 mg./l. Specific activity is expressed as units/mg. protein, one unit being defined as an initial rate of increase in extinction at 240 mμ of 0·001/min./ml. enzyme extract.

Effect of fluoroacetate on citric acid yield

Fluoroacetate is converted in mammalian tissues to fluorocitrate which is a potent inhibitor of aconitase (Morrison & Peters, 1954). The effect of fluoroacetate on both the parent and mutant *Aspergillus niger* strains was examined under conditions for citric acid accumulation to see whether the yields could be increased by the inhibition of aconitase. Fluoroacetate is most effective when present in the undissociated form (Aldous & Rozee, 1956). The pH value of medium B during growth of *A. niger* varied from pH 3·5 to 2·0, so that fluoroacetate would be present largely in the undissociated form. Sodium monofluoroacetate was added at the rate of 20 μmoles and 200 μmoles/flask at various stages during the incubation of *A. niger* cultures. At the end of the period, 8 days after inoculation, the flasks were analysed for yield of organism and citric acid, and for sugar utilization. Fluoroacetate depressed the yield of organism and of citric acid and the sugar utilization in both organisms.

DISCUSSION

The present investigation was done with the object of detecting differences in enzymic constitution as between the parent and mutant strains of *Aspergillus niger* which might indicate why the mutant strain produces higher yields of citric acid on the medium used. Previous enzymic studies of *A. niger* had indicated that, from the time when citric acid began to accumulate, aconitase and isocitric dehydrogenase were not present (Ramakrishnan *et al.* 1955). However, in that work it was not stated which isocitric dehydrogenase was examined. In the present work, aconitase and both the NAD-linked and the NADP-linked isocitric dehydrogenases were studied. No difficulty was experienced in obtaining active preparations of these enzymes from mycelium grown on a medium which favoured growth rather than citric acid accumulation. Provided that precautions were taken to maintain the pH value near neutrality during preparation of the extracts, the enzymes were also found throughout the incubation period of 8 days in both strains grown on a citric acid accumulation medium, a result in contrast to that of Ramakrishnan *et al.* These workers gave no details of the preparation of their extracts, nor did they mention precautions to avoid denaturation during preparation. It seems possible that inactivation of the enzymes could account for their results.

In *Aspergillus niger* the increased yields of citric acid at lowered metal concentrations may result from diminution in synthesis of the enzymes or restriction of their activity, for example, through lack of a metal co-factor. Iron is known to be required for maximal activity of aconitase from mammalian sources and is one of the critical metals involved in citric acid accumulation. Since it might influence accumulation by restriction of the activity of aconitase, the relation between iron and aconitase was examined. The addition to the culture medium of twenty times the optimal concentration for citric acid accumulation by *A. niger* 72-44, roughly doubled the amount of aconitase activity in that organism. Nevertheless, citric acid production was still quite high, being about three-quarters of that formed in the control culture. The addition of monofluoroacetate to the culture medium did not stimulate citric acid yield though it markedly inhibited the growth of the organism. Its toxic effect may have resulted from interference with reactions not involving aconitase (see Elsden & Ormerod, 1956; Callely & Dagley, 1959).

There was no significant difference in the amounts of aconitase or of the isocitric dehydrogenases in the two strains of *Aspergillus niger* grown in a medium in which citric acid did not accumulate. These enzymes were also found, though at lower concentrations, in the two organisms throughout fermentation. Thus, it is possible that the tricarboxylic acid cycle may function during fermentation, although the work of Cleland & Johnson (1954) indicated that there was little re-cycling of citric acid. The investigation did not reveal any significant differences in the quantities of these enzymes in the two strains of *A. niger* which might account for the observed difference in their ability to accumulate citric acid.

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Infrared Spectra of some Sulphate-Reducing Bacteria

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SUMMARY

The infrared absorption spectra of forty strains of sulphate-reducing bacteria have been recorded over the range 1800–800 cm.$^{-1}$. It was possible to identify positively *Desulfovibrio gigas* and the thermophilic *Desulfotomaculum nigrificans*. A broad differentiation was possible between salt-water and fresh-water forms of *Desulfovibrio desulfuricans*. *Desulfotomaculum orientis* and *D. ruminis* showed few characteristic differences from the fresh-water strains of *D. desulfuricans* but there was an indication that, with a more refined technique, at least one of the features otherwise characteristic of the other (thermophilic) desulfotomaculum species might be discerned. The results support to a considerable extent current views on the classification of the sulphate-reducers.

INTRODUCTION

A number of workers (e.g. Thomas & Greenstreet, 1954; Riddle *et al.* 1956; Rideal & Adams, 1957; Goulden & Sharpe, 1958; Scopes, 1962) have considered the use of infrared (i.r.) spectroscopy as an aid to the identification of bacterial species or strains. While i.r. spectra of bacteria are very similar from organism to organism, small differences are detectable both in intensities and in the presence or absence of weak absorption bands. The present work was designed to investigate the i.r. spectra of a range of sulphate-reducing bacteria and to determine whether the organisms could be grouped in a manner consistent with their i.r. spectra, regardless of their current taxonomic classification.

METHODS

Choice and preparation of bacteria. Forty strains of sulphate-reducing bacteria were examined, the choice being determined by availability. These are listed below with the NCIB number in brackets.

Desulfovibrio desulfuricans

Group 1. Fresh-water strains: (Saunders, Campbell & Postgate, 1964 and Dr L. L. Campbell, personal communication). Teddington M (8302), Hildenborough (8303), Wandle (8305), Holland D6 (8311), Beckton (8319), Marseilles (8386), Llanelly (8446), Monticello 2 (9442).
Salt-water strain: Walvis Bay (8397).

Group 2. Fresh-water strains: Essex 6 (8307), Teddington R (8312), Holland C6 (8372), Berre Eau (8387), Berre Sol (8388), 'Vibrio cholinicus' (9467). Salt-water strains: Norway 4 (8310), Venice 1 (8322), Canet 40 (8363), El Agheila Z (8380), Canet 20 (8391), Canet 32 (8392), Canet 41 (8393), Sylt 3 (9335), Aberdovey (9492).

Group 3. (Salt-requiring) El Agheila C (8308), California 43:63 (8364), Louisiana 43:11 (8365), Avonmouth (8398), British Guiana (8403).

Desulfovibrio gigas

Unnamed strain (9332).

Desulfotomaculum orientis (formerly *Desulfovibrio orientis*) (Campbell & Postgate, 1965)

Singapore I (8382), Singapore II (8445).

Desulfotomaculum nigrificans (formerly *Clostridium nigrificans* thermophilic)

Teddington garden (8351), Staines G (8353), Holland CT (8356), Delft 3T (8359), Delft 15T (8361), Delft 74T (8395), unnamed strain (8788).

Desulfotomaculum ruminis

Coleman 42 (8452).

The cultures were grown from the freeze-dried preparations at 30° or 55° as appropriate in Professor J. R. Postgate's modification of the medium C of Butlin, Adams & Thomas (1949; see Baker, Papiska & Campbell, 1962). NaCl 25 g./l. was added for salt-water forms. In each case, 1 ml. of stock culture (3–5 days old) was used as inoculum for 100 ml. of the above medium which was then incubated at the appropriate temperature under an atmosphere of nitrogen. The organisms were harvested by centrifugation for 7 min. at 16,000*g*, resuspended in 0·85% NaCl (w/v) and recentrifuged. The washing procedure was repeated 3 times and the final washed organisms suspended in 0·85% (w/v) NaCl sufficient to ensure a concentration of 10 mg. dry wt. organisms/ml. The spectrum was then determined immediately. Experience showed that no substantial differences were observed in the spectra obtained from 1-, 3- or 6-day cultures; 3 days was therefore taken as a standard incubation time.

Measurement of infrared spectra. The spectra were recorded on a Grubb–Parsons GS3 spectrometer. Programmed slits were used and loop gain and scanning speed were kept constant to minimize differences in response. The films were prepared on plates of the Kodak infrared optical material Irtran 2. This has advantages over the silver chloride plates used by earlier workers: a low background adsorption is obtainable and the use of a compensating plate in the reference beam is consequently unnecessary; in addition the material is quite stable and insensitive to light.

The suspensions of bacteria in 0·85 % (w/v) aqueous saline were evaporated on the Irtran plates under infrared lamps. The plates were tilted slightly to form a concentration gradient of organisms across the plates. The plate carrying the dried film was mounted in the spectrometer and rotated until the absorption was $90 \pm 2\%$ at 1650 cm.$^{-1}$ and 5% at 1800 cm.$^{-1}$.

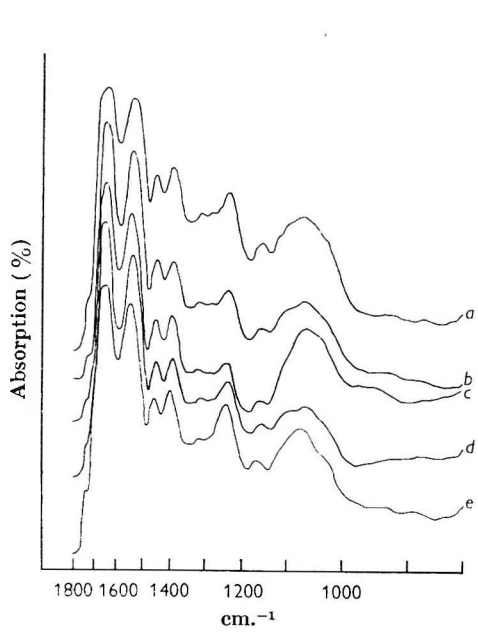

Fig. 1

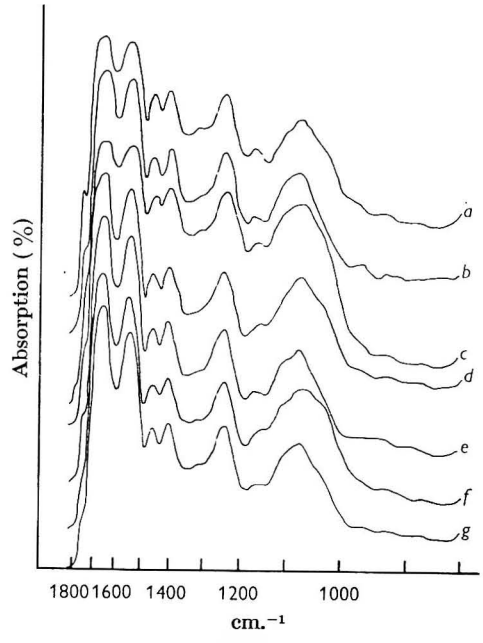

Fig. 2

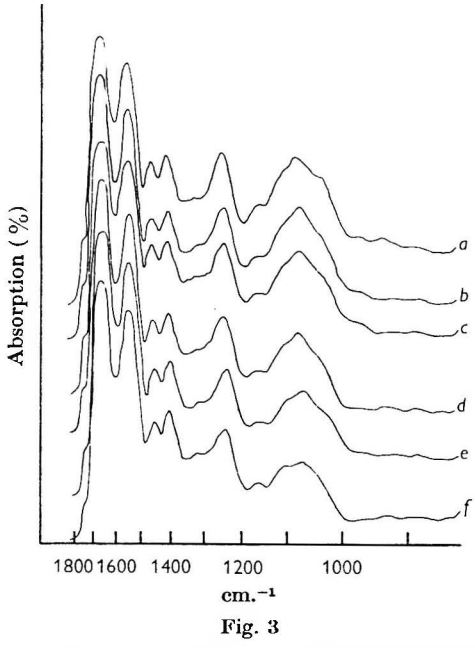

Fig. 3

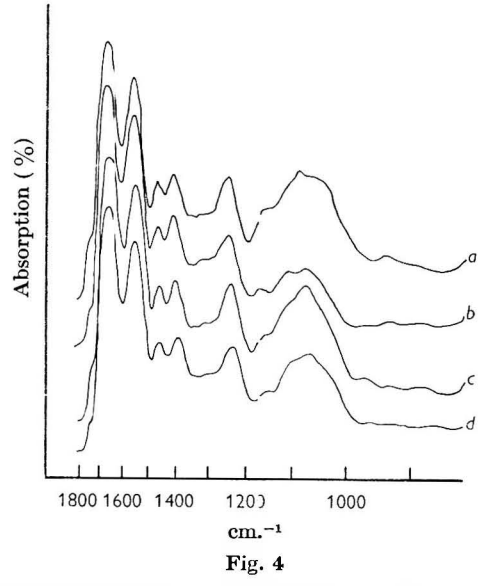

Fig. 4

Fig. 1. Typical infrared absorption spectra for thermophilic sulphate reducers, *Desulfotomaculum nigrificans*. *a*, Delft 15 T; *b*, Staines G; *c*, Teddington garden; *d*, unnamed; *e*, Aberdovey (mesophilic).

Fig. 2. Typical i.r. absorption spectra for mesophilic salt-water sulphate reducers *Desulfovibrio desulfuricans*. *a*, British Guiana; *b*, El Agheila C; *c*, Louisiana 43:11; *d*, El Agheila Z; *e*, Sylt 3; *f*, Canet 20; g, Norway 4.

Fig. 3. Typical i.r. absorption spectra for mesophilic, fresh-water sulphate reducers *Desulfovibrio desulfuricans*. *a*, Hildenborough; *b*, '*Vibrio cholinicus*'; *c*, Essex 6; *d*, Becton; *e*, Berre Eau; *f*, Singapore I.

Fig. 4. Infrared absorption spectra for some other sulphate reducers. *a*, *Desulfovibrio gigas*; *b*, *Desulfotomaculum orientis* Singapore I; *c*, *D. orientis* Singapore II; *d*, *D. ruminis*.

RESULTS

Figures 1–4 show examples of the spectra obtained, grouped according to their points of similarity. The spectra have been spread along the ordinate axis to avoid superposition.

Thermophilic organisms

In the 1350–1200 cm.$^{-1}$ region, the spectra of nearly all of the sulphate-reducing bacteria examined had a weak band at 1312 cm.$^{-1}$, followed by a plateau leading into the band of relatively moderate intensity at 1240 cm.$^{-1}$. A second, very weak, band was sometimes detectable at 1285 cm.$^{-1}$. In the present series this band appeared convincingly only in the thermophiles, with the single exception of strain Aberdovey. This organism is a mesophilic salt-water form and we were unable to obtain growth at 55°. *Desulfotomaculum ruminis* and the two examples of *D. orientis* showed a trace of the band at 1285 cm.$^{-1}$.

Mesophilic organisms

Salt-water strains. In all the spectra examined, the intense band at 1650 cm.$^{-1}$ had a higher frequency shoulder at about 1735 cm.$^{-1}$. This band varied slightly in frequency (it was at 1745 cm.$^{-1}$ for strain British Guiana) but more importantly in intensity. In most of the spectra, it varied between 15 and 25% absorption. In a small number of cases, it was in the range 25–40% and in these cases the broad weak band at 940 cm.$^{-1}$ also had a significantly higher intensity. The salt-water strains all had this characteristic with the exception of Norway 4. This strain is abnormal in other respects also (Miller & Saleh, 1964), specifically in that it lacks the usual pigment desulfoviridin.

Between the bands at 1650 and 1355 cm.$^{-1}$, the absorption generally fell to between 60 and 65%. However, in three cases, British Guiana, El Agheila C, and Louisiana 43:11 it fell to only 75–80%. This particular subgroup of the salt-water strains also exhibits different behaviour to inhibitors (Saleh, 1964): all three strains are members of Group 3 of Saunders *et al.* (1964), and are obligate salt-water forms.

Fresh-water strains. The spectra of more than half of all the organisms examined did not show any of the above characteristics. These were all in the group of mesophilic, fresh-water strains and included the organism '*Vibrio cholinicus*' (Hayward & Stadtman, 1959) that was reassigned to the species *Desulfovibrio desulfuricans* by Baker *et al.* (1962).

The three organisms *Desulfotomaculum orientis* strain Singapore I and Singapore II and *D. ruminis* (8452) also fell into this group, except that they gave some slight indication of the very weak band at 1285 cm.$^{-1}$ which was otherwise a characteristic of the thermophilic group. This subgroup of organisms have other properties distinguishing them from the 'normal' mesophilic, fresh-water sulphate-reducers, for example, they form spores (Adams & Postgate, 1961). The close resemblance between the i.r. spectra of *D. orientis* and the *D. ruminis* is of particular interest, since, at their original classification, all three were assigned to the same species, *Desulfovibrio orientis.*

The most exceptional sulphate-reducing bacterium is *Desulfovibrio gigas.* In the spectrum of this organism, a quite sharp band appears at 1080 cm.$^{-1}$ above the contour of the broad band extending from 1140 to 1000 cm.$^{-1}$. In no other spectrum was this band observed.

CONCLUSIONS

The sulphate-reducing bacteria can be divided into several reasonably well defined groups on the basis of their infrared absorption spectra. The groups correspond broadly with the current taxonomic classification of the organisms except that the members of the species of *Desulfovibrio desulfuricans* fall into two groups according to their marine or fresh-water origin, but without distinguishing between the groups of Saunders *et al.* (1964). *D. gigas* and *Desulfotomaculum nigrificans* are clearly distinguishable from other species. The other desulfotomaculum species are difficult to distinguish from the fresh-water desulfovibrio strains but there is some evidence that this might be possible with refined techniques. 'Cleaner' preparations, i.e. less contaminated with ferrous sulphide, and higher resolution spectroscopy are suggestions for refinement.

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Behaviour of *Paramecium aurelia* in Solutions of Purified and Fluorescent Labelled Tetanus Neurotoxin

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SUMMARY

An investigation of the effects on *Paramecium aurelia* of crude and a highly purified tetanus neurotoxin preparation free from oxidase and haemolysin activity was made to assess the value of this organism in a bioassay system and as a convenient organism for the study of the mode of action of the toxin. Whereas crude tetanus toxin produced cytotoxic changes in *P. aurelia*, purified tetanus neurotoxin did not even when the paramecia were exposed to ten human lethal doses.

Incubation of the paramecia with fluorescent-labelled toxin revealed that it accumulated within food vacuoles and in focal areas of cytoplasm but not in the cell membrane. Cytologic studies of the paramecia by means of light and electron microscopy revealed no abnormalities.

INTRODUCTION

Recent studies by Roux & Serre (1962, 1964*a*, *b*) have demonstrated cytotoxic effects on *Paramecium aurelia* exposed to partially purified tetanus toxin. If paramecia were specifically sensitive to tetanus neurotoxin in low concentrations, they would be valuable for bioassay of toxin fractions and serve as convenient material for the study of the mode of action of the toxin using cyto- and biochemical methods. For these reasons, the action of crude and highly purified tetanus toxin on *P. aurelia* was investigated.

METHODS

Paramecium aurelia containing cytoplasmic DNA was cultured by the method of Sonneborn (1947) and fed with *Aerobacter aerogense*. In the majority of the experiments, actively swimming organisms were used 12–24 hr after the last feeding.

Toxin preparations. Three preparations of tetanus toxin were utilized in these experiments.

1. Crude toxin; this was a filtrate of *Clostridium tetani* cultures obtained from Wyeth Laboratories, Radnor, Pennsylvania, containing 600,000 m.l.d./ml. In our assay system, one mouse m.l.d. was equal to $2{\cdot}5 \times 10^{-6}$ m.s.d. (minimum saturation dose: Sheff, Perry & Zacks, 1965). This material (referred to as crude toxin) contained 12–16 protein components, only one of which has neurotoxic activity (Sheff *et al.* 1965). The crude toxin also contained a highly active oxidase and was capable of partly uncoupling oxidative phosphorylation in isolated liver mitochondria (Sheff & Zacks, unpublished).

2. Purified toxin; this purified neurotoxin was obtained by chromatography on DEAE cellulose according to the method of Sheff *et al.* (1965) and had a m.s.d. of less than 10 μg./mouse, a dose equivalent to 4×10^4 m.l.d. This material was free from oxidase and haemolysin activity. Stock solutions containing 10 μg./ml. were made up in NaCl solution (0·85 g./100 ml.) and serially diluted before addition to the paramecia.

3. Fluorescent labelled toxin; purified tetanus toxin was labelled with rhodamine B200 chloride by the method of Rinderknecht (1962) without significant loss of activity. All unreated rhodamine was removed by column chromatography on G-25 Sephadex.

In some experiments, toxin was added directly to 10 ml. samples of the culture containing both bacteria and protozoa, but in most of the experiments the bacteria were removed before adding the toxin by sedimentation of the paramecia at 1000*g*. The protozoa were then sucked up with a Pasteur pipette and placed in culture medium free from bacteria. To observe the action of the tetanus toxin on the protozoa, approximately 50 actively swimming *Paramecia aurelia* were placed in small partially-covered watch glasses containing 10 ml. culture water. After a 60 min. period of temperature equilibration at 25°, 0·1 ml. of toxin preparation in saline was added by means of a syringe. Doses of 0·12–8 m.s.d. (0·0012–0·080 mg. toxin/ml.) were tested. After addition of the toxin, the paramecia were observed at 5 min. intervals for the first few hours and then intermittently up to 24 hr. The cultures were arbitrarily graded (on a scale of 0–5+) for motility. Mortality and morphological abnormalities were looked for under stereomicroscopic magnifications of $\times$10–30. The microscopic illumination was turned off between observations to avoid undue heating of the paramecia. The temperature remained at the range of 24° to 28° throughout most of the experiments; in a few experiments, the temperature was maintained at 37°.

Cytologic methods. After the bacteria-free cultures of paramecia were exposed to the action of tetanus toxin and observed for several hours, the protozoa were concentrated by slow speed centrifugation to a volume of 0·5 ml. and fixed by the addition of 40% formaldehyde to obtain a final concentration of 10% (v/v) or with 25% glutaraldehyde to obtain a final concentration of 6% (v/v) for fine structure studies. The fixed paramecia were washed and embedded in paraffin or Epon 812 by standard methods.

Fluorescent-labelled tetanus toxin. After exposing the protozoa to rhodamine-labelled toxin for various times, the paramecia were collected with a Pasteur pipette and examined in a Reichert ultraviolet microscope using a cardioid darkfield condenser. An ultraviolet pass filter IS/UGL was used at the ultraviolet source and 2XI/GG and Wratten 2B filters were used at the eyepiece. In some experiments, after exposure to labelled toxin, the paramecia were killed by osmium tetroxide or glutaraldehyde vapour and examined in ultraviolet radiation.

Detoxification. As it was possible that the paramecia were capable of detoxifying the tetanus toxin, the incubation medium was tested for toxicity after the paramecia had been exposed to the toxin for 6–24 hr. The protozoa were sedimented a 1000*g* and samples of the supernatant fluids were injected into mice which were then observed for signs of tetanus intoxication.

RESULTS

Effects of crude tetanus toxin

One to two minutes after addition of crude tetanus toxin to actively swimming paramecia, we observed a consistent slowing of their swimming. The cilia continued to beat and they continued to swim in a characteristic spiral pattern but at decreased velocity. Parallel control preparations showed free and active swimming. The organisms in the toxin solution also tended to hug the periphery of the watch glass and many organisms were immobile by 3–4 min. after addition of the toxin. By 30–40 min. all the organisms were dead and many showed clear blebs projecting from the periphery of the organisms similar to those described by Roux & Serre (1962). The control protozoa continued to swim at their initial velocity.

Effects of purified unlabelled toxin

When highly purified tetanus toxin in a concentration from 0·25 to 8 m.s.d./ml. was added to freely swimming paramecia, there was an initial slowing of swimming beginning 5–20 min. after exposure of the organisms to the toxin; this was followed by partial recovery. With the highest concentration (8 m.s.d.), onset of decreased activity occurred after 5 min.; and after partial recovery at 20 min., there was a second period of slowing followed by partial recovery (Fig. 1 A). Complete recovery had not occurred at the time the experiment was terminated. Paramecia in less concentrated toxin solutions (0·25–1 m.s.d.) required 15 min. to show decreased swimming activity and demonstrated a tendency to recover after 5½ hr of exposure to the toxin solutions (Fig. 1 B, C). Paraffin (7 μ) and Epon (1 μ) embedded sections from the sedimented paramecia collected at the termination of this experiment did not show any cytological abnormalities when examined either by light or electron microscopy.

When paramecia were kept for 24 hr in purified toxin solutions containing 0·5–0·25 m.s.d., occasional dead organisms were seen but not cytotoxic changes such as swelling or vacuolization were observed. In one experiment, ten human lethal doses (0·1 m.s.d.) was added to the paramecia and the preparation was observed for 24 hr. At the end of this period, they continued to swim actively without evidence of cytotoxic changes.

When samples of culture medium obtained after incubating paramecia with high concentrations of tetanus toxin (0·1–0·5 m.s.d.) was injected into groups of mice, all the mice died with signs of generalized tetanus in the time expected for equivalent doses of fresh purified neurotoxin.

Effects of fluorescent-labelled tetanus toxin

When doses of rhodamine-labelled purified tetanus toxin (0·1–1·0 m.s.d.) were added, the protozoa showed an initial slowing reaction as before but there was no swelling, vacuolization or death of organisms. When examined under ultraviolet radiation in the fresh state or after fixation with glutaraldehyde vapour, one to three vacuoles (10–12 μ diam.) filled with fluorescent label were observed (Pl. 1, fig. 1). These vacuoles corresponded in appearance and location with the food vacuoles observed in sectioned organisms. The cell membrane showed no labelled toxin but focal areas of the cytoplasm contained fluorescent-labelled toxin adjacent to the

food vacuoles. Paramecia which had not been exposed to fluorescent toxin showed diffuse blue autofluorescence of the cytoplasm and bright blue fluorescence in the macronucleus. Bright yellow autofluorescent granules (1–2 μ) were present in the cytoplasm and, in some areas, formed aggregates measuring up to 7 μ (Pl. 1, fig. 2).

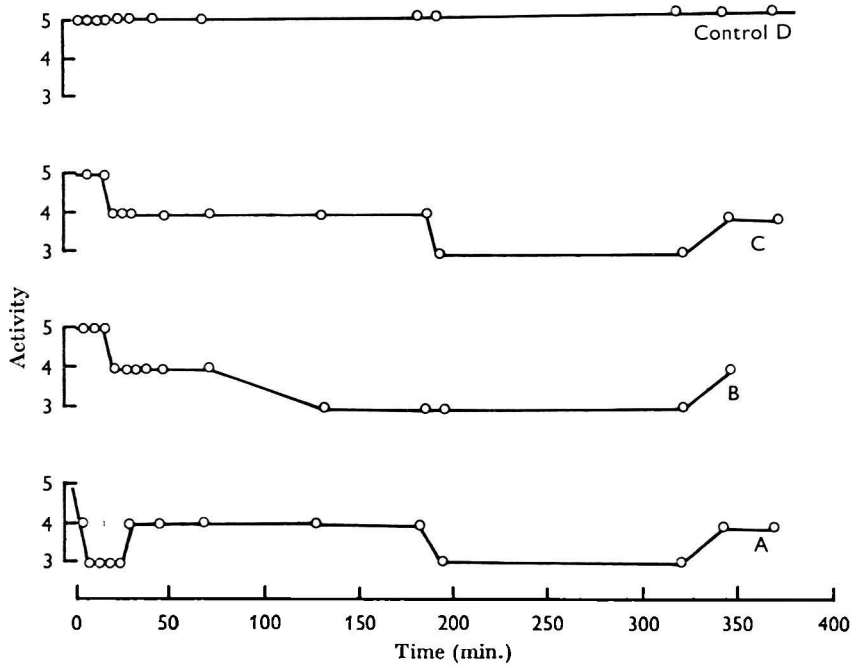

Fig. 1. A. Graph showing the effect of purified tetanus neurotoxin (8 m.s.d.) on the motility of *Paramecium aurelia*. Note the prompt decrease in swimming velocity followed by partial recovery.

B. Graph showing the effect of a lesser concentration of tetanus neurotoxin (1 m.s.d.) on the motility of Paramecia.

C. Graph showing the effect of tetanus neurotoxin (0·25 m.s.d.) on the motility of paramecia. Note the delayed onset of slowing of swimming and the tendency to recover.

D. Graph showing the motility of the control group of paramecia observed at the same time as the paramecia exposed to tetanus toxin (A, B, C). Activity is expressed on an arbitrary scale of 5 to 0, with 5 representing the motility of non-intoxicated paramecia.

DISCUSSION

This work has clearly shown that although crude tetanus toxin preparations containing numerous components had a cytotoxic effect on paramecia, a highly purified preparation which contained the neurotoxic principle of tetanus toxin does not. When exposed to crude toxic preparations paramecia showed swelling, bleb formation and cell death as reported by Roux & Serre (1962). We have previously observed marked swelling of isolated liver mitochrondria *in vitro* when they were exposed to similar concentrations of crude toxin (Zacks & Sheff, 1963). However, purified tetanus neurotoxin failed to produce mitochondrial swelling.

Cytologic changes were not found in the poisoned paramecia. The cytoplasmic mitochondria did not show abnormalities that have previously been observed in mouse and human muscle in tetanus poisoning (Zacks & Sheff, 1964; Zacks, Hall & Sheff, 1966).

Since the purified tetanus toxin solutions retained their lethality when injected into mice after incubation with paramecia, it is clear that the lack of cytotoxic changes during exposure to high concentrations of purified toxin was not due to destruction of toxin by the protozoa.

Experiments with fluorescent-labelled purified toxin demonstrated accumulation of the toxin within vesicles interpreted as food vacuoles and in focal areas of the cytoplasm. There was no binding to the pellicle. Beale & Kaeser (1957), using fluorescent-labelled antibodies to cilia proteins, found fluorescent labelled protein within food vacuoles and on the cell surface.

It would seem that the results of Roux & Serre (1962, 1964*a*, *b*) were probably due to a component of the crude tetanus toxic preparation used, rather than to the active neurotoxic principle itself. It is not surprising that Roux & Serre (1962) were able to show that tetanus antitoxin prevented the effects shown by their toxin preparations, because the usual commercial tetanus antitoxin preparation contains a large number of antibodies against many components of crude toxin as well as antibodies against the neurotoxic component (Sheff *et al.* 1965). We conclude that *Paramecium aurelia* cannot be used as a sensitive bioassay for tetanus neurotoxin.

I wish to thank Professor John R. Preer, Jr., of The University of Pennsylvania for supplying the initial samples of paramecia and information on their culture. Also, we wish to acknowledge the technical assistance of Miss Marilyn Salscheider and Mrs Steven Weiss and Miss Vicki Lavalle who helped in the preparation of the manuscript. This investigation was supported by a grant to The Pennsylvania Hospital by the John A. Hartford Foundation, Inc., New York.

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EXPLANATION OF PLATE

Fig. 1. Photomicrograph of a paramecium prepared as a whole mount and photographed in ultraviolet light. The bright objects (arrow) are yellow autofluorescent lipochrome granules. The cytoplasm shows pale autofluorescence. The line indicates 16 μ.

Fig. 2. Photomicrograph of a paramecium prepared as a whole mount and photographed in ultraviolet light after incubation in purified tetanus neurotoxin labelled with Rhodamine. The three bright objects (arrows) represent concentrations of red fluorescent tracer and the smaller bright objects in the cytoplasm are yellow pigment granules. The line indicates 15 μ.

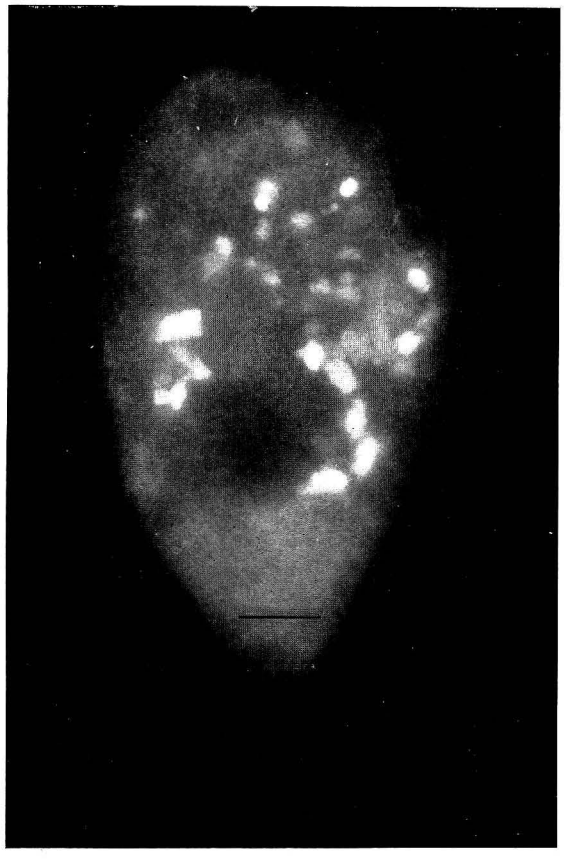

Fig. 1

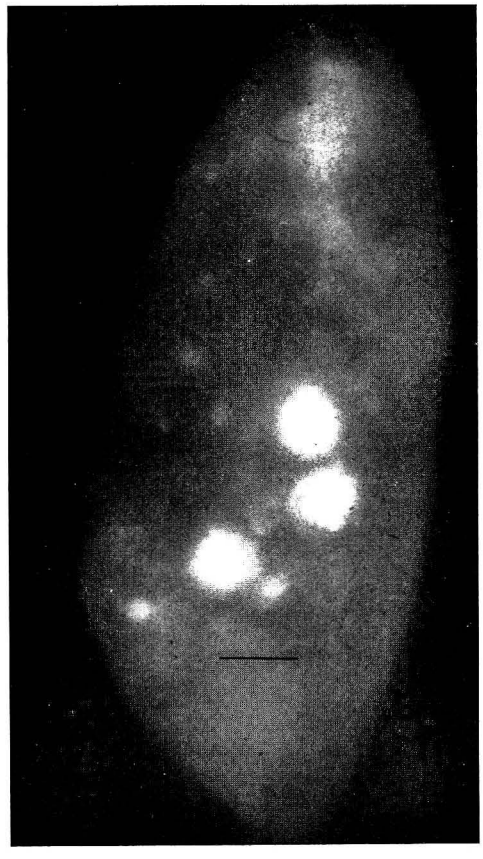

Fig. 2

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The Electrophoretic Movement of Proteins from Various Streptomyces Species as a Taxonomic Criterion

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SUMMARY

Attempts have been made to use the protein composition of streptomycetes as a criterion in their classification. By using polyacrylamide gel electrophoresis, negatively charged proteins in extracts of various streptomycete strains were separated. For each strain a distinctive pattern of protein bands was obtained. Protein bands were mapped for a number of strains, including several each of *Streptomyces venezuelae* and *S. griseus*. Maps were compared and contrasted: in general there was more similarity between strains within one species than between strains of different species. Although the polyacrylamide gel technique cannot yet be used as a major criterion in classification, it can serve, in conjunction with other criteria, as an aid in streptomycete taxonomy.

INTRODUCTION

The taxonomy of *Streptomyces* is still difficult and often uncertain, but progress is being made to evolve a convenient, workable and comprehensive means of classifying the streptomycetes. Taxonomic criteria such as sporophore morphology, spore wall structure, colour, and utilization of carbon sources are used with moderate success. For examples of this type of characterization see Hesseltine, Benedict & Pridham (1954), Pridham, Hesseltine & Benedict (1958), Gottlieb (1959), Pridham (1964), Pridham & Lyons (1956). Nevertheless, there is still an urgent need for new criteria that might aid in the recognition of species. Recently techniques of polyacrylamide gel electrophoresis have been developed to a point where the separation of proteins in relatively small amounts of extract can be achieved with good reproducibility. Studies on *Neurospora crassa* and *N. sitophila*, for example, indicated that mutant strains of these species could be distinguished from the wild type by their protein pattern (Chang, Srb & Steward, 1962). These intraspecific differences were smaller than interspecific differences. If similar relationships existed among the streptomycetes, the protein patterns could be used to aid in the characterization of species. The work reported in this paper was undertaken to ascertain whether the protein pattern of streptomyces species were stable characteristics of the species, and whether these characteristics were greater than intraspecific differences.

METHODS

The cultures used, their source, and the growth media are given in Table 1. One hundred ml. of liquid medium in 500 ml. Erlenmeyer flasks were used in all experiments. The streptomycete inoculum and the mycelium to be extracted were always grown in the same type of medium. Each strain was grown and extracted on at least two occasions but usually three times. The inoculum was a 48 hr shaken culture that had been homogenized for 10 sec. in a Waring blendor. Either 0·1 ml. or 0·2 ml. of the homogenate was added to each flask of fresh medium to grow mycelium for the experiments, and 15 or more flasks of a strain were used to obtain sufficient mycelium for each experiment. Inoculated flasks were incubated for 48 hr at 25° on a reciprocal shaker. The mycelium was harvested by centrifugation, washed three times with cold distilled water, and either stored at −10° or used immediately.

Table 1. *Streptomycetes studied and media on which they were grown*

The composition of the media were:

GA. Glucose asparagine medium: asparagine, 1·0 g.; $K_2HPO_4.3H_2O$, 4·0 g.; KH_2PO_4, 0·7 g.; $MgSO_4.7H_2O$, 2·0 g.; glucose, 10·0 g.; distilled water, 1000 ml.; adjusted to pH 7·0.

SM. Defined medium for *S. venezuelae*: KH_2PO_4, 0·7 g.; $K_2HPO_4.7H_2O$, 4·0 g.; NaCl, 0·3 g.; $MgSO_4.7H_2O$, 2·0 g.; $NaNO_3$, 2·0 g.; glycerol, 10·0 g.; sodium lactate (60% w/v), 13·3 g.; minor elements solution, 1·0 ml.; distilled water, 1000 ml.

E. Emerson solution: yeast extract, 1·0 g.; Bactopeptone, 4·0 g.; beef extract, 4·0 g.; sodium chloride, 2·5 g.; cerelose, 10·0 g.; distilled water, 1000 ml.

For stock cultures, 2% agar was included in the medium.

Species	Strain designation	Medium for stock cultures	Medium for experiments
Streptomyces griseus	NRRL B 1073	GA	GA, SM, E
S. griseus	NRRL B 2027	GA	GA, SM, E
S. griseus	NRRL B 1076	GA	GA, SM, E
S. griseus	NRRL B 1549	E	GA, SM, E
S. griseus	PD 04833	GA	GA, SM, E
S. venezuelae	NRRL B 902	E	GA
S. venezuelae	NRRL B 2277	GA	GA, SM, E
S. venezuelae	UC 2282	GA	GA, SM, E
S. venezuelae	UC 2014	GA	GA
S. orientalis	NRRL B 2450	E	SM, E
S. ramulosus	NRRL B 2714	E	SM, E
S. antibioticus	NRRL B 1793	E	SM, E
Streptoverticillium reticulatum subsp. *azocolutum*	NRRL B 1699	E	SM, E

* Cultures labelled NRRL were received from Dr T. G. Pridham (Northern Regional Laboratories); those labelled UC from Miss Alma Dietz (The Upjohn Co.) and the one labelled PD from Parke, Davis and Co., Detroit, Mich., U.S.A.

Five g. fresh weight of washed mycelium were ground for at least 7 min. with 10 g. acid-washed sand in a pre-cooled mortar, at 4°; 10 ml. cold water were then added gradually and grinding continued for 2 min. more. Sand and large debris were removed by centrifuging at about 2000*g* for 15 min. The supernatant fluid was centrifuged for 1 hr at 100,000*g*. Protein in the final supernatant fluid was determined by the method of Lowry, Rosebrough, Farr & Randall, 1951. For some experiments, cold phosphate buffer (0·1 M, pH 7·0) was used instead of water. In

preliminary experiments, extracts were dialysed against water or against 0·1 M-calcium chloride at 4°, but this procedure was abandoned in later experiments.

When the protein in the final supernatant fluid was less than 0·85–1·0 mg./ml. the solution was further concentrated. 'Aquacide' (California Corporation for Biochemical Research) was at first used; it removed up to 50% of the water in 1·5 hr, but the resulting protein bands on the stained gels were not sharp. Thereafter, concentration was achieved by leaving the dialysis bags containing extracts in an evacuated desiccator at 4° overnight.

'Canalco' Model 12 electrophoresis apparatus was used, and ready mixed stock solutions of polyacrylamide gels and of electrophoresis buffer for standard 7½% gels were obtained from Canal Industrial Corporation Ltd. (Bethesda, Md. U.S.A.). The procedure for preparation and polymerization of gels was similar to that described in the Canalco instructions. Glass tubes 6 cm. long with an internal diameter of 0·5 mm. were used to prepare the columns. The lower or separating gel was added first, 0·8 ml. to each tube, and these tubes were exposed to a fluorescent lamp for 40 min. Spacer gel, 0·1 ml., was then added and the tubes again exposed but for only 15 min. The streptomyces extract was then mixed with the upper gel solution so that there was at least one part gel to one part sample. The protein concentration of the extract had been previously adjusted with water so that 100–200 μg. protein was applied to each gel column, usually as 0·1 ml. of gel.

An electrophoresis buffer at pH 8·2–8·5 was used and a current of 4–5 mA per tube was applied to the column. The sample front, marked by the bromophenol blue tracking dye, usually reached 0·25 in. from the bottom of the tube in 25–35 min. At this stage, tubes were removed and immediately laid in an ice bath.

Removal of gels. Many gels broke when the recommended reaming needles were used. Instead, the tip of a fine hypodermic needle was ground flat to remove the point and the other end was attached by a Tygon tube to a water source. With water flowing, this needle was carefully inserted between the glass wall and the gel; the needle was turned as it was worked all round the column so that the water stream loosened the gel. This procedure was repeated from the opposite end of the tube and when the gel had loosened from the walls of the tube, it was blown out into water.

The bromophenol blue line marking the electrophoretic front disappeared after the staining and de-staining procedure routinely used. Since this front was used in making measurements of relative mobilities of protein bands, it was essential to know its position. To give reference for all measurements, the unstained gels were always cut at the final position of the tracking dye after their removal from the tubes and before staining them.

Amido black (0·5% in 7% acetic acid) was used in all experiments as a general protein stain. Washed gels were immersed and shaken in stain for at least 1 hr, then de-stained in several changes of 10% acetic acid until no more dye diffused from the gel. The de-staining was done in small Erlenmeyer flasks on a shaker, during a period of 6–24 hr. Ideally, the protein bands stained dark blue or pale blue in a relatively colourless gel.

No attempt was made to measure the band density quantitatively except to record them as light or dark (L or D). The position of the bands was the important criterion. For every gel stained satisfactory, the final protein band positions were

mapped and measured. Their positions were calculated as the ratio of the distance moved by the protein to the distance moved by the front (E_F). For any series of gels representing one particular streptomycete strain, certain dark stained or wide bands were easily recognizable as identical on all or most gels, with their E_F value in good agreement. The identities of some of the bands, particularly the lighter ones, were more difficult to determine, either because their position could not be accurately measured or because they were not consistently present (Table 2). The

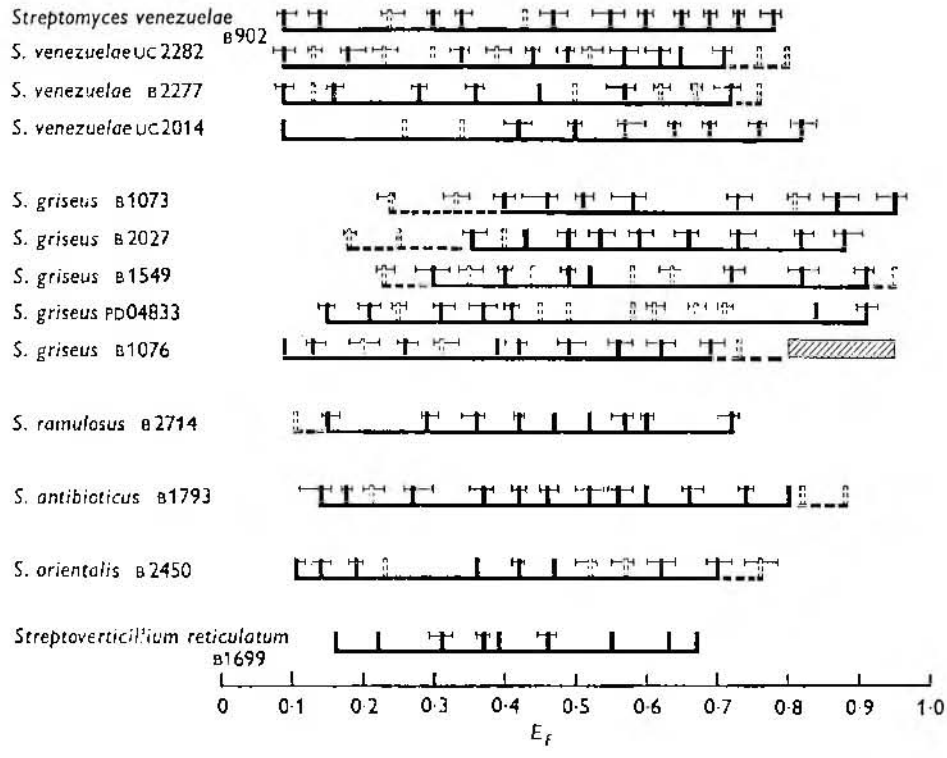

Fig. 1. Profile patterns of all protein bands observed in different species and strains of *Streptomyces*. The E_F values are the average for all experiments and replicates. Broad solid vertical lines represent bands occurring in more than 50 % of the gels. Broken lines represent bands occurring in less than 50 % of the gels. Actual limits of departures from the mean of each band is indicated by thin horizontal lines that cross the mean E_F lines of the profiles.

heavy bands were, therefore, used as markers. By knowing the relationship between the position of these light bands and the markers one could ascertain the identity of the light bands. Where there was reasonable agreement within and between experiments, and where a band occurred in more than 50 % of the gels for any one isolate, the band was established. Bands which occurred in less than 50 % of the gels for one strain have also been recorded. These are shown as broken lines in Fig. 1.

This method of recording was used to establish a series of protein bands as typical for each strain. The distribution of protein bands for each strain was plotted as average E_F value for each band. Thus one could distinguish established definite bands in a streptomyces isolate from those of more doubtful reliability, i.e. occurring

in fewer than 50% of gels, although in good agreement when they did occur. The resulting profile maps of the soluble proteins in various species or isolates could be rapidly compared with each other (Fig. 1).

RESULTS

The reproducibility of protein bands from any one isolate was generally good. However, the distance moved by the front in a given time varied, even within three replicate tubes of the same experiment. The calculation of the movement of a protein band as the ratio of the movement of the band to that of the front obviated this difficulty.

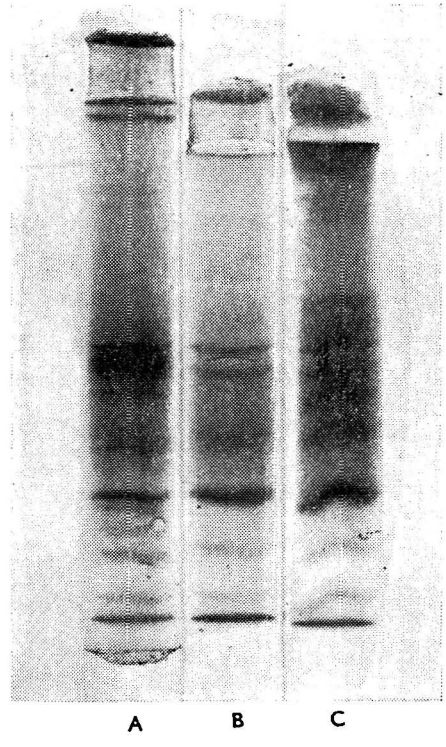

Fig. 2. Stained gels of extracts of *Streptomyces griseus* B 1073 that were grown and run on separate occasions.

Another problem was that the positions of the fainter bands sometimes could not be accurately measured and therefore were not always consistent, e.g. Table 2, band a. However, the E_F values of the main bands were readily measured and in good agreement even when the patterns of the gels did not look alike superficially. Figure 2 shows stained gels from three separate extracts of one strain of *Streptomyces griseus* NRRL B 1073, run on three separate occasions, A, B and C. Table 2 shows E_F values for bands on gels run from extracts of *S. griseus* B 1076 grown at different times. The main bands varied only little when extracts were made of any one isolate grown on different media.

The fresh weight of mycelium varied with the media; that grown in Emerson's

medium, for example, was as much as 10 times the weight of mycelium from the defined medium, after similar growth periods. Nevertheless, the protein band pattern was not affected by the medium in which the cultures were grown.

The extracts could be stored for at least 7 days in the cold with no apparent change

Table 2. *E_F values for a series of protein bands resolved on gels from four different extracts of one strain of Streptomyces griseus; strain B 1076*

		Protein bands											
		a	b	c	d	e	f	g	h	i	j	k	l
Extract*	Replicate runs†	E_F value of bands											
H	(i)	0·10	0·13	0·18	0·25	–	–	0·44	0·47	0·56	0·60	–	–
	(ii)	0·10	0·15	0·20	0·27	–	0·39	0·40	0·50	0·57	–	–	–
K	(i)	0·09	0·14	–	0·26	–	0·39	0·41	0·51	0·57	–	0·71	–
Z	(i)	0·10	0·12	–	0·27	0·30	0·38	0·42	–	0·58	0·64	0·70	0·72
	(ii)	–	0·13	0·18	0·27	0·30	0·39	0·42	0·48	0·56	–	0·70	–
	(iii)	0·09	0·12	–	0·25	–	0·38	0·43	0·50	0·54	0·61	0·70	–
AA	(i)	–	0·12	0·20	0·25	–	–	0·42	0·46	0·57	0·64	0·68	0·72
	(ii)	–	0·13	–	0·26	0·34	–	0·40	0·51	0·55	0·63	0·68	0·72

* Each lettered extract was from *S. griseus* B 1076 grown on different occasions.

† Each run had at least two replicate tubes. –, No band observed in the E_F range examined.

Table 3. *Average E_F values for each protein band observed in various strains of Streptomyces griseus*

	Strain of *S. griseus*				
	B 1073	B 2027	B 1549	PD 04833	B 1076
Band	E_F value				
a	–*	–	–	–	0·9 (62 %) D
b	–	–	–	–	0·13 (82 %) L
c	–	–	–	0·15 (100 %) L	–
d	–	0·18 (40 %) L	–	–	–
e	–	–	–	0·21 (55 %) L	0·20 (50 %) L
f	0·24 (36 %) D†	0·25 (40 %) D	0·23 (33 %) D	0·25 (27 %) D	0·26 (100 %) D
g	0·33 (35 %) L	–	0·30 (67 %) L	0·31 (67 %) L	0·31 (37 %) L
h	–	0·36 (60 %) L	0·35 (33 %) L	0·37 (66 %) L	0·39 (62 %) L
i	0·40 (78 %) D	0·40 (40 %) D	0·40 (83 %) D	0·41 (64 %) D	0·42 (100 %) D
j	0·46 (78 %) L	0·43 (60 %) L	0·44 (50 %)L	0·45 (33 %) L	–
k	0·51 (78 %) D	0·49 (60 %) D	0·49 (83 %) D	0·49 (18 %) D	0·49 (37 %) D
l	–	0·54 (80 %) D	0·52 (66 %) D	–	–
m	0·58 (78 %) D	0·59 (80 %) D	0·58 (33 %) L	0·58 (33 %) L	0·56 (100 %) D
n	–	0·66 (100 %) L	0·64 (50 %) L	0·61 (27 %) L	0·62 (62 %) L
o	–	–	–	0·67 (45 %) D	0·69 (75 %) D
p	0·73 (78 %) D	0·73 (100 %) D	0·72 (83 %) D	0·71 (27 %) L	0·73 (37 %) L
q	0·81 (35 %) L	0·82 (80 %) L	0·82 (83 %) L	0·84 (82 %) L	–
r	0·87 (85 %) D	0·88 (80 %) D	–	–	–
s	–	–	0·91 (66 %) L	0·91 (54 %) L	–
t	0·95 (57 %) L	–	0·95 (42 %) L	–	–

* The symbol – signifies that no bands were observed in this position.

† D and L refer to the intensity of the protein band as dark and light, respectively. Parentheses enclose the percentage of times a band could be observed in gels of all experiments on the isolate.

in pattern of protein bands. To test for denaturation of proteins, one extract was stored at 4° and run after 1, 3 and 8 days, respectively; there was no change in pattern even after 8 days, provided that bacterial contamination was avoided. In practice, all the runs on any one extract were completed within 3 days. The ability to store these extracts allows the comparison on the same day of cultures harvested at different times.

Other investigators (Chang *et al.* 1962) have extracted protein from fungi in phosphate buffer (pH 7·0). However, cold distilled water did as well phosphate buffer for these streptomycetes and water at 4° was routinely used for extraction. Dialysis against water or against salt solutions did not change the protein pattern, so this procedure was discontinued.

Table 4. *Average E_F values for each protein band observed in strains of Streptomyces venezuelae*

	Strain of *S. venezuelae*			
	B 902	UC 2282	B 2277	UC 2014
Band	E_F value			
a	0·09 (75 %) D†	0·09 (80 %) D	0·09 (75 %) D	0·09 (100 %) D
b	0·14 (88 %) D	0·13 (40 %) L	0·13 (15 %) L	–
c	–*	0·18 (60 %) L	0·16 (75 %) D	–
d	0·24 (50 %) L	0·23 (40 %) L	–	–
e	0·30 (75 %) L	0·30 (20 %) L	0·28 (63 %) L	0·26 (20 %) L
f	0·34 (75 %) D	0·34 (60 %) D	0·36 (88 %) D	0·34 (20 %) L
g	–	0·39 (47 %) L	–	–
h	0·43 (50 %) L	0·44 (73 %) D	0·45 (63 %) D	0·42 (100 %) D
i	0·47 (75 %) D	0·49 (53 %) D	0·50 (50 %) D	0·50 (60 %) D
j	0·55 (63 %) L	0·52 (40 %) L	–	–
k	0·60 (75 %) D	0·57 (60 %) D	0·57 (100 %) D	0·57 (100 %) D
l	–	0·62 (60 %) D	0·62 (50 %) D	–
m	0·65 (75 %) D	0·65 (60 %) D	–	0·64 (100 %) D
n	0·69 (63 %) L	–	0·67 (37 %) L	0·69 (80 %) L
o	0·73 (75 %) D	0·71 (63 %) D	0·72 (69 %) D	–
p	0·78 (63 %) L	0·76 (50 %) L	0·76 (47 %) L	0·76 (80 %) L
q	–	0·80 (33 %) L	–	0·82 (80 %) L

* The symbol – signifies that no bands were observed in this position.

† D and L refer to the intensity of the protein band as dark and light, respectively. Parentheses enclose the percentage of times a band could be observed in gels of all experiments on the isolate.

A comparison of the E_F values of the bands in different isolates of *Streptomyces griseus* (see Table 3) revealed a number of bands common to all or most of the five strains. For example, f, i, k, m and p occurred in all five strains and g, h, j, n and q in four strains. For any named band, the frequency of occurrence and the intensity of staining (dark or light) was not the same for all isolates. The frequency of band k was low, only 18 % for PD 04833, and high for all other isolates. Band p was light in PD 04833 and B 1076 but dark in all others. This difference in intensity of colour is related to the concentration of a specific protein in the extract. A few bands have been observed in one or two strains only, e.g. band a. It is reasonable to expect such qualitative and quantitative differences between strains of a species. In four of the isolates there were no bands with E_F values less than 0·15 and the upper range varied from 0·88 to 0·95. Isolate B 1076, however, had some slower moving

proteins, but none of the faster moving ones, above E_F 0·73. The number of missing bands varied with the isolate. There was good agreement among the E_F values (1) in the replicates in one experiment, (2) in repeated studies of the same extract, (3) in different extracts of one isolate, (4) among the different isolates. No one isolate of *S. griseus* contained all the possible bands.

Four isolates of *Streptomyces venezuelae* were thoroughly tested for the pattern of negatively charged soluble proteins; their E_F values and densities, dark or light, are listed in Table 4. The data show that many proteins were common to all the isolates of *S. venezuelae*, bands a, b, e, f, h, i, k, etc. Isolate UC 2282 contained the greatest number of soluble protein bands; it did not have band n which was present in the other three isolates. Isolate UC 2014 lacked seven bands which were present in UC 2282, whereas B 902 and B 2277 lacked four and five bands, respectively. The movement of the proteins ranged from E_F 0·09 to 0·82.

The band pattern for *Streptomyces ramulosus*, *S. antibioticus*, *S. orientalis* and *Streptoverticillium reticulatum* are shown in Fig. 1. Among these three *Streptomyces* species there is again a distinct and definite distribution of soluble proteins to form a series of bands which are different from other species in the genus. *Streptoverticillium reticulatum* had a relatively small number of discernible protein bands whose E_F values ranged only from 0·16 to 0·67 (Fig. 1).

DISCUSSION

As with other criteria that have been used in the description of streptomyces, the protein pattern of their extracts cannot be described in absolutes. Many of the protein bands are distinguishable with various degrees of resolution for there is a gradation from those protein bands whose identity is always unmistakable, to others that are too faint to make their identification certain. Even the absolute length of movement may vary from tube to tube in any one experiment. Best of all characteristics is the ratio between the movement of a band and the movement of the front, the E_F value.

For all gels from the four extracts of *Streptomyces griseus* strain B 1076, the variation of E_F of a protein band which occurred in all tubes was only slight (see for example, Table 2, band b). The main source of variation was in the absence of certain bands in some of the replicates, e.g. band j in Table 2. Even when a band was absent from some tubes, there were bands in other tubes whose E_F values were sufficiently alike to indicate that a protein regularly occupied that region (see band a in Table 2). Results from the other strains showed similar departures from the ideal agreement for E_F values of all bands. The soluble proteins seem to have relatively fixed E_F values under the conditions of the experiment, but many extracts would have to be run to establish this absolute E_F value for each protein. The departure from the mean E_F value of a band (shown for each band in Fig. 1) never overlapped with any of the other bands for that isolate. This individuality of each band justified considering it a distinct protein.

A survey of the protein band patterns of all the *Streptomyces griseus* isolates showed that strains B 1073, B 2027 and B 1549 were very similar (see Fig. 1 and Table 3). Their bands ranged from E_F 0·15 to 0·95. On the other hand, strain B 1076 had bands with a very different range of E_F values, 0·09–0·74, and also a region of

diffuse staining with no detectable bands from 0·77 to 0·98. In strain B 1076, the 'missing' bands were c, d, j, l, q, r, s and t; the 'new' bands were a and b. (Lower case letters here refer to Table 3.) This marked difference in protein bands in B 1076 strengthens the separation made by Pridham (1964) when he placed this grisein-producing strain in a subspecies—*griseinus*. Pridham also classified PD 04833 in another subspecies, *S. griseus* sub.sp. *purpureus*; however, the protein profile of PD 04833 is not as distinct and more closely resembles the remaining three strains of *S. griseus* than does B 1076.

Among the *Streptomyces venezuelae* extracts, the profiles of B 902, UC 2282 and B 2277 were very similar (see Fig. 1), whereas the strain UC 2014 differed from them in the absence of some bands (see Table 4, bands b, c, d, g, j, l and o). This is puzzling, since strains UC 2014 and B 902 both originated as transfers from *S. venezuelae* U.I. 8–44, more than 15 years ago. Subcultures were sent to different collections but this period was before the freeze-dry storage procedures of today were commonly used, and variation among the subcultures is not unexpected. Both strains are still similar in their own identifying characters. There are of course numerous examples of a change in some strain characteristics with time in culture or storage.

The similarities between patterns of strains of one species are different enough from those of other species to serve as species characteristics. Thus, the protein bands of *Streptomyces venezuelae* strains all have E_F values in the range 0·09–0·82, whereas *S. griseus* isolates (except B 1076) have values 0·15–0·95. Furthermore, no bands have been observed which are common to all strains of both species.

The protein band patterns of *Streptomyces orientalis* and *S. ramulosus* resemble that of *S. venezuelae* more than *S. griseus*. In addition, *S. orientalis* has some bands in common with *S. venezuelae* UC 2282. Generally the differences are sufficiently great to distinguish these species from either *S. griseus* or *S. venezuelae*. *S. antibioticus* and *Streptoverticillium reticulatum* (Fig. 1) had few bands in common with any other species, and each had E_F values in a range distinct from each other and from other species. Their bands thus also could aid in distinguishing them as definite species.

It is obvious that the polyacrylamide gel technique could not be used at present as a general procedure in which all protein bands from one extract were compared with all those from extracts of many other species. It can serve, however, to help separate or identify one streptomycete from another or a few other closely related streptomycetes. For this purpose both the range of E_F values and the presence or absence of particular well defined bands could be used.

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The Amino Acid Composition of Algal Cell Walls

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SUMMARY

The cell walls of six different species of algae were prepared in highly purified form and their amino acid and amino sugar contents determined qualitatively. A limited number of ninhydrin-positive components was found, implying that the walls contain peptide rather than protein. In five of the algal walls, those of the Chlorella species, a Scenedesmus and a Lyngbya, there were eight amino acids found in common: aspartic acid, glutamic acid, glycine, alanine, serine, valine, leucine, isoleucine. In addition to these common amino acids, the walls contained sometimes proline or hydroxyproline and different combinations of amino sugar which were characteristic of the species. In the sixth algal wall preparation, that of Nostoc, only five of the eight common amino acids were found along with hydroxyproline. Muramic acid and diaminopimelic acid were found in Lyngbya walls but in none of the others, excepting Nostoc walls which may have contained small amounts of diaminopimelic acid. Carotenoids were found associated with crude wall preparations of some of these algae. The above is taken as evidence for the existence of wall peptide in algae similar to that found in bacteria. This finding strengthens the phylogenetic relationship between the blue-green algae and the other algae, and it raises the possibility that classification of the algae may be aided by knowledge of the chemistry of the wall.

INTRODUCTION

In most investigations of the chemistry of algal cell walls, complex amino acid and carbohydrate patterns have been found and have been ascribed to the existence of protein and mixtures of polysaccharides in the walls. In those investigations the analyses were performed either on whole algae extracted with water and alkali (e.g. Cronshaw, Myers & Preston, 1958; Iriki & Miwa, 1960) or on mechanically disrupted organisms washed with water (Northcote, Goulding & Horne, 1958, 1960). The use of such preparative procedures leaves open the question of whether the walls are intrinsically complex or whether a simpler amino acid and carbohydrate composition is masked by cytoplasmic contamination of the wall preparations. In comparable studies of the walls of bacteria, water washing was seldom adequate to remove such contamination and more drastic procedures had to be adopted (Salton, 1953; Cummins & Harris, 1956*a*). In the case of the very complex walls of Gram-negative bacteria, treatment with detergents, phenol and other reagents had

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to be used to remove some of the wall components and reveal the simpler, underlying mucopeptide (Weidel & Primosigh, 1957; Mandelstam, 1961).

If algal cell walls are as difficult to purify as bacterial walls, then perhaps some of the algal 'wall proteins' and 'wall polysaccharides' previously described actually arose from contaminating membranes or from insoluble portions of organelles that could not be removed by centrifugal water washing of extracted whole or punctured organisms. In this case, more extensive washing of algal cell-wall preparations might reveal simple amino acid and carbohydrate compositions. The present work was done to see whether this was the case. A preliminary report of the results obtained has appeared elsewhere (Punnett & Grieg, 1961).

METHODS

The algae used in this study were obtained from the Algal Culture Collection at Indiana University and are identified by their I.U. culture collection numbers. They were grown in inorganic media with gas phase air + 4% (v/v) CO_2 over a fluorescent light source. The culture media had the following compositions (concentrations in mM unless stated otherwise): for the chlorella species (Emerson–Chalmers medium) KH_2PO_4 1·5, KH_2PO_4 2·5, KNO_3 10, $MgSO_4$ 6, NaCl 0·5, $Ca(NO_3)_2$ 0·5, $FeSO_4$ 20 μM/l, A_5, B_9 (the A_5 and B_9 micronutrient solutions described by Brody and Emerson, 1959); for *Scenedesmus obliquus* (Gaffron medium) K_2HPO_4 1·0, K_2CO_3 1·0, NH_4NO_3 2·5, $MgSO_4$ 0·2, NaCl 0·15, $Ca(NO_3)_2$ 0·1, $FeSO_4$ 20 μM/l., A_5 B_9; for the lyngbya, anabaena, nostoc species; KH_2PO_4 1·0, tris (OH form) 5·0, Na_2CO_3 15·0, KNO_3 10·0, $MgSO_4$ 1·0, $Ca(NO_3)_2$ 0·3, $FeSO_4$ 20 μM/l. A_5, B_9.

The algae were harvested by centrifugation and washed several times with 0·05 M-KCl. The washed algae were made to a 50% (v/v) or more suspension in water, mixed with three to four weights of glass beads and ground for 15–30 min. in a Virtis homogenizer (Model 23). After the beads settled out, the suspension of broken algae was decanted, the beads washed several times with small samples of distilled water and the washes pooled with the broken algae. This suspension was centrifuged for 10 min. at 8000 to 10,000*g* and the supernatant fluid discarded. This degree of centrifugal force was needed to sediment the cleanest walls. The pellet, which contained some whole algae, the broken algae and other cellular debris, was washed 4 to 8 times with distilled water at 10,000*g*. It was then washed with M-NaCl 4 to 6 times. The wall suspensions were next washed with water and then 0·1% Na laurylsulphate 2 to 4 times. The detergent was removed by two water washes. This salt and detergent washing cycle was often repeated 3 to 5 times.

Following this treatment, the crude wall preparation was treated in one of two ways. Some samples were extracted with a 1+3 mixture of 82% (w/v) formic acid + dimethyl formamide at room temperature for 2–4 hr and re-extracted several times with this solvent. They were then water washed and fractionated by high-speed centrifugation as described below. Other samples were fractionated directly without the organic solvent extraction. The crude wall preparations were centrifuged at 20,000*g* for 45–60 min. in narrow diameter tubes so that the heterogeneous particles were packed into different layers. The top layer was white and loose-packed, while the layers beneath were successively tan, brownish green and green. These layers were removed from the pellet one at a time by gently scraping and

rinsing with distilled water. Care was taken to prevent mixing of the layers. The separate fractions so obtained were refractionated in the same manner two or three times more. The white pure wall fractions obtained in this way were lyophilized and weighed. The yields varied from 2 to 5 mg. dry weight for each 400 mg. dry wt. starting material (about 4 g. wet weight). During the preparation, sodium azide was added to the wall suspensions whenever they were left standing in water to prevent bacterial growth (Cummins & Harris, 1956*a*).

The criteria for the purity of the wall fractions were: (1) the white colour of the fraction when packed by centrifugation; (2) absence of particulate material inside or around the broken walls when examined microscopically. The brown colour found in some fractions was almost always correlated with the presence of small particles trapped inside the algal walls. These particles showed up especially clearly when viewed with a phase-contrast microscope and in white light phase contrast frequently took on a different hue from that of the walls.

For amino acid analysis, 3–5 mg. samples of the pure walls were hydrolysed in 5–6 N-HCl for 18 hr. The hydrolysates were dried in vacuum over P_2O_5 and NaOH, taken up in water and re-evaporated several times to remove HCl. Qualitative analysis of the amino acids was done by two dimensional paper chromatography with the solvent system 1 of Redfield (1953) developed by ninhydrin + acetic acid at room temperature. In most cases, the solvent methanol + pyridine + water was used in one dimension and phenol + water (Smith, 1960) in the other, to check the identity of the amino acids in the serine, glycine, alanine region of the chromatogram.

RESULTS

Of the ten algae investigated in a preliminary survey, four were found to be experimentally unsuitable. When *Anabaena variabilis* (I.U. 394) was broken, the intracellular contents formed a gel which was not dissolved by any procedure except great dilution which resulted in loss of the walls. The walls of *Chlamydomonas reinhardii* (I.U. 90) proved to be so fine that the wall fraction was lost when attempts were made to remove other organelles by repeated centrifugation. The crude wall fractions of a spirogyra and a cladophora dissolved and were lost when suspended in sodium laurylsulphate, a reagent found necessary in this study if thoroughly clean walls were to be obtained. These difficulties were not insuperable, but in view of the limited amounts of crude wall fractions available and the abundance of more suitable algae, no further attempts were made to purify the walls of these four algae. The other six algae *Chlorella pyrenoidosa* (I.U. 252), *C. vulgaris* (I.U. 398), *C. ellipsoidea* (I.U. 247), *Scenedesmus obliquus* (I.U. 393), *Lyngbya* sp. (I.U. 622) and *Nostoc* sp. (I.U. 387) yielded purified walls in quantities sufficient for qualitative analysis of their amino acid composition.

Amino acid composition

In chromatograms of acid hydrolysates of the purified walls, an unmistakable relation between amino acid content and species was found (Table 1). Considering first the four green algae, eight amino acids occurred in high concentration in all four. These were aspartic acid, glutamic acid, glycine, alanine, leucine, isoleucine, valine and, in lesser amount, serine. In addition to the eight common amino acids,

Table 1. *Qualitative amino acid and amino sugar composition of algal cell-wall hydrolysates*

Major components	*Chlorella pyrenoidosa*	*C. vulgaris*	*C ellipsoidea*	*Scenedesmus obliquus*	*Lyngbya* sp.	*Nostoc* sp.	*Platymonas subcordiformis**
Glutamic acid	+	+	+	+	+	+	+
Aspartic acid	+	+	+	+	+	+	+
Alanine	+	+	+	+	+	+	+
Glycine	+	+	+	+	+	+	+
Valine	+	+	+	+	+	+	+†
Leucine	+	(+)	+	+	+	−?	+†
Isoleucine	+	+	+	+	+	−?	+†
Serine	+	+	+	+	+	−?	+
Proline	+	−	−	+	+	−	+
Hydroxyproline	−	+	−	−	−	+	−
Diaminopimelic acid	−	−	−	−	+	.?‡	−
Arginine	−	−	−	−	−	−	+
Threonine	−	−	−	−	−	−	+
Glucosamine	+	+	+	−	−	−	−
Galactosamine	−	+	+	−	−	−	−
Yellow unknown§	+	+	+	−	−	−	−
Muramic acid	−	−	−	−	+	−	−
Late-appearing unknown	−	−	+	−	−	−	−
Minor components							
Phenylalanine	+	−	−	−	−	+	−
Tryosine	+	+	+	−	−	−	−
Methionine	−	+	+	−	−	−	−†
Cysteine	−	−	−	+	+	−	−
Lysine	−	−	−	−	−	.?‡	−

* Results of R. A. Lewin (1958).

† Methionine/valine and leucine/isoleucine not resolved.

‡ Small amounts of lysine or possibly DAPA were found in nostoc wall hydrolysates.

§ 'The $R_{alanine}$ values for the yellow unknown were 1·00 in the methanol/pyridine solvent and 0·18 in the tert-butanol 1/2 butane/diethylamine solvent. The values for the late-appearing unknown were 1·35 and 0·09 respectively.'

each species had no (*Chlorella ellipsoida*) or one additional amino acid: *C. vulgaris*, hydroxyproline; *C. pyrenoidosa* and *Scenedesmus obliquus*, proline. The three chlorellas had two or three additional ninhydrin positive components. Two of them were common to the three species: an unknown giving a yellow colour with ninhydrin and glucosamine. The third, galactosamine, was found only in *C. vulgaris* and *C. ellipsoidea*. Another major component of *C. ellipsoidea* walls did not appear on the unheated chromatogram until 4–6 months after the ninhydrin treatment.

Of the two blue-green algae studied, the walls of lyngbya had the same '8 × X' pattern as found in the green algae. In addition to the 8 common amino acids listed above, these contained diaminopimelic acid (DAPA) and proline. Another ninhydrin positive unknown was found near alanine. This compound was probably muramic acid which has already been reported as a component of the walls of the closely related blue green phormidium (Frank, Lefort & Martin, 1962), and of microcoleus (Salton, 1964). In one preliminary experiment, the walls of nostoc were found to be different from the others in that they did not have the same 8+X pattern of amino acids. Only six of the 8 'common' amino acids were found: leucine and isoleucine were apparently absent and serine was present in greatly reduced amounts; hydroxyproline was present with traces of lysine (DAPA ?) but muramic acid was definitely not present in this alga.

Also included in Table 1 are the amino acids found in the walls of platymonas by Lewin (1958) because, despite the lack of resolution of leucine/isoleucine and valine/methionine by the method used, the similarity to the amino acid distribution patterns found in the present work is striking.

The amount of serine found in the wall hydrolysates of all these organisms was always roughly half that of the other major components to judge by the intensity of the colour of the spot on the chromatogram compared with standard chromatograms.

Minor components

When the amino acids designated 'minor components' in Table 1 were found, they were always present in much smaller amounts than the major components. They were variable constituents of the different wall preparations, both qualitatively and quantitatively, and were sometimes entirely absent. Impure wall preparations were always found to contain these minor components. In one series of experiments, samples of impure wall preparations of scenedesmus and lyngbya were analysed before and after successive washes with formic acid + dimethyl formamide. The major components were qualitatively and quantitatively unaffected by these washings but the minor components were removed. These observations lead to the conclusion that the minor amino acids probably arose from protein contamination and were not part of the wall itself. Critical evidence ruling out the possibility that they were wall components has not yet been obtained.

Pigments

Carotenoids were associated with walls of several of the algae. This was most easily seen when the preparation procedure was modified as follows. After breakage and extensive water washing, the crude walls were washed with M-NaCl and finally with water. At this stage, the wall fraction prepared from *Chlorella pyrenoidosa* and *Scenedesmus obliquus* were pink while the lyngbya and nostoc preparations were

orange. These pigments were removed from nostoc and lyngbya walls by shaking them either with 0·1 % sodium laurylsulphate or formic acid + dimethylformamide. *Chlorella pyrenoidosa* walls were not rendered completely colourless by the detergent treatment but the residual pigment was removed with formic acid + formamide or phenol. The crude wall fraction prepared from *C. ellipsoidea*, the smallest of this group of algae, was usually pale green, probably because of chloroplast fragment inclusions which were not removed by simple washing.

Preliminary evidence about the nature of these carotenoid pigments was obtained from reversed phase paper chromatography (Angapindee, Silberman, Tantivatana & Kaplan, 1958). The pink pigment from *Chlorella pyrenoidosa* and the orange nostoc pigment appeared to be carotenols, while the pink scenedesmus pigment and the orange lyngbya pigment were carotenes. This latter orange pigment changed to a pink carotenol during the running of the chromatogram. The only absorption spectra determined were those of the nostoc carotenol which had a single symmetrical band at 467–470 mμ in methanol, and the lyngbya carotene which had peaks at 442, 470 and 500 mμ in methanol.

These pigments do not seem to have originated from the chloroplast or photosynthetic lamellae. In one experiment, the 10,000*g* supernatant fluid from lyngbya was extracted and the pigments chromatographed and compared to the wall pigment. The pigments from the photosynthetic lamellae were chromatographically different from the corresponding wall pigments.

DISCUSSION

The results obtained in this work of the amino acids found in algal cell walls differ substantially from those obtained in earlier studies. The reason for this difference was probably the preparative technique used, in particular the washing with M-NaCl, which presumably removed nucleoproteins, and with detergent which removed adsorbed protein. Washing these walls with water alone did not yield clean preparations. Another significant difference in preparation was the removal of walls containing contaminating inclusions by centrifugal layering, a technique described by Miller & Phaff (1958). Unless both these procedures were used, the amino acid pattern obtained showed the degree of complexity expected of a protein hydrolysate.

Because of the relatively simple amino acid composition found in thoroughly washed algal walls, it is clear that they contain peptide but do not contain protein. Some protein was found adsorbed to water-washed walls, as already suggested by Northcote (1963), but it was removed by treatment not likely to break covalent bonds. By contrast, the limited numbers of amino acids found in the walls resisted extraction with detergent, phenol and formic acid + dimethylformamide and were undoubtedly covalently bonded. Simple amino acid compositions such as these were reported previously for walls of platymonas released during cell division (Lewin, 1958).

It should be noted that these procedures yielded wall preparations which were analogous to those of Cummins & Harris (1956*a*) in that all easily soluble constituents were removed and only the tough, insoluble wall fraction was analysed. The efforts to remove contaminating membranes and organelles may also have

removed loosely bound components of the envelope, such as an outer slime layer. For this reason, the thoroughly washed material which we define as the wall of the alga may represent something less than the complete envelope of the organism.

The existence of simple amino acid patterns in cell walls has thus been extended from Gram-positive bacteria (Salton, 1953) to Gram-negative bacteria (Weidel & Primosigh, 1957) and actinomycetes (Cummins & Harris, 1956*b*) to unicellular and filamentous algae. It is tempting to speculate that purified walls of yeasts and fungi prepared in the same way will also be found to possess simple amino acid patterns. In this regard, the amino acid content of the walls of trigonopsis reported by SentheShanmuganathan & Nickerson (1962) were similar to those reported here, in that nine or ten amino acids were present. In addition, Northcote & Horne (1952) found only six amino acids in a saccharomyces wall preparation. These latter results may have been due to incomplete hydrolysis, however, because in later reports from the same laboratory (Korn & Northcote, 1960) 18 to 20 amino acids are reported in yeast walls. Protein was found by Dyke (1964) in nadsonia wall preparations which had been washed with dilute NaCl, which does not dissolve nucleoproteins, but not with detergent. These walls were prepared in such a manner that they would still possess their mannan-protein complex and, in addition, may have had some cytoplasmic membrane contamination. Dyke's experimental criteria for lack of cytoplasmic contamination depended on two assumptions; that 0·155 M-NaCl removed cytoplasmic protein and that added cytoplasmic protein was in equilibrium with broken cell walls. The first assumption would not have been valid if nadsonia walls are as difficult to purify as algal walls and the second assumption is not likely to have been correct because the walls of the freshly broken cells must already have had membranes tightly adsorbed to them. Since these are among the most extensively washed yeast walls that have been analysed, it is clear that a simple amino acid pattern will not be found in yeasts, if it exists, unless more drastic procedures are used to purify them. The amino acid compositior of yeast walls will be further complicated in those many cases in which protein-polysaccharide complexes (Falcone & Nickerson, 1956) or enzymes (Wilkes & Palmer, 1932) are located in the wall itself.

The pattern of the distribution of amino acids in algal walls was surprisingly regular. The finding that eight amino acids were common to three chlorella species, a scenedesmus and a lyngbya is reminiscent of the situation found in the Gram-negative bacteria in which all genera have the same amino acids in the walls. The distribution of amino acids in algal walls is also similar to that found in the walls of Gram-positive bacteria in that the amino sugars and the amino acids other than the common eight were characteristic of genus and species. It may be that there is a common basal structure in the walls of algae, except for some blue-green algae (see below), as has been proposed for bacteria (Work, 1957). The hope that the minor variations in this regular pattern can be used as an aid in classifying the algae is premature, because examples of only five or six species have been studied. Furthermore, the only amino acids found so far, in addition to the common eight, have been proline and hydroxyproline: enough to distinguish only four species. Lewin's finding of arginine and threonine in platymonas walls (1958) suggests that other patterns will be found.

The failure to find diaminopimelic acid (DAPA) in the chlorella species was rather

surprising in light of the report of its presence in *Chlorella ellipsoidea* (Fujiwara & Akabori, 1954) which was confirmed by Hoare & Work (1957). Perhaps the DAPA is present in chlorella only as an intermediate in the biosynthesis of lysine (Vogel 1959) rather than as a wall component. The contrast between the unusual amino acid distribution and the presence of unknowns reported in this study and the conventional distribution found by Fowden (1954) and by Wagner (1962) in their studies of algal amino acids was to be expected, however, because these authors examined the extractable amino acids in the algae, and in some instances discarded the wall fraction.

The two blue-green algae examined have already provided evidence of heterogeneity even though the nostoc results are preliminary. DAPA, muramic acid and the common eight amino acids were present in lyngbya, but muramic acid was not found in nostoc. Nostoc did contain proline and traces of lysine or possibly DAPA while serine, leucine and isoleucine appeared to be missing from the common eight. The absence of muramic acid and possibly DAPA from nostoc distinguishes this organism from the other blue-green algae which have been examined. The significance of this finding, if confirmed, is that the phylogenetic relation between the blue-green algae and the bacteria is not so clear and simple as has been claimed. When first DAPA (Work & Dewey, 1953) and then muramic acid (Frank *et al.* 1962) were found in blue-green algae, these results were taken as confirming the proposed close phylogenetic relation between these groups. The earlier arguments in favour of this relation used by Stanier & Van Niel (1941) were that in both groups there is: (1) lack of organized nuclei; (2) lack of chloroplasts; (3) lack of sexual reproduction. The opposite point of view was presented by Pringsheim (1949) who pointed out that the bacteria and the blue-green algae differ; (1) morphologically (2) in photosynthetic pigments; (3) cytologically; (4) in not showing any striking physiological similarities; (5) in modes of locomotion. Many of the arguments supporting both sides of this question must be modified because of recent developments, especially in the areas of sexual reproduction, cytological fine structure and mechanism of photosynthesis. On balance, recent investigations, with the possible exception of the studies of cell walls, have tended to emphasize the differences between the two groups. The finding in the present work that muramic acid and DAPA are probably absent from the walls of some blue-green algae weakens the argument for close relationship based on cell-wall chemistry. Certainly the flat statement that the blue-green algae stem directly from a bacterial line (Frank *et al.* 1962) is too strong. The blue-green algae are undoubtedly transition organisms, but they have a closer relation to the other oxygen-evolving photosynthetic algae than they do to bacteria.

The carotenoids found in the crude wall preparations of algae most likely originated in the limiting membrane. That they did not come from the chloroplasts was shown by the evidence given above. They were associated with the walls during the early stages of fractionation and were removed by the detergent wash or by phenol treatment. This conclusion is tentative, however, because there is no way to rule out the possibility of the adsorption of other pigmented organelles to the crude walls. The association of pigments with cell walls of other organisms is known from other studies (Mason & Powelson, 1958; Salton, 1960). The significance of this pigmented layer around the periphery of some algae is that the photosynthetic action

spectra of these organisms will be depressed in the region of carotenoid absorption. Such depressions have often been reported but have been ascribed to the low photosynthetic efficiency of the carotenoids in the chloroplast. This conclusion may have to be modified in cases in which algae with pigmented walls were used. Significantly, the strain of *Chlorella pyrenoidosa* analysed was very possibly Emerson's strain 3. Unfortunately, there is no evidence from this study which allows an estimation of the proportion of total carotenoids found in the membrane or the absorption it causes.

There are many questions left unanswered in this initial study: the nature of the sugars in the walls, the nature of the unknowns, the configuration of the amino acids and their linkages to the rest of the wall and the overall amino acid and carbohydrate distributions in other algal genera. Until methods for the preparation of larger amounts of pure material are developed, however, progress will be slow.

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Effects of Polyene Antibiotics on Growth and Sterol-induction of Oospore Formation by *Pythium periplocum*

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SUMMARY

The effect of polyene antibiotics upon growth and sterol-induction of oospore production by *Pythium periplocum* was studied. Filipin and fungichromin markedly inhibited growth and prevented reproduction. Amphotericin B also prevented oospore production but only slightly inhibited growth. Pimaricin slightly inhibited growth and greatly decreased reproduction. Nystatin usually had no effect on growth and slightly stimulated reproduction. At 4 μg./ml., amphotericin B and fungichromin prevented oospore induction by 1 μg. cholesterol/ml. but had no effect on growth. Growth inhibition by amphotericin B and fungichromin was almost completely annulled by higher cholesterol concentrations, and prevention of oospore formation was partially annulled. These results generally support the hypothesis that polyene antibiotics act by interfering with the action of cellular sterols, but the growth inhibition which occurred on sterol-free medium suggests that a second mechanism may be operative.

INTRODUCTION

Pythium and phytophthora species, which comprise a destructive family of plant pathogenic fungi, require an exogenous source of sterol for reproduction by sporangia or oospores (Chee & Turner, 1965; Elliott, Hendrie, Knights & Parker, 1964; Hendrix, 1965; Klemmer & Lenney, 1965). Growth of some (Chee & Turner, 1965; Hendrix, 1964, 1965), but not all (Hendrix, 1965), species was stimulated by sterols such as cholesterol; also growth of some species may be stimulated by cholesterol but not by other sterols (Hendrix, 1964). In a liquid medium growth stimulation of *Phytophthora parasitica* var. *nicotianae* was dependent upon good aeration (Hendrix, Norman & Apple, 1966). Species of Pythiaceae appear to be unable to synthesize the 3-β-hydroxy sterols predominant in plants, animals, or fungi. Sterols such as cholesterol, ergosterol, stigmasterol and the sitosterols are readily precipitated by digitonin; yet digitonin-precipitable compounds were not found in extracts of pythiaceous fungi grown on a sterol-free medium (Elliott *et al.* 1964; Hendrix, 1966). *Pythium aphanidermatum* and *Phytophthora palmivora*, unlike related fungi, were unable to incorporate acetate into digitonin-precipitable compounds (Hendrix, 1966).

Polyene antibiotics, which inhibit a wide spectrum of fungi, had little effect on pythium and phytophthora species and were used in selective media for isolating these fungi (see Eckert & Tsao, 1962; Vaartaja & Rumbeeris, 1964). Gottlieb *et al.*

(1961) first proposed that the mechanism of action of polyene antibiotics involves an interference by the antibiotic with an essential cellular function of sterols. Since then considerable evidence (reviewed in detail by Demel, van Deenen & Kinsky, 1965) has indicated that polyene antibiotics alter permeability by interacting with membrane sterols. The present experiments were designed to test the hypothesis that polyene antibiotics would not affect growth of a pythium species but would prevent oospore formation.

METHODS

Organism. The test fungus *Pythium periplocum* Drechs. was chosen because its rapid but sparse linear growth on the medium used facilitated direct microscopic observation. Under the conditions of these experiments, it produced oospores in response to cholesterol (Hendrix, 1965) or other sterols (Hendrix, 1964); but on sterol-free media the growth was completely vegetative.

Chemicals. Polyene antibiotics used and their sources were: fungichromin (Merck Sharp & Dohme Research Laboratories, Rahway, N.J.); filipin (potency 1000 μg./mg.; The Upjohn Company, Kalamazoo, Mich.); nystatin (Mycostatin Process C), potency 4400 units/mg., and amphotericin B Type 1, potency 842 μg./mg. (The Squibb Institute for Medical Research, New Brunswick, N.J.); pimaricin (Myprozine), potency 975 μg./mg. (American Cyanamid Company, Princeton, N.J.). Cholesterol (U.S.P.) was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The cholesterol preparation used had the same effects on growth and reproduction as a highly purified sample (Hendrix, 1964).

Medium and cultivation. The medium contained 5·4 g. glucose, 1·5 g. $NaNO_3$, 1·0 g. KH_2PO_4, 0·5 g. $MgSO_4.7H_2O$, 2 mg. thiamine HCl, 17 g. agar/l. deionized water. The medium was adjusted to pH 6·0 before the medium was autoclaved for 10 min. at 121°. The medium was cooled to 55°, and the antibiotics and cholesterol were added with rapid agitation as dimethyl sulphoxide and anhydrous ethanol solutions, respectively. The media were then pipetted into 9 cm. Petri plates (25 ml./plate), and the plates seeded after the agar had solidified.

Inoculum was prepared by transferring the fungus growing on water agar to a small piece of thebasal medium agar placed on another water agar plate. The inoculum (3 mm. discs) was taken from the border of the resulting colony after about one week. The fungal material used had been transferred 5–10 times on sterol-free medium.

The plates were incubated at 22° in paper bags in closed cabinets because of the light sensitivity of polyene antibiotics. They were exposed to light no more than 10 min. daily for growth measurements or oospore counts.

Oospore counts. All oospores in a single ×100 microscope field (2·63 mm.^{2}) of each of the five to seven replicates of each treatment were counted. Counts were made about 1 cm. from the inoculation point. When considerable growth inhibition had occurred, this distance was decreased proportionally. All oospores from the bottom of the plate to the upper surface of the agar were counted. Eventually the mycelia underwent autolysis and the medium became cloudy. Oospore numbers then seemed to decrease, but we believe the apparent decrease was due to the opacity of the medium, not to an actual decrease. Thus the maximum number of

oospores recorded are given here. The time required for maximal oospore production ranged from 5 to 12 days and averaged 8 days.

Solvent effects. Final solvent concentrations for all treatments, whether or not antibiotics or cholesterol was present, were 1 μl. ethanol/ml. and 5 μl. dimethyl sulphoxide/ml. Solvent controls were included. Growth was not affected or was stimulated 5–10% by 1 μl. ethanol/ml. sterol-free medium. Dimethyl sulphoxide was usually 5–10% inhibitory whether or not cholesterol or ethanol was present. Neither solvent appeared to affect reproduction.

Statistics. Variances of various treatments for growth data were found to be equal; therefore an analysis of variance and least significant difference (L.S.D.) for each experiment was computed. Because variances of various treatments for oospore data were unequal, standard errors for each treatment are given.

RESULTS

Effect of polyene antibiotics on growth and reproduction

With the exception of nystatin, all five antibiotics inhibited growth (Table 1). When this experiment was repeated, nystatin was not always inhibitory; the other antibiotics were. Filipin and fungichromin were markedly inhibitory. The toxicity of fungichromin, but not filipin, was partially annulled by cholesterol. Oospore formation was completely blocked by amphotericin B, filipin and fungichromin (Table 1). Oospore production was greatly decreased and was erratic in the presence of pimaricin (Fig. 1). Nystatin initially retarded but eventually slightly stimulated oospore formation (Fig. 1, Table 1).

Table 1. *Effect of polyene antibiotics on growth and oospore production by Pythium periplocum on a medium without and with cholesterol*

Growth measurements were at 71 hr after inoculation. Concentrations of antibiotics and cholesterol were 15 μg./ml. and 1 μg./ml. respectively.

	No cholesterol		Cholesterol	
Antibiotic	Growth (mm.)	Oospores/field	Growth (mm.)	Oospores/field
Amphotericin B	45	0	60	0
Filipin	23	0	17	0
Fungichromin	7	0	26	0
Nystatin	57	0	58	74
Pimaricin	49	0	57	22
None	61	0	67	67
L.S.D. 0·05	4	.	4	.
L.S.D. 0·01	5	.	5	.

Effect of antibiotic concentration on growth and reproduction

Amphotericin B and fungichromin were chosen for further study because amphotericin B blocked reproduction but had relatively little effect on growth and fungichromin blocked reproduction and greatly inhibited growth. Concentrations of 1 μg./ml. or less of both antibiotics had no effect either on growth or reproduction (Table 2). Both antibiotics at 4 μg./ml. had no effect on growth but completely blocked oospore production on medium containing 1 μg. cholesterol/ml. At 16 μg./ml., both antibiotics inhibited growth.

Table 2. *Effect of antibiotic concentration on growth and oospore production by Pythium periplocum on a medium without and with cholesterol*

Growth measurements were made at 72 hr after inoculation. Oospore numbers with standard errors are maximal values which occurred 7 or 8 days after inoculation. Cholesterol concentration was 1 μg./ml.

		No cholesterol		Cholesterol	
Antibiotic	Antibiotic (μg./ml.)	Growth (mm.)	Oospores/ field	Growth (mm.)	Oospores/ field
Amphotericin B	0	54	0	67	55 ± 15
	0·25	54	0	69	51 ± 15
	1	51	0	71	45 ± 14
	4	48	0	70	0
	16	39	0	47	0
Fungichromin	0	54	0	67	55 ± 15
	0·25	53	0	64	48 ± 10
	1	53	0	68	60 ± 10
	4	49	0	65	0
	16	5	0	27	0
L.S.D. 0·05		8		8	
L.S.D. 0·01		10		10	

Effect of cholesterol concentration on antibiotic influences

The toxicity of fungichromin and amphotericin B to growth was considerably decreased as the cholesterol concentration was increased to 10 μg./ml. (Fig. 2). The block to oospore production produced by fungichromin but not amphotericin B was partially annulled by cholesterol at 10 μg./ml. (Table 3). In other experiments in which the ethanol concentration was raised to permit higher concentrations of

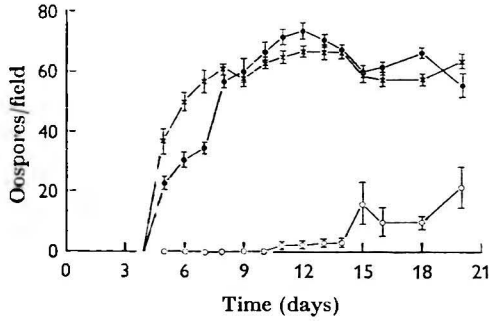

Fig. 1

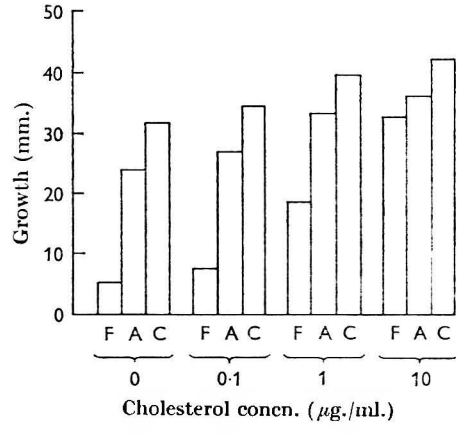

Fig. 2

Fig. 1. Oospore development by *Pythium periplocum* on a cholesterol-containing medium supplemented by polyene antibiotics. Pimaricin (○), nystatin (●), no antibiotic (×). Brackets enclose ± standard errors.

Fig. 2. Effect of cholesterol concentration on growth of *Pythium periplocum* on a medium with and without polyene antibiotics. Fungichromin (F), amphotericin B (A), control (C). Growth measurements were at 47 hr after inoculation. Antibiotic concentrations were 15 μg./ml. L.S.D. 0·05, 2·0 mm. L.S.D. 0·01, 2·7 mm.

cholesterol, limited oospore formation occurred on media containing 15 μg. amphotericin B/ml. and 50 μg. cholesterol/ml. However, these experiments are difficult to interpret because the higher ethanol concentrations (5 μl./ml.) decreased growth and blocked reproduction at low concentrations of cholesterol.

Table 3. *Effect of cholesterol concentration on oospore production by Pythium periplocum on a medium without and with antibiotics*

Oospore numbers with standard errors are maximal values which occurred 7 days after inoculation. Antibiotic concentrations were 15 μg./ml.

	Cholesterol (μg./ml.)			
	0	0·1	1	10
Antibiotic	Oospores/field			
Amphotericin B	0	0	0	0
Fungichromin	0	0	0	27 ± 10
None	0	0	114 ± 18	207 ± 10

DISCUSSION

The results generally support the sterol hypothesis about the mode of action of polyene antibiotics since three of the five antibiotics completely blocked oospore formation, a fourth significantly decreased oospore formation and the fifth retarded the initial rate of oospore formation. The eventual stimulation of oospore formation by nystatin is consistent with the experience of Vaartaja & Brambeeris (1964), who found that high nystatin concentrations (500 μg./ml.) stimulated oospore formation by some pythium species. These data agree with the generalization that filipin is the most potent, and nystatin the least potent, of the common polyene antibiotics, amphotericin B and pimaricin occupying intermediate positions (Demel *et al.* 1965).

The growth responses of *Pythium periplocum* to polyene antibiotics support the sterol hypothesis in some cases but suggest some other mechanism in others. The extreme growth inhibition on sterol-free medium by filipin and fungichromin suggests another mechanism. Growth inhibition by amphotericin B and fungichromin was annulled by higher concentrations of cholesterol, but the cholesterol may have been preventing toxicity by forming a sterol+antibiotic complex, as suggested by Lampen, Arnow & Safferman (1960). Fungichromin was considerably more inhibitory to growth than amphotericin B, but inhibition of oospore formation by fungichromin was more easily annulled by cholesterol than by amphotericin B. At 4 μg./ml., neither amphotericin B nor fungichromin affected growth, but both at this concentration blocked oospore production. However, this may merely reflect different sensitivities of growth and reproduction to stimulatory or toxic compounds, for growth stimulation was observed at cholesterol concentrations too small to induce oospore formation.

In most cases the polyene antibiotics did not mask the stimulatory effect of cholesterol on growth. With filipin, however, the fungus grew better on sterol-free medium than on medium containing cholesterol, suggesting an analogy with lysis of *Mycoplasma laidlawii* by filipin only when the mycoplasma was cultured in the presence of cholesterol (Weber & Kinsky, 1965). However, amphotericin B

had an effect similar to that of filipin upon *M. laidlawii* (Feingold, 1965), while in the present work cholesterol appeared to annul to some extent the toxic effect of amphotericin B.

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Isolation and Serological Analysis of Mutant Forms of Flagellar Antigen *i* of *Salmonella typhimurium*

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SUMMARY

Nine spontaneous mutants with altered forms of flagellar antigen *i* were obtained by picking more rapidly spreading swarms from growth in semi-solid medium containing enough anti-*i* serum to retard spreading growth. One mutant was in a line of *Salmonella typhi* given antigen *i* by transduction, the rest in *S. typhimurium* strain LT2 *adeC-7 proA-46*. Two mutants of independent origin were serologically identical and presumably arose by a recurrence of the same mutation. Bacteria expressing the mutant phase-1 antigen were normally motile and the LT2 mutants showed normal phase-variation, to give cultures with an apparently unaltered phase-2 antigen, *1,2,3*. Flagellate bacteria with flagella of two of the mutant types, *i*M6 and *i*M9, were agglutinated to titres 8–16 by sera from uninoculated rabbits and to titres 50–100 by sera from rabbits immunized with unrelated antigens; suspensions of flagella of these types, but not of others, caused flocculation of indian ink.

The residual activity of anti-*i* (wild type) sera fully absorbed with mutant antigens showed that each mutant antigen had lost some of the serological specificity of the wild-type antigen; the complex pattern of residual activity on mutant and wild-type antigens of anti-*i* (wild type) sera absorbed with pairs of mutant antigens indicated the existence of at least 13 antigenic factors in the wild-type antigen, and that each of the serologically distinct mutant antigens lacked a different combination of these factors. The residual activities on the homologous antigens of anti-mutant sera fully absorbed with wild-type antigen showed that all the mutant antigens, except perhaps *i*M6 and *i*M9, had antigenic specificities absent from the wild-type antigen. Each of the 8 serologically different antigens had a unique new specificity, but antigens *i*M7, *i*M10 and *i*M12 shared some new factors.

Attempts to infer the linear order of the presumed sites of amino acid substitution in the polypeptide chain of flagellin from the serological data were unsuccessful; this probably indicates the incorrectness of an assumption involved: namely, that anti-flagellar antibodies have an absolute affinity for, and only for, all of the amino acid side-chains (or all of a reactive subset of them) in a relevant length of polypeptide chain.

INTRODUCTION

The numerous phase 1 and phase 2 flagellar antigens of different salmonella serotypes or species, used for their classification in the Kauffmann–White table,

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are determined by a series of allelic genes at two loci, *H1* and *H2* (Lederberg & Edwards, 1953) which are widely separated on the linkage map (Smith & Stocker, 1962; Mäkelä, 1964). The flagella of salmonellas are composed of a single protein, flagellin, probably made of a single polypeptide chain. It was therefore surmized that the serological differences between, for instance, phase-1 flagella of antigenic character *i* and those of antigenic character *g,p*, reflected differences in the primary structure, that is the amino acid sequence of the two sorts of flagellin, determined by differences in the base sequence in the DNA of their structural genes, the *H1* alleles *H1-i* and *H1-g,p*. This assumption has been confirmed by the demonstration of considerable differences in amino acid composition between these two antigenically distinct salmonella flagellins (McDonough, 1962).

Variants of altered H antigenic constitution have been obtained from many salmonella species by growth in the presence of antiserum for the expressed H antigen(s), either growth in serum broth (Scott, 1926) or the more efficient technique of growth in semi-solid medium containing serum (Gard, 1937; Edwards & Bruner, 1959*a*; Bruner & Edwards, 1939). Bacteria whose flagella combine with the antibody present are immobilized, and so grow only at the site of inoculation; whereas those whose flagella do not react remain motile and spread through the medium as they multiply. The presence of the antiserum thus permits detection of rare variants; there is no reason to suppose that it directly invokes them. In some of the many reported instances of changes in H antigenic constitution (Kauffmann, 1951; Joys, 1961) the new flagellar antigen obtained was a previously undetected alternate phase (Edwards & Bruner, 1939*b*). In others the new antigen was a modified form of the original phase-1 or phase-2 antigen. Thus Gnosspelius (1939) obtained in *Salmonella stanley* (4, 5, 12: *d* ↔ *1,2*) a variant with a modified antigen *d*, and another with a modified antigen *1,2*, the other phase in each variant being unaltered.

We investigated spontaneous variation of the phase-1 antigen *i* of *Salmonella typhimurium* strain LT2 (4, 5, 12: *i* ↔ *1,2*). In some previous investigations prolonged cultivation, sometimes with repeated subculture, in the presence of a high concentration of antibody has been used. Such methods presumably detect only variants with a considerably altered H antigen, perhaps resulting from the cumulative effect of more than one mutation. The commonest class of mutational alteration of flagellin which permitted normal locomotor function of the flagella would presumably be a single amino acid substitution. This would presumably cause only a minor change in serological character. To permit the detection of slightly altered forms of antigen and to minimize the risk of selecting double mutants, we used a concentration of anti-*i* serum sufficient only to retard the spreading growth of bacteria expressing antigen *i*, and we picked faster-spreading 'swarms' after only 1 or 2 days of incubation. All of 8 such swarms tested proved to have modified forms of antigen *i*, as a result of spontaneous mutation at the *H1* locus. Preliminary accounts of the isolation, serology and genetics of the mutants (Joys & Stocker, 1963) and of chemical investigations of their flagellins (McDonough, 1962) have been published.

METHODS

Strains. A serologically mutated form of antigen *i* was first encountered in an experiment with strain SW520, a derivative of *Salmonella typhi* strain Watson in which antigen *d* had been replaced by antigen *i*, transduced by phage P22 from *S. typhimurium* strain LT7 (Zinder & Lederberg, 1952). Serological mutants were later sought in strain LT2 *adeC-7 proA-46*, a purine-exacting and proline-exacting double mutant of strain LT2. *S. paratyphi B* O strain SW543, a monophasic phase-1 strain lacking flagella through mutation at a locus co-transducible with *H1* (Stocker, Zinder & Lederberg, 1953), was used as recipient when it was desired to transduce the *H1* locus of a serological mutant into a different genetic background.

Media and cultural methods. Nutrient broth and agar were made from a tryptic digest of meat. Large quantities of bacteria for absorbing sera were grown on a buffered peptone agar fortified with glycerol (1%, v/v), sodium lactate (0·5%, w/v) and Marmite autolysed yeast (0·5%, w/v). A nutrient gelatin agar (Edwards & Bruner, 1942; Stocker *et al.* 1953), which is semi-solid at 37° but solid at room temperature, was used for the selection of highly motile bacteria and of serological mutants. Cultures were incubated at 37°; broth cultures were incubated unshaken.

Serological methods. Strains of bacteria to be used for preparation of agglutinable suspensions, absorbing suspensions or vaccines were first passaged through semi-solid medium, to ensure good development of flagella. A discrete colony, shown by slide agglutination to be in the required H antigenic phase, was inoculated into broth; after incubation for a few hours formalin was added, to give a formaldehyde concentration of 0·16% (w/v) for agglutinable suspension, and 0·8% (w/v) for vaccines. Agglutinable suspensions were adjusted to match the turbidity of the standard suspensions of the Serological Standards Laboratory, Colindale, London, N.W. 9. Vaccines after sterility tests were inoculated intravenously into rabbits, four doses being given at 6-day intervals (Mackie & McCartney, 1953). Sera were titrated by twofold steps, by the ordinary tube method or by a micromethod (Arkwright, 1927; Mandelbaum, 1932) to economize low-titre absorbed sera. In the micromethod a standard loopful (about 0·01 ml.) of agglutinable suspension as used for the tube tests was mixed with a standard loopful of a serum dilution on a glass plate which was incubated in a damp chamber (16 cm. Petri dish containing damp filter paper and cottonwool, enclosed in a plastic bag). After 2 hr at 37° and 1 hr at room temperature, the droplets were examined for agglutination by naked eye and by low-power dark-ground microscopy. To avoid non-specific clumping due to the bacteria sticking to the glass, the plates were cleaned with chromic + sulphuric acid, rinsed in water and ethanol, dried with a fluff-free cloth and flamed before use. The micromethod was about one dilution more sensitive than the tube test. The titres by the micromethod were therefore recorded as the last positive serum dilution, disregarding the further approximate twofold dilution involved in mixing with the agglutinable suspension, so that titres recorded by the micro- and macro-methods were about the same.

For growth of absorbing suspensions, cultures of highly motile organisms were inoculated to nutrient agar in 1 l. volumes in trays. Since the adenine-exacting strain gave rather poor growth on the medium used, derivative lines not exacting for adenine, obtained by transduction with phage P22, were generally used.

After 48 hr at 37° the growth from a tray was harvested into 20 ml. saline with phenol (0·5%, w/v), or of saline without phenol, because serum absorbed with phenol-killed bacteria was too toxic to use in semi-solid media for the selection of mutants. One volume of undiluted serum was mixed with 5 vol. of the dense unwashed suspension; after 2 hr at 37° the bacteria were centrifuged down. When the undiluted once-absorbed serum still agglutinated the absorbing strain this process was repeated once or more, using at each step equal volumes of absorbed serum and bacterial suspension, until no activity for the absorbing strain remained. When live bacteria had been used for absorption the fully absorbed serum was sterilized by Seitz filtration.

Serum number 713, used in isolation of the mutants, was an anti-*i* serum prepared by immunization of a rabbit with a formalin-killed broth culture of a motile derivative of *Salmonella paratyphi B* O strain SW 543, given antigen *i* (wild type) from *S. typhimurium* strain LT2 by transduction with phage P22. Sera nos. 131 and 133 were from rabbits inoculated with a formalin-killed phase-1 culture of *S. typhimurium* LT2 *adeC-7 proA-46*, i.e. contained antibody evoked by wild-type antigen *i*. Serum GL74 was an anti-*i* serum received from the Serological Standards Laboratory, Central Public Health Laboratory, Colindale, London, N.W. 9.

RESULTS

Isolation of mutants with altered antigen i

A serologically altered form of antigen *i* was first detected in an experiment in which salmonella strain SW520 (*Salmonella typhi* given antigen *i* by transduction) was inoculated into semi-solid medium containing anti-*i* serum no. 713. A swarm spreading faster than the partly immobilized parent strain yielded a strain with a form of antigen *i*, labelled *i*2, detectably different from the wild-type antigen *i* of the parent and of strain LT2; for anti-*i* serum no. 713, after complete absorption with antigen *i*2, still agglutinated wild-type antigen *i* to a titre of 400. Anti-*i* serum no. 713, unabsorbed or fully absorbed with *i*2, was next used to isolate mutants with altered antigen *i* from strain LT2 *adeC-7 proA-46*. Inocula from nine different single-colony stocks, previously passaged through semi-solid medium to enhance their motility, were stabbed into plates of semi-solid medium containing anti-*1,2* serum, to immobilize bacteria in phase 2, and anti-*i* serum, absorbed or unabsorbed, at a final concentration three times the agglutinating titre for wild-type *i*. At this concentration the growth of the inoculated strains spread slowly, taking about 60 hr at 37° to progress across a 5 cm. plate. After incubation for 24 or 48 hr, two faster-spreading swarms were detected on plates containing unabsorbed serum and eight on plates containing absorbed serum (Table 1). These swarms, like the slow-spreading growth of the parent strains, had sharp edges, indicating that the flagellate bacteria of the swarm combined with, and were immobilized by, some antibody component in the anti-*i* serum used (Stocker, 1956; Joys, 1961). The isolates from the ten faster-spreading swarms, labelled M4 to M13, all proved to have modified forms of antigen *i*, termed antigens *i*M4–*i*M13. The serological and biochemical characters of isolate M8 showed that it was a re-isolate of the strain (*S. typhi* strain SW520-*i*2) used to absorb serum 713, and evidently derived from a rare bacterium in the absorbing suspension which had survived contact with phenol.

Strain M8 was included in the serological analysis, though it could not be used in later genetical experiments (Joys & Stocker, 1963). Of the nine other isolates (all proven to have arisen from LT2) five (M6, M9, M10, M11, M12) clearly resulted from independent mutations, since each was isolated in a different single-colony stock. Isolate M7 was obtained by selection with unabsorbed serum from a stock which in a plate containing absorbed serum yielded M4 and M5; but since it differed serologically from them M7 must have resulted from an independent mutation.

Table 1. *Origin of the serological variants, and their agglutination by various anti-i (wild type) sera*

Strain Variants	Obtained from*	Serum used to select	Titre† to which agglutinated by anti-*i* serum no. 713	713/*i*2	131	133
M 4	LT 2 *ade pro* P8	713/*i*2	5,000	24	40,000	20,000
M 5	LT 2 *ade pro* P8	713/*i*2	5,000	24	40,000	20,000
M 6	LT 2 *ade pro* P4	713	1,280	192	20,000	40,000
M 7	LT 2 *ade pro* P8	713	1,280	24	20,000	10,000
M 8	LT 2 *ade pro* P3‡	713/*i*2	5,000	< 6	40,000	20,000
M 9	LT 2 *ade pro* P5	713/*i*2	2,500	24	20,000	20,000
M 10	LT 2 *ade pro* P7	713/*i*2	5,000	196	40,000	40,000
M 11	LT 2 *ade pro* P2	713/*i*2	10,000	24	40,000	20,000
M 12	LT 2 *ade pro* P9	713/*i*2	20,000	196	40,000	20,000
M 13	LT 2 *ade pro* P6	713/*i*2	10,000	24	20,000	20,000
*i*2	*S. typhi-i* (SW 520)	713	5,000	< 6	40,000	20,000
Parent strains						
S. typhimurium LT 2 *ade pro*	.	.	20,000	≤84	40,000	20,000
S. typhi-i (SW 520)	.	.	20,000	≤84	.	.

* The variants were selected from single-colony isolates, P2–P9, of *Salmonella typhimurium* LT 2 *adeC-7 proA-46* by selection in semi-solid medium containing a just immobilizing amount of anti-*i* serum no. 713, unabsorbed or fully absorbed with *S. typhi-i*2 (termed serum 713/*i*2).

† Titres by tube agglutination, except for serum 713/*i*2, titrated by the micromethod; the titres of this serum (diluted 1/6 in the course of absorbtion) are expressed in terms of content of original serum.

‡ M 8 was isolated from a swarm on a plate inoculated with LT 2 *ade pro* P3, but proved identical with *S. typhi-i*2, which had been used to make the absorbed serum incorporated in the medium.

Isolates M4 and M5, though picked from two apparently separate swarms on a single plate, were serologically identical; probably they were two isolates from a single mutant clone. Isolate M13 arose by mutation in a different single-colony stock but proved serologically identical with M4 (= M5); it presumably resulted from a recurrence of the mutation which produced M4 (= M5). The other seven isolates all differed serologically from M4 (= M5 = M13) and from each other.

Properties of serological mutants

All the serological mutants were normally motile in phase 1 and the phase-1 suspensions were agglutinated to high titres by various anti-*i* (wild type) sera (Table 1). All the LT2 mutants showed normal change of flagellar antigenic phase, to show an apparently unaltered phase-2 antigen, *1,2,3*. Subsequent serological and chemical examination showed that none of them were *nml*⁻ mutants, with the (serologically distinguishable) flagella which lack the ε-*N*-methyllysine which is

present in the flagella of wild-type *Salmonella typhimurium* (Stocker, McDonough & Ambler, 1961; Mrs U. B. Pearce, T. M. Joys & B. A. D. Stocker, unpublished). All the mutants gave stable H suspensions; but the phase-1 suspensions of M6 and M9 showed an abnormal reactivity with some 'non-specific' reagents.

Special properties of M6 and M9. Formalinized phase-1 suspensions of M6 and M9, but not their phase-2 suspensions nor phase-1 suspensions of the other mutants, were agglutinated by all of ten sera from uninoculated rabbits, to titres of 8–16; washed deflagellated M6 and M9 phase-1 suspensions were unaffected. The *i*M6 and *i*M9 suspensions were likewise agglutinated, to titres of 50–100, by four sera from rabbits inoculated with salmonellas having no O or phase-1 H antigens in common with *Salmonella typhimurium*; a wild-type *i* suspension was not agglutinated by these sera, even undiluted. *Salmonella paratyphi B* SW543 transductants given antigens *i*M6 or *i*M9 were similarly agglutinated by 'non-specific' sera; transductants given antigen *i* (wild type) or *i*M10 were unaffected. Evidently the phase-1 flagella are the site of the abnormal agglutinability of mutants M6 and M9. In experiments on the agglutination of suspensions of detached flagella by sera we added indian ink to enhance the visibility of the flocculated flagella (Craigie, 1931). When 10 vol. of diluted indian ink (3 drops of ink in 5 ml. saline) and 1 vol. of *i*M6 or *i*M9 flagellar suspensions were mixed, large black granules rapidly formed; flagella from the *i*M6 and *i*M9 *S. paratyphi B* transductants had the same effect. Even tenfold higher concentrations of *i* (wild type) flagella or flagella of the other mutant-*i* types did not cause such flocculation. The flocculation of ink by *i*M6 and *i*M9 flagella was obtained only at NaCl concentrations > 0·3% (w/v); > 1% NaCl caused flocculation of the ink even in the absence of flagella. The antigenic analysis of *i*M6 and *i*M9 was complicated by their agglutinability by non-specific components of anti-*i* sera—that is, components not able to combine with the immunizing antigen. Thus anti-*i* (wild type) serum no. 131 and anti-*i*M10 serum no. 57 after complete absorption with their homologous antigens still agglutinated *i*M6 and *i*M9 suspensions, despite dilution to 1/24 and 1/12, respectively, in the course of absorption. As *i*M6 and *i*M9 flagella were alike in their reaction with indian ink and with components present in the serum of uninoculated rabbits (and, in higher concentrations, in sera of rabbits immunized with related or unrelated antigens) it was expected that the same normal serum component reacted with both *i*M6 and *i*M9. However, a sample of serum from an uninoculated rabbit after complete absorption with *i*M6 still agglutinated *i*M9; and a sample fully absorbed with *i*M9 still agglutinated *i*M6.

New serological specificities of mutant antigens

Two rabbits were immunized with antigen *i*M6, two with *i*M12 and one each with *i*M5, *i*M7, *i*M8, *i*M9, *i*M10, *i*M11 and *i*M13; *i*M4 and *i*2 were not used, being regarded as identical with *i*M5 and *i*M8, respectively. All the anti-mutant sera obtained agglutinated the homologous antigens to titres of 10,000–40,000, except the two anti-*i*M6 sera, which agglutinated the homologous suspensions to titres of only 1000–3000 (Table 2). All the sera agglutinated wild-type *i* and the remaining antigens to about the same titre as the immunizing antigen. Since the unabsorbed anti-mutant sera did not distinguish their homologous antigens from the wild-type and the other mutant antigens, each anti-mutant serum was fully absorbed with the wild-type antigen. Each of the fully absorbed anti-mutant sera still agglutinated

its homologous antigen, to titres of between 200 (anti-*i*M5 serum no. 48) and 6000 (anti-*i*M8 serum no. 85). The absorbed sera did not agglutinate salmonella H suspensions representing the following phase-1 antigens: *a*; *b*; *c*; *d*; *e,h*; *e,n,x*; *g,p*; and *r*. The two anti-*i*M6 sera and the anti-*i*M9 serum after absorption with wild-type antigen had titres on their homologous antigens of only 200–400, not much greater than the titres of 50–100 on the same suspensions of sera from rabbits immunized with antigenically unrelated salmonellas. With this exception, the results

Table 2. *Log titres (to base 2) on mutant antigens of anti-mutant sera fully absorbed with wild-type antigen*

Serum	Titre* before absorption	Dilution during absorption	Log titre of absorbed serum on								
			M5†	M13	M6	M9	M7	M10	M12	M8‡	M11
anti-M 5 no. 48	10,000	1/48	**2**	**2**	.	.	.	.	.	.	.
anti-M 13 no. 58	20,000	1/24	**4**	**4**	**1**	**2**	.	.	.	.	.
anti-M 6 no. 55	1,000	1/12	.	.	**5**	**4**	.	.	.	.	.
anti-M 6 no. 132	3,000	1/12	.	.	**4**	**4**	.	.	.	.	.
anti-M 9 no. 56	10,000	1/12	.	.	**4**	**4**	.	.	.	.	.
anti-M 7 no. 84	10,000	1/24	.	.	**4**	**3**	**8**	**6**	**5**	.	.
anti-M 10 no. 57	40,000	1/24	.	.	**3**	**3**	**1**	**6**	**2**	.	.
anti-M 12 no. 128	10,000	1/24	.	.	**4**	**2**	.	**1**	**6**	.	.
anti-M 12 no. 22	10,000	1/12	.	.	**3**	**3**	**1**	**2**	**7**	.	.
anti-M 8 no. 85	20,000	1/24	.	.	**2**	**3**	.	.	.	**8**	.
anti-M 11 no. 86	10,000	1/24	.	.	**1**	**1**	.	.	.	.	**5**
anti-*i* (wild-type) no. 131	20,000	1/24	.	.	**3**	**2**	.	.	.	.	.

The anti-sera after complete absorption with wild-type antigen were tested by the micromethod on the indicated antigens. Titres are stated as last effective dilution, expressed as log to base 2, of the absorbed antiserum itself; titres in terms of content of original antiserum can be calculated from dilution involved in absorption (col. 3). Bold-face figure = titre on homologous antigen. . = no agglutination in undiluted absorbed antiserum. Antigens arranged in an order which brings together mutants with cross-reactive mutant specificities (M 5 and M 13; M 6 and M 9; M 7, M 10 and M 12). The last antiserum listed is an anti-*i* (wild type) serum, illustrating the agglutination of *i*M6 and *i*M9 suspensions by sera fully absorbed with their homologous antigens.

* Titre on homologous antigen, wild-type antigen and most non-homologous mutant antigens.

† A suspension of M 4, which is probably the same mutant as M 5, gave the same results as the M 5 suspension with all sera.

‡ A suspension of *S. typhi-i* 2, which is probably the same strain as M 8, gave the same results as the M 8 suspensions with all sera.

indicate that each mutant antigen has a specificity or antigenic subfactor absent in wild-type antigen *i*. Some of the absorbed anti-mutant sera agglutinated mutant antigens other than their homologous antigen. We attribute the agglutination of the *i*M6 and *i*M9 suspensions by nearly all the absorbed anti-mutant sera, to titres of 50–400, to the 'non-specific' agglutinability of these antigens by unrelated immune sera, discussed above. The other cross-reactions, presumably indicative of antigenic relatedness of the mutant specificities of the mutants concerned, were investigated by cross-absorption.

Cross-reactions of iM4, iM5 and iM13. These three suspensions were agglutinated to equal titres by the absorbed anti-*i*M5 serum no. 48 and also by the absorbed anti-*i*M13 serum no. 58. To test the antigenic relationship of *i*M5 and *i*M13, known to be of independent origin, unabsorbed anti-*i*M13 serum was absorbed with *i*M5 and unabsorbed anti-*i*M5 serum with *i*M13. With each successive absorption

the titres of each serum on *i*M13 and *i*M5 decreased *pari-passu*, and the fully cross-absorbed sera did not agglutinate their homologous suspensions. Thus the mutant specificity of *i*M13 was indistinguishable from that of *i*M5 by the 'mirror' absorption test; this and the similar activities of anti-*i* (wild-type) sera absorbed with *i*M5 and *i*M13 show that these two antigens are serologically identical. (The *i*M4 suspension was agglutinated to the same titres as the *i*M5 and *i*M13 suspensions at each stage, as expected in view of the probable origin of M4 from the same mutant clone as M5.)

iM8 and i2. The agglutination of the *Salmonella typhi-i2* suspension by anti-*i*M8 serum absorbed by wild-type antigen *i* is accounted for by the identity of M8 and *S. typhi-i2*, noted above.

iM7, iM10 and iM12. The fully absorbed sera against these mutant antigens agglutinated all three of them, in each case the homologous antigen to a higher titre than the two heterologous antigens. Appropriate cross-absorption tests were made. If an antigenic subfactor is postulated to account for each distinct type of antibody recognized by the reactions of the cross-absorbed sera, the results indicate the presence of mutant subfactors as follows:

$$\begin{aligned} i\text{M7} &= a\ b\ c\ -\ -\ - \\ i\text{M10} &= a\ b\ -\ d\ e\ - \\ i\text{M12} &= a\ -\ -\ d\ -\ f \end{aligned}$$

Absorption of anti-i (wild-type) sera by mutant antigens

Several anti-*i* (wild-type) sera were titrated on wild-type and all the mutant antigens (Table 1). As was expected antisera 713 and 713 absorbed with *i*2 (the sera used in selection of the mutants) agglutinated nearly all the mutant suspensions to lower titres than the wild-type antigen; so also did one other anti-*i* (wild-type) serum tested later (no. GL74). Two other anti-*i* sera (nos. 131, 133) had, however, about equal titres on all the mutant and the wild-type antigens. Several anti-*i* (wild-type) sera were next absorbed with each serological mutant, and tested for activity on wild-type antigen, to look for subfactors present in the wild-type antigen but absent from the mutants. Samples of three sera, nos. 131, 133 and GL74, were fully absorbed with each of the mutant antigens, *i*M4 to *i*M13 and *i*2; serum no. 713 because of its scarcity was absorbed only with *i*2, *i*M9, *i*M10 and *i*M13. The absorbed samples, diluted to 1/12–1/48 in the course of absorption, were tested on suspensions of wild-type *i* and non-homologous mutant antigens by the micromethod and titrated on those suspensions which were agglutinated. The titres are recorded in Table 3 as logs to the base 2 of the dilutions of the absorbed sera. Complete absorption of any of the four sera with any mutant antigen except *i*M12 left titres of at least 1/32 of the absorbed serum for wild-type *i*, and variable titres for some of the heterologous mutant antigens. After absorption with *i*M12 only a low titre for wild-type *i* remained. Thus each mutant antigen did not absorb some species of antibody active on the wild-type antigen, and so was inferred to have lost some antigenic specificity.

Each of the serum samples, unabsorbed or absorbed, had equal titres on the three suspensions *i*M4, *i*M5 and *i*M13, later found to be serologically identical; but all the other suspensions differed in their agglutinability. Correspondingly, each serum had

an essentially similar spectrum of residual activity after absorption with either *i*M4 or *i*M5 or *i*M13; whereas absorption with each of the other mutants revealed a unique spectrum of residual activity. Thus all the mutant antigens except these three differed from each other. The four anti-*i* sera after similar absorptions had

Table 3. *Log titres (to base 2) on wild-type and mutant antigens of several anti-i (wild type) sera fully absorbed with different mutant antigens*

Serum no.	Absorbed with	Dilution during absorption	Log titre of absorbed serum on									
			i (w.t.)	M5*	M13	M6	M9	M7	M10	M12	M8	M11
131	M 4	1/24	9	< 0	< 0	8	3	< 0	6	9	4	4
133	M 4	1/24	8	< 0	< 0	6	2	< 0	5	6	3	2
GL 74	M 4	1/12	6	< 0	< 0	4	0	< 0	3	4	2	0
131	M 5	1/24	9	< 0	< 0	9	1	< 0	6	9	4	1
133	M 5	1/24	8	< 0	< 0	7	3	< 0	6	7	3	2
GL 74	M 5	1/12	6	< 0	< 0	4	0	< 0	2	4	4	0
131	M 13	1/24	9	< 0	< 0	9	0	< 0	7	9	5	4
133	M 13	1/24	8	< 0	< 0	7	2	< 0	5	7	3	2
GL 74	M 13	1/12	6	< 0	< 0	4	0	< 0	3	4	0	0
713	M 13	1/24	10	< 0	< 0	10	5	< 0	9	10	7	2
131	M 6	1/24	10	10	10	< 0	< 0	9	10	10	10	10
133	M 6	1/24	8	7	7	< 0	< 0	7	7	7	7	7
GL 74	M 6	1/12	7	6	6	< 0	< 0	6	6	6	6	6
131	M 9	1/12	10	10	10	10	< 0	10	10	10	10	10
133	M 9	1/12	9	9	9	9	< 0	9	9	9	9	9
GL 74	M 9	1/12	8	6	6	6	< 0	6	6	6	6	6
713	M 9	1/24	10	9	9	10	< 0	6	9	9	9	9
131	M 7	1/12	10	8	8	10	5	< 0	9	10	8	9
133	M 7	1/12	10	6	6	9	5	< 0	7	9	6	6
GL 74	M 7	1/12	7	4	4	6	0	< 0	5	6	4	5
131	M 10	1/48	6	5	5	6	2	< 0	< 0	6	3	4
133	M 10	1/24	6	5	5	5	2	< 0	< 0	5	2	5
GL 74	M 10	1/12	5	3	3	3	2	< 0	< 0	3	2	3
713	M 10	1/48	7	0	0	4	3	< 0	< 0	7	3	3
131	M 12	1/48	1	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0
133	M 12	1/24	1	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0	< 0
GL 74	M 12	1/12	3	3	3	< 0	< 0	< 0	< 0	< 0	< 0	< 0
131	M 8	1/12	9	7	7	9	6	6	9	9	< 0	6
133	M 8	1/12	8	6	6	7	5	5	7	7	< 0	6
GL 74	M 8	1/12	6	2	2	4	1	1	4	4	< 0	2
713	*i*2(= M8)	1/6	7	3	3	6	3	3	6	6	< 0	3
131	M 11	1/24	9	6	6	9	4	1	8	9	8	< 0
133	M 11	1/24	8	3	3	7	3	< 0	6	7	5	< 0
GL 74	M 11	1/12	7	2	2	5	1	< 0	3	5	2	< 0

The antisera before absorption had titres on *i* (wild type) of 40,000 (no. 131), 20,000 (no. 133), 10,000 (no. GL 74) and 20,000 (no. 713); the mutant antigens were agglutinated to about the same titres, except by antiserum 713, which had lower titres on most of them (Table 1). The antisera after complete absorption with the indicated antigen were tested by the micromethod. Their titres are stated as last effective dilution, expressed as log to base 2, of the absorbed antiserum itself; titres in terms of content of original serum can be calculated from dilution involved in absorption (col. 3).

0 = agglutination only in undiluted absorbed antiserum. < 0 = no agglutination in undiluted absorbed antiserum. < 0 = negative reaction with antigen used for absorption.

* A suspension of M 4, which is probably the same mutant as M 5, gave the same results as the M 5 suspension with all sera.

patterns of residual activity which were qualitatively similar though not quantitatively identical. For instance after complete absorption with *i*M13 all four sera had a titre on *i*M6 = titre on *i*M12 > titre on *i*M10 > titre on *i*M8; and did not agglutinate *i*M7.

Double absorptions of anti-i (wild type) serum. The complex pattern of activity on heterologous mutant antigens of the absorbed samples of anti-*i* (wild type) sera suggested that the wild-type antigen *i* was complex. One anti-*i* (wild type) serum, no. 133, was therefore fully absorbed with all possible pairs of the eight serologically different mutant antigens, the serologically identical set *i*M4, *i*M5 and *i*M13 being represented by *i*M4 alone. Each double absorption was made in two different orders, e.g. a sample absorbed with *i*M4 was further absorbed with *i*M6, and a sample absorbed with *i*M6 was further absorbed with *i*M4. The doubly absorbed sera, diluted to 1/12–1/96 in the course of absorption, were tested without further dilution by the micromethod on all the *i* antigens. In every test the two different samples of doubly absorbed serum behaved alike (Table 4). All the doubly absorbed sera still agglutinated the wild-type antigen, but they differed widely in their activities on the mutant antigens not used for absorption. Thus, some agglutinated none of the mutant antigens, e.g. serum absorbed with *i*M6 and *i*M8; whereas others, for instance that absorbed with *i*M4 and *i*M7, agglutinated all the mutant antigens except the two absorbing antigens. We did not proceed to further absorptions of those doubly absorbed sera which still agglutinated more than one antigen. Partly for this reason it was difficult to analyse the data in the ordinary way, postulating antigenic subfactors in the wild-type antigen, each defined by its reaction with a particular species of antibody left as the sole antibody remaining after single, double, triple, etc. absorption. However, we attempted to find the distribution in the mutants of the minimum number of subfactors to account for the activities of the singly and doubly absorbed samples. The scheme arrived at (Table 5) is based on the assumption that the unabsorbed anti-*i* serum no. 133 contains antibodies specific for each of the thirteen postulated subfactors of antigen *i* and that after single or double absorption it will still agglutinate all mutant antigens containing a given factor if the absorbing suspension(s) lacked that factor.

The full argument leading to the scheme is too lengthy to reproduce here, but may be illustrated as follows. Consider those absorbed samples which agglutinate only the wild-type antigen, i.e. antiserum absorbed with *i*M12 alone, or with *i*M4 + *i*M6, or with *i*M6 + *i*M8, etc. We ascribe their activity to residual antibody for a factor, G, present in the wild-type antigen and absent from all the mutant antigens. Consider next the absorbed samples with the next most restricted range, those absorbed with *i*M4 + *i*M10, and with *i*M10 + *i*M11, which agglutinate only *i*M6, *i*M12 and wild-type *i*. We postulate a factor, J, present in *i*M6 and *i*M12, but absent in *i*M4, *i*M10 and *i*M11 (since absorption with them did not remove the anti-J activity), and absent also from the remaining mutant antigens because they were not agglutinated by the doubly absorbed antisera having anti-J activity. Since absorption with the wild-type antigen removed all activity from the antiserum this also must possess factor J—and, by the same argument, all the other factors postulated. (The doubly absorbed antisera had been diluted during absorption beyond the range of activity of the non-specific agglutinins for *i*M6 and *i*M9). If, as postulated, all the mutant antigens except *i*M6 and *i*M12 lacked factor J,

Table 4. *Activity on mutant and wild-type antigens of anti-i (wild-type) antiserum no. 133 and anti-iM 13 antiserum no. 58 after absorption with different mutant antigens and pairs of antigens*

Absorption: Antigen(s) used	Absorption: Dilution involved	Activity of absorbed antiserum on: *i*(w.-t.)	M 4*	M 6	M 7	M 8	M 9	M 10	M 11	M 12
M 4	24/.	++/.	−/.	++/.	−/.	++/.	+/.	++/.	++/.	++/.
M 6	24/24	++/++	++/++	−/−	++/++	++/++	−/−	++/++	++/++	++/++
M 7	12/24	++/++	++/++	++/++	−/−	++/++	++/++	++/++	++/++	++/++
M 8	12/24	++/++	++/++	++/++	++/++	−/−	++/++	++/++	++/++	++/++
M 9	12/12	++/++	++/++	++/++	++/++	++/++	−/−	++/++	++/++	++/++
M 10	24/24	++/++	++/++	++/++	−/++	++/++	++/++	−/−	++/++	++/++
M 11	24/24	++/++	++/++	++/++	−/++	++/++	++/++	++/++	−/−	++/++
M 12	24/24	+/++	−/++	−/++	−/++	−/++	−/++	−/++	−/++	−/−
M 4 and M 6	96/48	+/.	−/.	−/.	−/.	−/.	−/.	−/.	−/.	−/.
M 4 and M 7	12/48	++/.	−/.	++/.	−/.	++/.	++/.	++/.	++/.	++/.
M 4 and M 8	48/48	++/.	−/.	++/.	−/.	−/.	−/.	++/.	−/.	++/.
M 4 and M 9	48/48	++/.	−/.	++/.	−/.	++/.	−/.	++/.	−/.	++/.
M 4 and M 10	48/48	++/.	−/.	++/.	−/.	−/.	−/.	−/.	−/.	++/.
M 4 and M 11	48/48	++/.	−/.	++/.	−/.	++/.	−/.	++/.	−/.	++/.
M 4 and M 12	48/48	+/.	−/.	−/.	−/.	−/.	−/.	−/.	−/.	−/.
M 6 and M 7	48/48	++/++	++/++	−/−	−/−	++/++	−/−	++/++	++/++	++/++
M 6 and M 8	48/48	+/++	−/++	−/−	−/−	−/−	−/−	−/++	−/++	−/++
M 6 and M 9	24/48	++/++	++/++	−/−	++/++	++/++	−/−	++/++	++/++	++/++
M 6 and M 10	48/48	+/++	−/++	−/−	−/−	−/++	−/−	−/−	−/++	−/++
M 6 and M 11	48/48	+/−	−/++	−/−	−/−	−/−	−/−	−/−	−/−	−/−
M 6 and M 12	48/48	+/++	−/++	−/−	−/−	−/−	−/−	−/−	−/++	−/−
M 7 and M 8	48/48	++/++	++/++	++/−	−/−	−/−	−/−	++/++	++/++	++/++
M 7 and M 9	48/48	++/++	++/++	++/++	−/−	++/++	−/−	++/++	++/++	++/++
M 7 and M 10	48/48	++/++	++/++	++/++	−/−	+/++	+/−	−/−	++/++	++/++
M 7 and M 11	48/48	++/−	++/++	++/−	−/−	++/−	+/−	++/−	−/−	++/−
M 7 and M 12	48/48	+/++	−/++	−/−	−/−	−/−	−/−	−/−	−/++	−/−
M 8 and M 9	48/48	++/++	++/++	++/−	−/−	/	/−	++/++	++/++	++/++
M 8 and M 10	48/48	++/++	++/++	++/−	−/−	−/−	−/−	++/++	++/++	++/++
M 8 and M 11	48/48	++/−	−/++	++/−	−/−	−/−	−/−	++/−	/	++/−
M 8 and M 12	48/48	+/++	−/++	−/−	−/−	−/−	−/−	−/−	−/++	−/−
M 9 and M 10	48/48	++/++	++/++	++/++	−/++	−/++	−/−	−/−	++/++	++/++
M 9 and M 11	48/48	++/−	++/++	++/−	/−	++/−	−/−	++/−	−/−	++/−
M 9 and M 12	48/48	+/++	−/++	−/−	−/−	−/−	−/−	−/−	−/++	−/−
M 10 and M 11	48/48	+/−	−/++	+/−	−/−	−/−	−/−	−/−	−/−	+/−
M 10 and M 12	48/48	+/++	−/++	−/−	−/−	−/−	−/−	−/−	−/++	−/−
M 11 and M 12	48/48	+/−	−/++	−/−	−/−	−/−	−/−	−/−	−/−	−/−

The first symbol in each column records the activity in micro-agglutination test of absorbed anti-*i* (wild type) antiserum no. 133, the second that of absorbed anti-*i*M 13 serum no. 58.

++ = strong agglutination. + = weak agglutination. − = no agglutination. . = not tested, because absorption of the anti-*i*M 13 serum with the serologically identical *i*M 4 removes all activity (except the 'non-specific' effect on *i*M 6 and *i*M 9).

* Antiserum no. 58 was tested on the serologically identical *i*M 13, instead of on *i*M 4.

then antiserum absorbed with any mutant or pair of mutants, not including either of these two, should still contain anti-J and should agglutinate *i*M6 and *i*M12; as was observed (Tables 3 and 4). Now consider the absorbed samples with the next most restricted activity, those absorbed with *i*M4+*i*M8, and with *i*M8+*i*M11, which agglutinate *i*M6, *i*M10 and *i*M12, as well as wild type. The activity of these samples on *i*M6 and *i*M12 is accounted for by the presence of anti-J antibody. To account for the activity on *i*M10 we postulate a further factor H, present in *i*M10,

Table 5. *Distribution in mutant antigens of subfactors of antigen i (wild type) inferred from activities of singly and doubly absorbed samples of anti-i (wild type) antiserum no. 133 and anti-iM13 serum no. 58*

	Factors of *i* (wild-type) antigen																					
Antigen i (w.-t.)	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
*i*M4 and *i*M13	B	C	.	E	F	.	.	.	K	L	M	.	O	P	Q	R	S	T	U	V	W	X
*i*M6	.	C	D	E	F	.	H	J	.	L	M	N	O	P	Q	R	.	.	.	V	.	X
*i*M7	B	C	.	.	.	.	.	.	.	.	.	.	.	P	Q	R	.	.	.	.	.	X
*i*M8	B	.	D	E	F	.	.	.	K	.	M	N	O	P	Q	R	.	T	.	V	.	X
*i*M9	.	C	D	E	F	.	.	.	.	.	.	.	O	P	Q	R	.	.	.	.	.	.
*i*M10	B	C	D	.	F	.	H	.	K	.	M	N	O	.	Q	R	S	.	.	.	.	.
*i*M11	B	C	D	E	.	.	.	.	K	L	.	.	O	P	.	R	S	T	U	V	W	X
*i*M12	B	C	D	E	F	.	H	J	K	L	M	N	O	P	Q	.	S	T	.	V	W	X
Serum detecting	133 and 58		133 only										58 only									

The activities on other mutant antigens of anti-*i* (wild type) antiserum no. 133 and anti-*i*M13 antiserum no. 58 absorbed with different mutant antigens and pairs of antigens are shown in Table 4; see text for method of inference of factors from the data. The antigenic specificity common to the wild type and all the mutant antigens (factor A) is not shown. The anti-*i*M13 antiserum also detected a mutant specificity present in *i*M4 (= *i*M5 = *i*M13) but absent from (wild type) and the other mutant antigens.

absent in *i*M4, *i*M8 and *i*M11. The sample absorbed with *i*M4 (which lacks H) and with *i*M6 does not agglutinate *i*M10, which has H. We infer that absorption with *i*M6 has removed anti-H and that H is therefore present in *i*M6. A similar argument indicates that *i*M12 also possesses factor H. Antigens *i*M7 and *i*M9 are unaffected by the samples containing anti-H and must therefore lack H. The inferred distribution of H predicts that all samples not absorbed with *i*M6, *i*M10 or *i*M12 will agglutinate all three of these antigens; as was observed. Successive consideration of the remaining singly and doubly absorbed sera requires the postulation of factors N, L, K, M, E, B, C, F and D, all present in wild type and distributed amongst the mutants as shown in Table 4. The scheme accounts for all the positive and negative results in Tables 3 and 4; for every factor postulated there is at least one positive result, generally several, which cannot be otherwise accounted for, so that it seems unlikely that any unnecessary factors have been proposed. Absorption with any mutant antigen greatly decreased the titre of the anti-*i* (wild type) serum on its homologous antigen. Since the anti-factor antibodies postulated are all of relatively low titre this implies that antibodies present in high concentration in the unabsorbed serum will combine with either the wild-type or with any mutant antigen. We assign the symbol A for the factor(s) common to the wild type and all the mutant antigens.

Attempt to map sites of amino acid substitution from serological results

If each mutant antigen differs from the wild-type antigen by a single amino acid substitution it might be possible on certain assumptions to infer the sequence of the sites of amino acid substitution from the serological data. Suppose that each antigenic factor in the wild-type antigen (i.e. each chemical feature recognized by a specific family of antibody molecules in an anti-*i* antiserum) was completely defined by a given sequence of amino acids in the *i* flagellin polypeptide chain, and that each anti-factor antibody had an absolutely specific affinity for all the amino acids in the corresponding sequence (or at least for all of a class of serologically important amino acids within it). The *i* flagellin and *i* antigen could then be represented by a single straight-line map of the amino acid sequence, with every wild-type factor shown by a single line covering a certain number of the amino acid sites. A serologically altered mutant flagellin resulting from a single amino acid change *ex hypothesi* would not combine with any anti-factor antibody with affinity for a sequence of amino acids which included the site of substitution, and the mutant antigen would therefore lack the corresponding wild-type factors. On such a map, and *a forteriori* on a simplified map, showing only in their correct sequence the sites of amino acid substitution of a series of mutants, all the wild-type factors absent from a given mutant would be represented by unbroken lines of appropriate length, all spanning the site of substitution of that mutant, and some of them spanning also one or more adjacent substitution sites, on one or both sides, corresponding to the other mutants which lacked the wild-type factor represented by the line in question. On the assumption that the eight serologically distinct mutant antigens each arose by a single amino acid substitution we tried to arrange the eight hypothetical substitution sites in a linear order such that all the wild-type factors inferred as absent from one or more mutants could be represented as described above. However, no order permitted representation of each missing factor by an unbroken line. Some factors of *i* might comprise the C-terminus of one flagellin molecule and the N-terminus of an adjacent molecule (cf. the antigenic factor present in tobacco mosaic virus (TMV) and re-aggregated TMV A-protein but absent from free TMV A-protein, Aach, 1959). The linear map of the antigen and its factors would then be a circle instead of a straight line. The missing factors of the mutants could not, however, be represented by single lines on such a map.

On the above assumptions the order of the sites of substitution in a series of mutant antigens could also be inferred directly from the activity or inactivity on other mutant antigens of anti-wild-type serum fully absorbed with different pairs of mutant antigens. Consider a mutant antigen, P, with a substitution at site p in the polypeptide chain and another mutant antigen, Q, with a substitution at site q. Absorption of an anti-wild type antiserum with antigens P and Q would leave only antibody with affinity for amino acid sequences which spanned both site p and site q. These residual antibodies would *ex hypothesi* have affinity for all the antigenically important sites between p and q in the wild-type antigen, and would therefore fail to agglutinate a third mutant, R, if its site of substitution, r, were between sites p and q. Thus if antiserum absorbed with mutant antigens P and Q agglutinated mutant antigen R then, on the assumptions stated, the order of the sites of their amino acid changes cannot be $p-r-q$. The data of Table 4 were therefore analysed

in respect of each possible set of three mutant antigens, drawn from the eight tested. For each set there were available observations on three different doubly absorbed samples of anti-wild-type antiserum, each capable of excluding one of the three possible orders. None of the 56 sets gave the self-contradictory result (all three orders excluded); two orders were excluded in 21 sets, one order in 30 sets and no order in the remaining 5 sets. However, no sequence of the eight sites of amino acid substitution was found which would satisfy all 21 of the unequivocally indicated orders within sets of three. A few discrepant results remained even when it was assumed that mutants M7 and M9, concerned in most of the contradictions, were double mutants, each having two different sites of amino acid substitution.

Analysis of wild-type antigen i by absorption of an anti-mutant serum

In view of the complexity of the wild-type antigen *i* revealed by the activities on the mutant antigens of an anti-*i* (wild type) antiserum absorbed by different mutant antigens and pairs of antigens (Tables 3, 4) it was decided to make a similar investigation with an anti-mutant antiserum. Anti-*i*M13 antiserum no. 58 was used; after complete absorption with wild-type antigen this antiserum agglutinated only its homologous antigen, *i*M13 (and the serologically identical antigens *i*M4 and *i*M5) (Table 2). Samples fully absorbed with each of the different mutant antigens other than the homologous antigen and with each pair of such mutant antigens were tested qualitatively on the wild-type and mutant antigens (Table 4). As was expected all the absorbed samples agglutinated the homologous suspension; as this reflected the presence of mutant-specific antibodies (Table 2) these reactions are omitted in Table 4. The activities of the absorbed samples of the anti-*i*M13 antiserum on mutants other than *i*M13 (Table 4) must have resulted from the presence of antibodies active on factors present in the wild-type antigen (since absorption with wild-type antigen removed all activity except that on the homologous antigen (Table 2)), and present also in antigen *i*M13, used for immunization and capable of removing all activity from the antiserum. It was therefore expected that the activities on the mutant antigens of the absorbed anti-*i*M13 antiserum would be similar to those of the absorbed anti-*i* (wild-type) sera (Tables 3 and 4), except for the absence of the positive reactions attributed to antibodies against the wild-type factors D, G, H, J, N, inferred to be absent from antigen *i*M13 (Table 5). Most of the reactions obtained agreed with this expectation. Some of the absorbed anti-mutant samples, however, were active on mutant antigens unaffected by the corresponding sample of anti-*i* (wild type) antiserum. Some such differences were apparent when the singly absorbed antisera were compared; for instance, the anti-*i*M13 antiserum absorbed with *i*M10, but not the similarly absorbed anti-*i* (wild type) antiserum, strongly agglutinated antigens *i*M7 and *i*M8. As four different anti-*i* (wild type) antisera after single absorption with different mutant antigens gave qualitatively identical results on the remaining mutant antigens (Table 3), it seems unlikely that the unexpected additional activity of some absorbed samples of anti-*i*M13 antiserum no. 58 reflected merely chance differences in the response of different rabbits to minor factors in antigens *i* (wild type) and *i*M13.

The data of Table 4 were analysed to find the minimum number of antigenic factors present in both wild-type *i* and *i*M13 antigens which would account for the observed activities of the absorbed samples of anti-*i*M13 antiserum, by using the

same kind of argument as was used for analysis of the activity of absorbed samples of anti-*i* (wild-type) antiserum no. 133. The results are recorded in Table 5. Of the seven wild-type factors previously inferred to be present in antigen *i*M13, only factors B and C were recognizable by analysis of the activities of the anti-*i*M13 antiserum. It was necessary to postulate an additional ten factors (O to X in Table 5), each present in the wild-type and *i*M13 antigens and each with a distinctive distribution amongst the other mutant antigens.

DISCUSSION

Analysis of a serum prepared against the wild-type phase-1 antigen *i* of *Salmonella typhimurium* strain LT2 by absorption and agglutination tests with mutant forms of this antigen showed that many different antigenic subfactors can be recognized in the wild-type antigen. The single symbol *i* recorded in the Kauffmann–White table for the phase-1 antigen of many species perhaps conceals some variation in the naturally occurring antigen. The phase-1 antigen *i* of *S. landau* is known to differ somewhat from the standard form (Günther & Hauser, 1954) and in the original descriptions of many other species the complete identity of the phase-1 antigen with standard antigen *i* was not demonstrated, since 'mirror' absorption tests were not made. It would not be surprising if some of the mutant forms of antigen *i* were to occur as the wild-type phase-1 antigen in some species other than *S. typhimurium* (or in some wild strains of this species); the previously undescribed antigen l,z_{40} of *S. rutgers* was found to be closely related to an unnamed mutant form of antigen *l,v* previously obtained in the laboratory by serum selection (Drs P. R. Edwards & D. W. Bruner, personal communication).

All the eight serologically distinct mutant antigens had lost some of the serological specificity of the wild-type antigen and were agglutinated to rather lower titres than it by the anti-*i* (wild-type) antiserum used in their isolation—as was to be expected since they were detected by their ability to spread more rapidly in the presence of this serum. One wild-type specificity (factor G, Table 5) was missing from all the mutant antigens; but each of the eight serologically different antigens also lacked a different combination of wild-type factors. Serological mutants were easily obtained by selection with serum 713, either unabsorbed or absorbed with *i*2 (= *i*M8), the first mutant antigen to be isolated. This perhaps indicates that in this antiserum the antibody with the highest immobilization titre was specific for a factor or factors easily lost by mutation. Later attempts to isolate serological mutants by selection with other anti-*i* sera failed.

On inoculation into rabbits all the mutant antigens (except perhaps the non-specifically agglutinable *i*M6 and *i*M9) evoked some antibody unable to combine with wild-type antigen. Perhaps any alteration in the amino acid sequence of an antigenically determinant part of the flagellin molecule (or at least any change in a serologically important amino acid in this sequence) results in the evocation of antibody molecules with affinity for the new features, presumably amino acid sequences, absent in the wild-type molecule. Rather similarly tobacco mosaic virus (TMV) from which the carboxy-terminal threonine of the constituent protein has been removed by digestion with carboxypeptidase differs from native TMV by the absence of one 'native' serological specificity and the appearance of a new specificity

(Harris & Knight, 1955). The mutant specificities common to *i*M7, *i*M10 and *i*M12 (Table 2) are unexpected if, as we suppose, each of these mutant antigens differs from the wild type by a single amino acid substitution at a different site. Possibly each of the three different substitutions causes a similar change in the tertiary structure of the *i* flagellin molecule, and thereby produces a similar new feature (e.g. the approximation of two antigenically important amino acid side-chains) in a part of the molecule distant from the site of amino acid substitution.

We do not know the cause of the curious agglutinability of *i*M6 and *i*M9 suspensions by the sera of non-immunized rabbits, and, to higher titres, by the sera of rabbits inoculated with related or unrelated antigens, even after complete absorption with the immunizing antigens. The flocculation of indian ink by these antigens suggests that a change in some physical property of the flagellar protein may be concerned; but, contrary to what would be expected if they were agglutinable by an exceptionally small amount of antibody, these antigens were not agglutinated to higher titres than other antigens by various anti-*i* (mutant or wild-type) sera (Tables 1 and 2).

In all the mutants the site of mutation is in *H1*, the structural gene for phase-1 flagellin (Joys & Stocker, 1963, and unpublished results). In four of the mutant flagellins a single change in the tryptic or peptic peptide 'map' has been detected (McDonough, 1962). It is reasonable to assume that in all the mutant antigens the difference from the wild type consists in a single amino acid substitution. On this assumption it follows that a single amino acid change can destroy the affinity of the wild-type antigen for several different families of antibody (since each mutant antigen has lost several different wild-type antigenic factors); this suggests that the antibodies evoked by a flagellar antigen include very many species, some with affinities for overlapping features of the antigenically determinant part of the flagellum. The failure of our attempt to discover the order of the sites of amino acid substitution in the different mutants, either by consideration of the factors inferred to be missing in different mutant antigens (Table 5) or directly from the activity or inactivity on the remaining mutant antigens of anti-wild-type antiserum absorbed with pairs of mutant antigens (Table 4), indicates the incorrectness of one of the assumptions involved—probably the assumption that every antigenic factor consists of a length of polypeptide chain and that change of any amino acid (or of any antigenically determinant amino acid) in this length would result in complete loss of affinity for the anti-factor antibody concerned. It is likely that some anti-protein antibodies are specific for a given short sequence of amino acids, regardless of their configuration in the complete antigen; for instance, an artificial antigen consisting of a carrier protein to which is attached a synthetic oligopeptide comprising the last 6, 5, 4 or 3 amino acids at the carboxy terminus of TMV protein evokes antibody which precipitates and neutralizes TMV (Anderer & Schlumberger, 1965). But it may well be that some species of antibody molecules have specificities for pairs of chemical features which are close together on the surface of the flagellum but far apart along the flagellin polypeptide chain; the existence of such antibodies in the anti-*i* antiserum examined would invalidate the attempted analysis. It is also possible that some of the serological changes caused by the mutations reflect not alterations in amino acids whose side-chains are themselves part of an antigenically determinant surface, but alterations of the configuration of the antigenically determinant part

of the flagellin molecule. However, as all the mutant flagellins support normal locomotor function it is unlikely that the amino acid substitutions cause any profound change in the tertiary structure of the flagellin molecule, at least within the complete flagellum.

In microbial genetics most mutations involving alteration of the structure of a protein are recognized by a defect in some function, which may reflect either the absence of a recognizable protein product, e.g. enzyme, or the production of an abnormal protein. Various abnormal enzymic proteins are recognized as cross-reactive material (CRM) by their reaction with antibody evoked by the wild-type antigen. In general the CRM was not distinguished serologically from the wild-type protein by the techniques employed; the use of quantitative complement fixation methods has shown that some mutant tryptophan-synthetase proteins of *Neurospora crassa* cross-react with, but are serologically distinguishable from, the wild-type enzyme, and has disclosed also a correlation of site of mutation within the structural gene with kind of alteration in serological character (Kaplan, Mills, Ensign & Bonner, 1964). The flagellar antigen of *Salmonella typhimurium*, though it lends itself exceptionally well to detailed serological analysis in terms of antigenic subfactors, is somewhat intractable material for fine-structure genetical mapping; and the sequence of the 330 or so amino acids in the molecule (McDonough, 1965) is as yet unknown. It is therefore unlikely that the mutational alterations in serological character described above will be interpretable in the near future by reference to a complete genetical and amino acid sequence map, such as is available for the tryptophan-synthetase proteins of *Escherichia coli* (Yanofsky *et al.*, 1964).

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The L Form of *Neisseria meningitidis*

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SUMMARY

L forms were produced by the penicillin gradient plate technique from four strains of *Neisseria meningitidis*. All strains were group B and had various sulphadiazine sensitivities. One parent strain had been propagated for many years in the laboratory, whereas the other three were isolated from recent cases of meningitis. To date, each L form has had more than 60 serial passages on medium containing penicillin. From two strains stabilized L form variants developed on penicillin-free medium following 30 or 40 such passages. Morphological characteristics of these organisms were similar to L forms of other bacteria. Medium and environmental conditions necessary for optimal growth included: brain heart infusion containing a final agar concentration of 1·2% (w/v), sucrose, 10% (w/v), horse serum, 10% (v/v), pH 7·4, temperature 37° and increased CO_2 tension (candle jar). The L forms were non-groupable and had various fermentative reactions, whereas their sensitivity to sulphadiazine was the same as that of their respective parents. Revertants were produced on penicillin-free medium following every tenth serial passage in the L-form state. Sensitivities to sulphadiazine, fermentative reactions and serological groups of the revertant strains were identical with those of their respective parents.

INTRODUCTION

The induction of L forms by modifications of the gradient plate technique described by Sharp (1954) has been reported by many investigators (Lederberg & St Clair, 1958; Madoff & Dienes, 1958). Penicillin has been the inducing agent most commonly used, though other antibiotics such as bacitracin (Rotta, Karakawa & Krause, 1965), cycloserine (Ward & Martin, 1962) and cephalosporin (Williams, 1963), have also been used. Although L forms from a number of bacteria have been produced by this method, the successful production and propagation of L forms of *Neisseria meningitidis* have not been previously reported. In recent years a high carrier rate and an increasing number of clinical meningococcal infections have been noted in the United States (Bristow, Van Peenen & Volk, 1965; Brown & Condit, 1965; Communicable Disease Center, 1964). Because of the possibility that L forms may persist both during prophylaxis and in recurrent meningococcal disease, the present study was undertaken. This paper describes the *in vitro* production and propagation of L forms from four strains of *Neisseria meningitidis*. Morphological characteristics and optimal growth requirements are described. Serological groups, carbohydrate fermentations, and sulphadiazine sensitivities of the parent organisms are compared with those of their respective L forms and revertants.

METHODS

Organisms. Three of the four strains of *Neisseria meningitidis* used in this study were obtained from the Department of Bacteriology, Walter Reed Army Institute of Research, Washington, D.C., U.S.A. These strains (78-Eur., 79-Eur., 55-III, hereafter designated 78, 79, 55) were recently isolated (April 1965) from the blood of patients with meningitis. Two of the patients were stationed in Europe, the third in the United States. The fourth strain (Ne 15) was kindly sent to us by Dr B. W. Catlin (Marquette University School of Medicine, Milwaukee, Wisconsin). This strain was originally isolated in 1955 from the spinal fluid of a fatal case of non-epidemic meningitis (Catlin & Cunningham, 1961). Since then it has been repeatedly subcultured on artificial media and is now considered to be a 'laboratory strain'. All four strains were group B and had identical fermentative reactions.

Media. The standard medium used for the production and propagation of the L forms was Difco brain heart infusion broth (BRHIB) to which was added Difco agar, 1·2% (w/v), sucrose, 10% (w/v), Difco yeast extract, 0·5% (w/v), and heated (60° for 30 min.) horse serum, 10% (v/v). Benzylpenicillin (Charles Pfizer and Co., Inc., New York), 1000 units/ml., was added to the complete medium to maintain unstable organisms in the L form. This enriched agar medium containing benzylpenicillin, unless otherwise noted, is designated BRHIA. Penicillin was not added to the medium for the propagation of stable L forms. BRHIA was prepared weekly. To test sterility freshly prepared medium was incubated at 37° for 24 hr before storage at 4°.

To determine range and optimal conditions necessary for growth of meningococcal L forms, colonies were transferred for five serial passages on various media and the degree of growth noted. Growth was tested on three types of base medium; BRHIA, Difco heart infusion agar (HIA) and Baltimore Biological Laboratories (BBL) Trypticase agar (TA). Sodium chloride, 2 or 4% (w/v), and sucrose, 10 or 20% (w/v), were compared for osmotic stabilization. The effect on L-form growth of sera from horse, rabbit and man was also compared. Growth was evaluated on agar concentrations of 0·7–2·4% (w/v), at temperatures of 25–41°, in three gaseous environments (candle jar, aerobic, anaerobic) and in the range pH 5·5–11·0. The pH value of the medium was adjusted by adding either 0·1 N- or 0·5 N-HCl or NaOH.

Parent and revertant meningococci were subcultured either in BBL Eugonbroth or on Difco Mueller–Hinton chocolate agar.

Production and propagation of L forms. As the initial step in the production of L forms, a 12–18 hr growth of meningococci was inoculated into 20 ml. of Eugonbroth. This broth was placed on an Eberbach reciprocating shaker (6500 osc./hr), and incubated aerobically at 37° for 6–8 hr. Penicillin-free BRHIA plates (100 × 15 mm.) in which a 5 × 50 mm. trough had previously been made were inoculated with 0·3 ml. of this broth culture. After distributing the inoculum over the surface of the agar, 0·25 ml. of benzylpenicillin (100,000 units/ml.) was placed in the trough. Plates were incubated at 37° in candle jars containing moist gauze and were examined each day for 10 days under a colony microscope. When L-form growth was present, blocks of agar containing L-form colonies were cut out with a small metal spatula, inverted onto fresh BRHIA plates and streaked with the spatula

or a sterile glass rod. Subsequent cultures were then transferred in duplicate every 2–3 days. For future studies, several agar blocks containing a heavy growth of L-form colonies were frozen with acetone and dry ice following each 10th serial passage and immediately stored at −20°.

Reversion of L forms. Following every 10th serial passage, agar blocks containing L-form colonies were transferred to BRHIA without penicillin and subcultured until bacterial forms appeared. These revertant organisms were then transferred to Mueller–Hinton chocolate agar and subsequently freeze-dried.

Morphology of L forms. The morphological characteristics of L-form colonies were studied by two methods: (1) examination of unstained colonies with the colony microscope (magnification ×45); (2) by the stained agar technique of Dienes (Madoff, 1960) with phase contrast microscopy.

Serological grouping, carbohydrate fermentation and sulphadiazine sensitivity of L forms and revertants. Two methods were used in the preparation of L-form antigens for group determinations. In the first method L-form colonies were cultured on BRHIA containing 10% (v/v) rabbit serum instead of horse serum. After three serial passages, growth was removed from the surface of the agar with a sterile cottonwool swab and suspended in normal saline.

The second method was as follows. After three serial passages on BRHIA containing 10% (v/v) rabbit serum, large agar blocks with L-form growth were transferred to flasks containing 25 ml. BRHIA. The inoculated agar was then overlayed with 10 ml. of BRHIB containing 0·01% (w/v) agar, 10% (w/v) sucrose, 0·5% (w/v) Difco yeast extract and 1000 units benzylpenicillin/ml. Both the agar and broth contained 10% (v/v) rabbit serum. On the third and sixth day after inoculation 0·2 ml. of broth was subcultured on BRHIA. Following incubation for 48 hr, these subcultures were in turn transferred to other flasks with diphasic media. This procedure was repeated until a heavy growth of L-form colonies appeared in the broth. Organisms from the 10 ml. of broth were sedimented by centrifugation, washed once and resuspended in physiological saline. The concentration of the final suspensions prepared by both methods was equivalent to a MacFarland optical density standard no. 3. Difco meningococcus antiserum was used for grouping by the usual macroscopic slide and tube agglutination techniques. Controls included both normal saline and normal rabbit serum.

Carbohydrate fermentations were determined on HIA medium by using the method of Edward (1950). The medium also contained NaCl 2% (w/v), horse serum 10% (v/v), test carbohydrate 1·0% (w/v), phenol red 0·005% (w/v), and benzylpenicillin 1000 units/ml. Carbohydrates were not added to the control medium.

Sensitivity to sulphadizaine was determined on BRHIA without penicillin. Both the L form and its respective parent were subcultured on medium containing various concentrations of sulphadiazine and the minimum inhibitory concentration (m.i.c.) determined.

All revertant meningococci were identified by the following methods: (1) Gram stain; (2) grouping with meningococcus antiserum; (3) fermentative reactions and sulphadiazine sensitivities determined by the method of Evans, Hunter, Cary & Rust (1964).

RESULTS

Production and propagation of L forms

Following the inoculation of penicillin gradient plates with a broth culture of the parent meningococcus, L-form colonies appeared in 3–5 days. The number of colony forming units varied from 1 to 30 per plate. When agar blocks containing colonies were transferred to BRHIA, propagation of these colonies generally did not occur. Organism 78 was an exception, and these L forms propagated well following the first passage.

When growth did not occur in 8–10 days, the previously transferred agar blocks were again streaked across the surface of the same agar plate. Within 4–6 days, growth appeared at the previous site of the block though colonies were not evident along the streak pathway. These L colonies were then transferred to BRHIA without penicillin for reversion. The revertants designated 79 R, Ne 15 R, 55 R were subcultured in Eugonbroth and inoculated onto gradient plates. Within 2–4 days, a heavy growth of L colonies appeared in a band-like pattern between the penicillin trough and bacterial growth (Pl. 1, fig. 1). Non-propagating, small, rough bacterial colonies were also seen in this area (Pl. 1, fig. 2). On subsequent transfer, the L-form colonies propagated well, growth being apparent within 24 hr, and well-developed colonies were present in 48 hr. At the time of writing this report these L forms had had at least 60 serial passages on BRHIA.

In general, all revertant bacteria produced a significantly greater number of L forms than their respective parents. This property was maintained even after repeated subculture (× 45) of the revertants on Mueller–Hinton chocolate agar.

To test L-form stability BRHIA cultures were transferred to penicillin-free medium from every 10th serial passage. Initially all L-form colonies reverted to bacteria. Selected stabilized variants of L forms 78 and 55 first developed in penicillin-free subcultures from the 30th and 40th serial passage, respectively, and at the time of this report these variants had had 30 transfers on BRHIA without penicillin. By the 60th serial passage on BRHIA, all L form colonies of strain 78 were stable following subsequent transfer to penicillin-free medium. To date all L form colonies of organisms 79 and Ne 15 have continued to revert to bacteria by the third passage on penicillin-free medium.

Growth requirements of L forms

Range and optimal conditions necessary for growth of meningococcal L forms are shown in Table 1. Though L forms propagated on all three types of base medium (BRHIA, HIA, TA , optimal growth occurred on BRHIA. Growth was also tested on media that contained final agar concentrations of 0·7–2·4% (w/v). Growth occurred over a range of 0·8–1·8% (w/v) agar with optimum growth on 1·0–1·6% (w/v). On 2·0–2·4% (w/v) agar medium, L forms reverted to bacteria even in the presence of penicillin.

L forms did not propagate unless an osmotic stabilizing agent was present in the medium. Although propagation occurred with a concentration of 2% (w/v) NaCl, 4% (w/v) NaCl completely inhibited L form growth. Sucrose, on the other hand, was equally effective in supporting growth at a 10% or 20% (w/v) concentration.

Serum was also necessary for the propagation of L forms. Unheated serum appeared to be as effective as heated serum, and growth occurred equally well on media containing serum derived from horse, rabbit or man. In the first several passages, growth was less abundant when L forms were transferred from medium containing one species of animal serum to another. Horse serum concentrations ranging from 1 to 20% (v/v) appeared to be equally effective in L-form propagation.

Table 1. *Growth requirements for L forms of Neisseria meningitidis*

Requirement	Range	Optimum
Base media	Brain heart infusion Heart infusion agar Trypticase agar	Brain heart infusion
Agar % (w/v)	0·8–1·8	1·0–1·6
Osmotic stabilization	Sucrose NaCl	Sucrose
Serum	Horse Rabbit Human	Horse Rabbit Human
pH	6·0–9·5	7·0–8·5
Environment	CO_2 (candle jar) Aerobic	CO_2 (candle jar)
Temperature	29–39°	34–38°

The range of pH values in which growth occurred was pH 6·0–9·5 with an optimum of pH 7·0–8·5. Reversion of L forms was common on media at a pH 9·5–10·5. Since the unaltered pH value of freshly prepared BRHIA was pH 7·3–7·4, no adjustment was necessary for the routine cultivation of these organisms. L form propagation was also tested in three different environments: candle jar, aerobic and anaerobic environment. Growth was superior in the presence of extra CO_2 (candle-jar), and L-form propagation did not take place in an anaerobic environment. The concentration of the CO_2 in candle jars was not measured. Finally, cultures were incubated at temperatures ranging from 25 to 41°. Growth occurred in the range 29–39°, though organisms did not propagate well at either temperature extreme. For optimal growth the temperature range was 34–38°.

Morphology of L forms

Two types of L-form colonies were seen. The first type of colony contained little or no periphery and had a poorly developed core which did not penetrate well into the agar. When transferred, these cores tended to break apart and propagation did not occur. This type of L-form colony was characteristic of those seen on gradient plates inoculated with the parent bacterium.

The second type of L-form colony had a well-demarcated core which penetrated into the agar and a sharply defined periphery (Pl. 1, fig. 3). The core of this type of colony remained intact during agar transfer and colony propagation was excellent. The appearance of these colonies and the relative size of the core to periphery varied widely in the same culture (Pl. 1, fig. 4). Colony size, determined by duration of growth and degree of colony crowding, varied between 50 and 500 μ. When stained and observed under higher magnification (Pl. 2, fig. 5), structural elements were

indistinguishable from those of L form colonies of other bacteria. These structures included granular bodies which characteristically were found in the core of the colony and vacuoles which were primarily seen in the periphery. Phase microscopy defined these structures even more clearly (Pl. 2, figs. 6–8). Granular bodies varied in size (Pl. 2, fig. 7) and were also present both between and within the peripheral vacuoles (Pl. 2, fig. 8). The density of these vacuoles appeared homogenous and their boundaries were distinct (Pl. 2, fig. 8). Intermediate sized, phase-dense bodies were also seen throughout the periphery (Pl. 2, fig. 6).

Serological grouping, fermentative reactions, sulphadiazine sensitivity and Gram staining of L forms and revertants

Comparison of serological grouping, fermentative reactions and sulphadiazine sensitivity of L forms and of their respective parents is shown in Table 2. Suspensions of parent bacteria agglutinated with group B antisera. L form antigens whether harvested from agar or from broth did not agglutinate with polyvalent or groups A, B, or C antisera. Both normal serum and saline controls were negative.

Table 2. *Comparison of parent meningococci with their respective L forms*

Organism no.	Group	Sugar fermentation			Sulphadiazine sensitivity‡ (mg./100 ml.)*
		Glucose	Maltose	Sucrose	
Parent 78	B	+	+	–	0·05
(1) L form	N-g.†	+	+	–	No change
(2) Stabilized L form	N-g.	+	+	–	No change
Parent 79	B	+	+	–	0·05
L form	N-g.	+	+	–	No change
Parent Ne 15	B	+	+	–	0·05
L form	N-g.	±	±	–	No change
Parent 55	B	+	+	–	4·0
(1) L form	N-g.	+	+	–	No change
(2) Stabilized L form	N-g.	+	+	–	No change

* Minimum inhibitory concentration.
† Non-groupable.
‡ Sensitivities determined on complex media.

All parent meningococci produced acid from glucose and maltose but not from sucrose. L forms from organisms 78, 79 and 55 had fermentative reactions identical to those of their respective parents. These reactions were manifested by a permanent yellow colour change of the entire medium within 2 days after plate inoculation. The L form of Ne 15 produced a colour change only beneath the inoculating agar block. Fermentative reactions were tested with both recently produced L forms and L forms which had had 50 serial passages. The degree of fermentation in each group was the same. All control plates were negative.

Initial sulphadiazine sensitivity values of the parent organisms were determined by the method of Evans *et al.* (1964). These results, expressed as minimal inhibitory dose (m.i.c.) in mg./100 ml., are shown in Table 2. L-form sensitivity values were determined on penicillin-free BRHIA plates which contained various concentrations of sulphadiazine. Each plate was also inoculated with the parent organism.

On this medium the L form and its parent bacterium had identical sulphadiazine sensitivities. A comparison of these three properties in revertant bacteria and parent organisms is shown in Table 3. Serological grouping, carbohydrate fermentation and sulphadiazine sensitivity of the revertant strains were identical to those of their respective parent bacterium.

Bacteria were not seen in repeated Gram stains of L-form colonies. When Gram stains were performed on recently reverted bacteria, these organisms varied in size, shape and intensity of staining. However, following several transfers on Mueller–Hinton chocolate agar, the morphology and staining properties of these organisms were identical with those of typical meningococci.

Table 3. *Comparison of parent meningococci with their respective revertants**

Organism no.	Group	Sugar fermentation			Sulphadiazine sensitivity (mg./100 ml.)†
		Glucose	Maltose	Sucrose	
Parent 78	B	+	+	–	0·05
Revertants	B	+	+	–	0·05
Parent 79	B	+	+	–	0·05
Revertants	B	+	+	–	0·05
Parent Ne 15	B	+	+	–	0·05
Revertants	B	+	+	–	0·05
Parent 55	B	+	+	–	4·0
Revertants	B	+	+	–	4·0

* Revertants were produced from every 10th serial passage of unstable L forms.
† Minimum inhibitory concentration.

DISCUSSION

The successful production of L forms of *Neisseria meningitidis* was achieved in this work by using the penicillin gradient plate technique. Possibly the single most important factor in obtaining augmented production and serial propagation of L forms was the use of revertant meningococci for re-inoculation of gradient plates. This *in vitro* method proved simple and generally effective, and L forms from both old and new isolates were readily obtained. More recent studies in this laboratory indicate that the technique is equally applicable to production of L forms from group A and group C meningococci. Our methods differed widely from those described in previous reports in which L forms of neisseria from clinical sources could not be isolated and subsequently propagated (Dienes & Weinberger, 1951; Dienes, Bandur & Madoff, 1964).

To facilitate biochemical and serological studies of these L forms, optimal growth requirements were determined. In some instances these requirements, such as animal serum, agar concentration, pH of medium and gaseous environment, were similar to those that have previously been described for other L forms. Though studies on nutritional requirements of L forms are few, the need for animal serum for growth of certain L forms has been well established (Edward, 1953) and has also proved necessary for the growth of meningococcal L forms. High concentrations of agar promoting L-form reversion were first described by Landman & Halle (1963). They reported that agar concentrations of 2·0–2·5% (w/v) favoured reversion of

L forms of *Bacillus subtilis*. Dr Gooder (personal communication) has found that reversion of *Streptococcus faecalis* L forms occurs on 2·5% (w/v) agar. These findings were also characteristic of L forms of meningococci. Edward (1950) reported that L forms of *Streptobacillus moniliformis* grew well on media in the range pH 6·8–9·2; growth on a more acid or alkaline medium was not tested. Smith (1964) stated that loss of L-form viability occurred when the medium was less than pH 7·0 or greater than 9·0. Our results were in general agreement with these two reports, since L-form propagation was observed in a range pH 6·0–9·5 and optimal growth occurred at pH 7·0–8·5. To our knowledge, reversion of L forms on a high alkaline medium (pH 9·5–10·5) has not previously been reported. It has also been stated that the gaseous requirement for L-form growth is the same as that for the parent bacterium (Dienes & Weinberger, 1951). The growth of parent meningococci and their respective L forms was superior in the presence of extra CO_2, whereas neither organism propagated in an anaerobic environment.

Differences in growth requirements between meningococcal and other L forms were also apparent. For growth of certain L forms, media with high concentrations of salt are required (Smith, 1964). NaCl 4% (w/v) inhibited growth of meningococcal L forms, whereas sucrose supported growth at both concentrations tested. The optimal temperature for growth of other L forms has not been reported, though it has been suggested that the temperature should be the same as that for the parent bacterium (Smith, 1964). Studies in this laboratory demonstrated that parent meningococci grow at temperatures of 25–40°. The range for growth of meningococcal L forms was 29–39°, though optimal growth occurred only at 34–38°. Quantitative studies to define further the growth requirements of meningococcal L forms must await adaptation of these organisms to a broth medium.

Colony morphology, when examined under low magnification ($\times 45$), varied both with L forms derived from different parents and with different L-form colonies from the same parent. Factors such as type of medium employed, age of culture and amount of inoculum were important determinants. Under higher magnification granular bodies, intermediate size bodies and peripheral vacuoles were similar to those seen in other L forms. Since colony appearance varies within a given strain and structural elements are similar to L forms of other bacteria, macroscopic and microscopic colony morphology are not reliable differential tools in the identification of L forms of meningococci.

The specificity of serological grouping of group B meningococci is probably determined by a polysaccharide-polypeptide complex (Menzel & Rake, 1942); however, its site within the bacterium has not been determined. Because L forms of group B meningococci did not agglutinate with known group B antisera, the L form may lack this complex; and since L forms do not have a cell wall, the site of this complex is probably in the cell wall of the parent group B meningococcus. Other immunological studies to define the antigenic complexes of both the parent and the L form of group B meningococci are in progress.

The importance of (1) the use of a simplified medium to exclude sulphadiazine competitors, and (2) the standardization of the inoculum size have been previously emphasized in the determination of sulphadiazine sensitivities of meningococci (Jewell, 1958; Feldman, 1965). Because a simplified medium does not support L-form growth, sulphadiazine sensitivities were determined on penicillin-free BRHIA.

The minimum inhibitory concentrations of the L form and its respective parent were identical on this complex medium. Subsequently the m.i.c., both of the same parent and of the revertant from the respective L form, was determined on Mueller–Hinton agar (Table 3). This value was approximately one-tenth that observed on BRHIA. The discrepancy in these values emphasizes the importance of considering the effects of complex media when determining sulphadiazine sensitivity of L forms. The authors hope that with the application of this information and the future elucidation of more definitive tests for identification, the possible role of these L forms in human meningococcal disease can be defined.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. A penicillin gradient plate showing a band of L-form colonies between the trough and bacterial growth; ×0·8.

Fig. 2. Enlargement of an area of L-form growth on a penicillin gradient plate. Non-propagating, small, rough, bacterial colonies also present; ×16.

Fig. 3. Two-day growth of L-form colonies with well-demarcated cores and sharply defined peripheries; ×24.

Fig. 4. Two-day growth of L-form colonies demonstrating variation in size and structure; ×16.

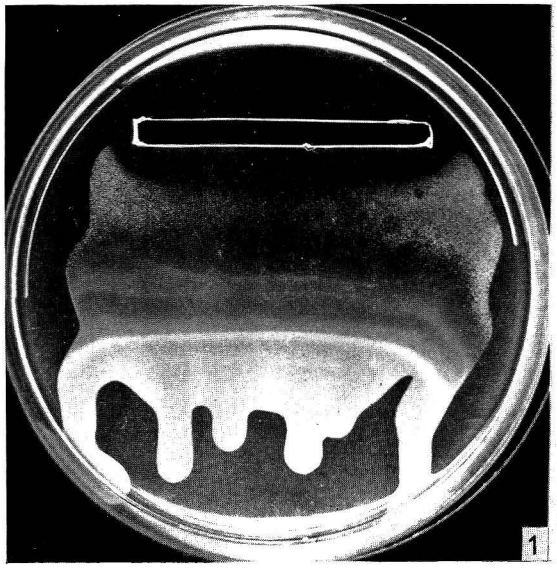
PLATE 2

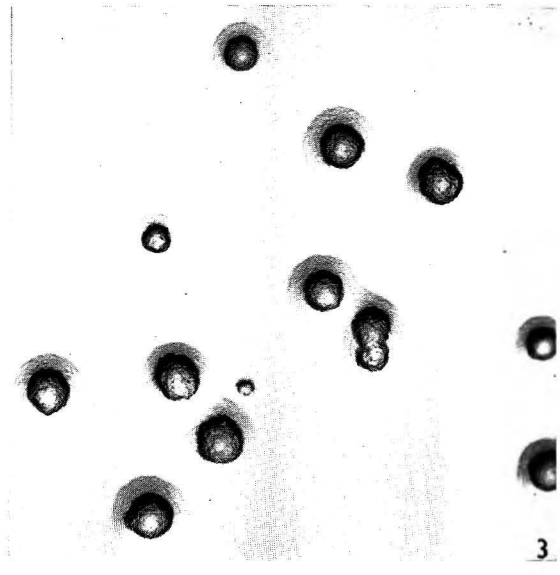
Fig. 5. An L-form colony with a double core and peripheral vacuoles. Dienes's stain with light microscopy; ×320.

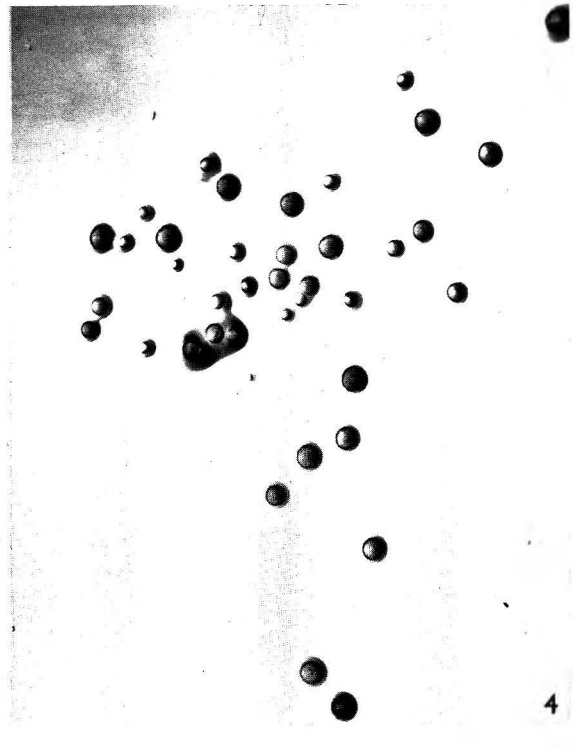
Fig. 6. One-day growth of an L-form colony. Periphery in focus demonstrating many intermediate size phase-dense bodies. Dienes's stain with phase microscopy; ×1600.

Fig. 7. One-day growth of the same L-form colony shown in Fig. 6. Core in focus demonstrating many granular bodies. Dienes's stain with phase microscopy; ×1600.

Fig. 8. Two-day growth of an L-form colony. Periphery in focus demonstrating well defined vacuoles. Dienes's stain with phase microscopy; ×1600.







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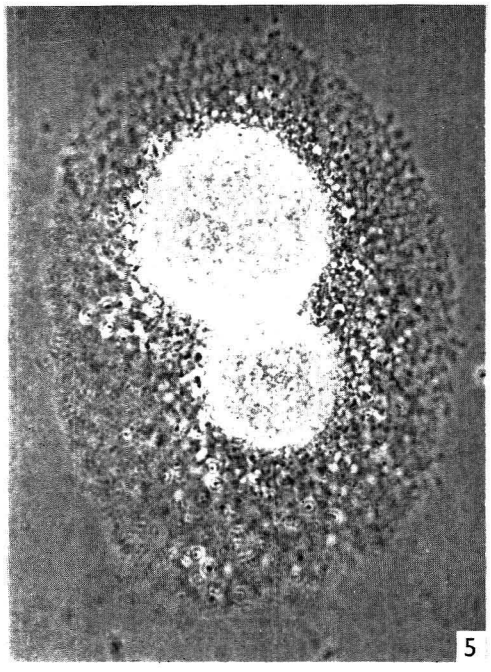
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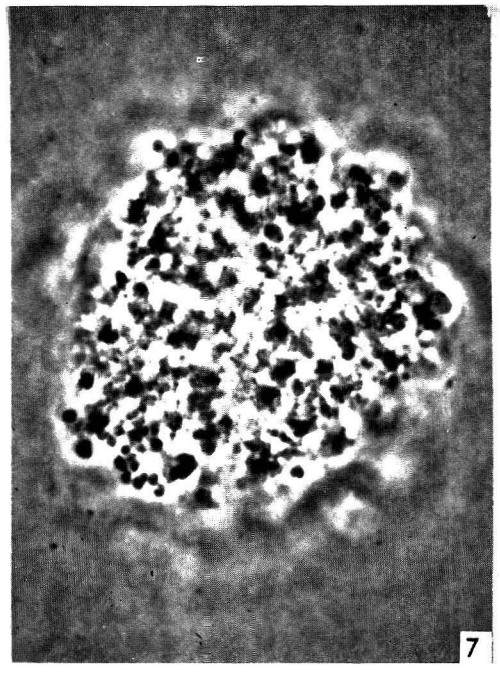
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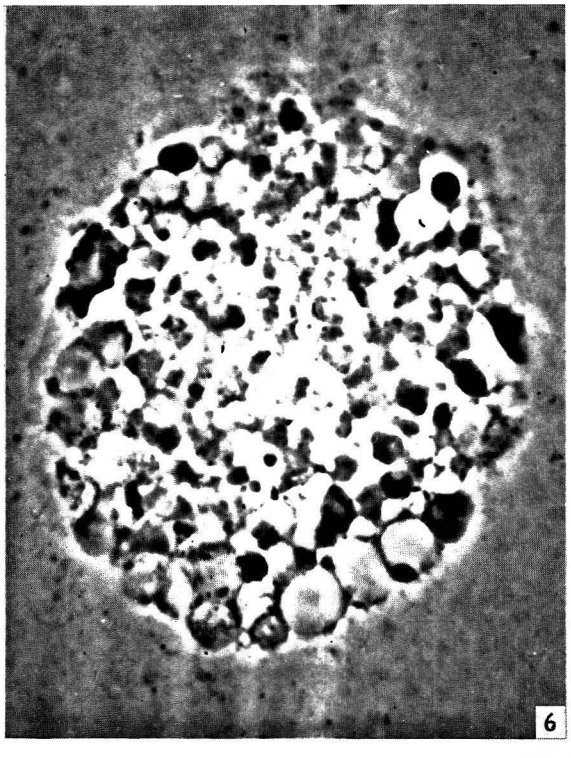
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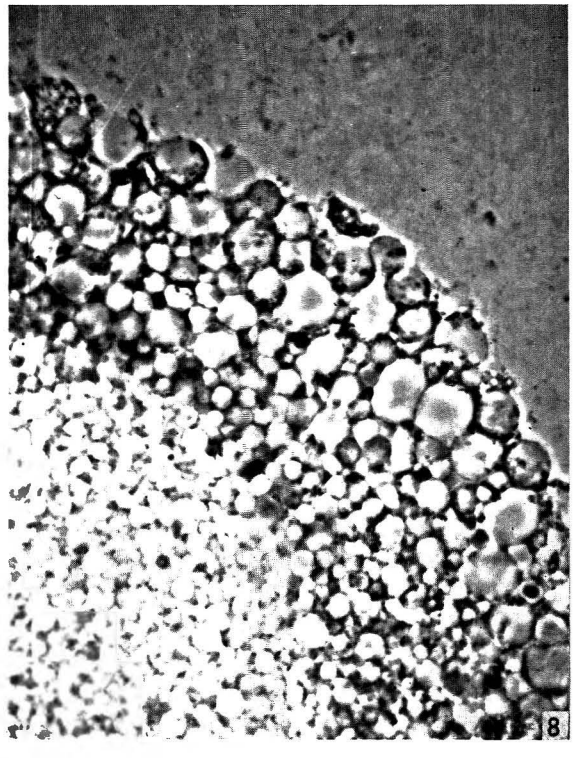
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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its forty-sixth General Meeting at the Royal Institution, London, on Monday, Tuesday and Wednesday, 4, 5 and 6 April 1966. *The following communications were made:*

ORIGINAL PAPERS

The Chemical Composition of Cell Walls from *Pediococcus cerevisiae* and from a Substrain that requires Methicillin for Growth. By P. J. WHITE (*Twyford Laboratories, Twyford Abbey Road, London, N.W.*10)

From *Pediococcus cerevisiae* ATCC 8081, a substrain (8081 CRD) was developed which grew only when the partially defined medium was supplemented with methicillin or other penicillins (White, P. J. (1962), *Abstracts, 8th Int. Congr. Microbiol., Montreal.* p. 75). Preparations of walls were obtained from: (1) *Pediococcus cerevisiae* 8081 grown without methicillin; (2) *P. cerevisiae* 8081 grown with a subinhibitory concentration of methicillin (10 μg./ml.); and (3) *P. cerevisiae* 8081 CRD grown with an optimal concentration of methicillin (100 μg./ml.).

About 35 % (dry wt.) of preparations 1 and 2 was removed by extraction with trichloroacetic acid (TCA) at 2°, whereas only 16 % (dry wt.) was removed from preparation 3. Walls (and the residue after TCA extraction) from the methicillin-requiring substrain were hydrolysed by lysozyme much more rapidly than were walls (or residues) of the parent strain.

Acid hydrolysates of all three wall preparations contained glucosamine, muramic acid (both probably *N*-acetylated in the wall), alanine, aspartic acid, glutamic acid and lysine, in proportions which suggested that they were components of a mucopeptide polymer (Perkins, H. R. & Rogers, H. J. (1959), *Biochem. J.* 72, 647). No *O*-acetyl groups were present. These mucopeptide components made up about 50 % by wt. of preparations (1) and (2), but about 80 % in preparation (3). Glucose and phosphorus were also present in the walls (in greater amounts in preparations (1) and (2) than in (3)), but were largely removed by TCA extraction. The residues after extraction all contained about 90 % (by wt.) of mucopeptide components.

Teichoic acids were isolated from each TCA extract: phosphorus, glucose, alanine and traces of glycerol were found in all. Teichoic acid from preparation (3) contained in addition about 7 % (by wt.) of mucopeptide components.

Capsular and Cell Wall Antigens of *Clostridium welchii*. By S. HAYTHORNTHWAITE and C. S. CUMMINS (*Bacteriology Department, The London Hospital Medical College, E.*1)

Cells of a capsulated strain of *Clostridium welchii* Type A (NCTC 2837) were extracted with M/30 phosphate buffer pH 8·0 for 1–2 hr at 100° C., and the extract precipitated with 3 vol. of methanol. The extracted organisms had lost most of their capsule. The methanol precipitate (1 mg./ml.) gave two lines against homologous serum on immuno-electrophoresis at pH 8·2. One of these moved towards the anode, the other towards the cathode. The same two lines were obtained in immuno-diffusion and immuno-electrophoretic tests with a trichloracetic acid extract (10 % TCA at 4° C.) of cell walls of NCTC 2837. A formamide extract of capsulated organisms (165° C. for 20 min.) also gave two lines on immuno-electrophoresis, one moving to the anode and one towards the cathode. The phosphate

buffer extract gave the same two lines after pronase treatment, but after periodate treatment one line had disappeared.

Acid hydrolysates of the pronase-treated capsular extract showed, on paper chromatography, only traces of amino acids, a large amount of galactose, traces of glucose, mannose and rhamnose, and two dense Elson-Morgan staining spots of hexosamines (neither glucosamine nor galactosamine).

The acidic antigen (moves towards anode) was isolated from the phosphate buffer extract by precipitation with 2% cetyl pyridinium chloride. The purified antigen contained no detectable hexosamine.

A Lipopolysaccharide-Lipoprotein Complex excreted by a Lysine-requiring mutant of *Escherichia coli*. By K. W. Knox and Elizabeth Work (*Twyford Laboratories, Twyford Abbey Road, London, N.W.*10)

A lipopolysaccharide is found in the culture filtrate of lysine-requiring *Escherichia coli* ATCC 12408 grown under lysine-limiting conditions (Knox, K. W., Taylor, A. & Work, E. (1965), *J. gen. Microbiol.* **41**, xvi; Bishop, D. G. & Work, E. (1965), *Biochem. J.* **96**, 567). This substance is excreted as a complex containing 60% lipopolysaccharide, 26% phospholipid and 10% protein. In the electron microscope, the isolated complex resembled the extracellular globules observed in close association with the cells in the process of excreting the complex (Vesk, M., Knox, K. W. & Work, E. (1965), *J. gen. Microbiol.* **41**, xvii). The phospholipid was mainly phosphatidylethanolamine; the protein contained all normal amino acids except cystine and had no diaminopimelic acid. The lipopolysaccharide had characteristic constituents of a typical lipopolysaccharide from rough strains of *E. coli* and was immunologically active. The polysaccharide moiety of the lipopolysaccharide (released by mild acid hydrolysis) was not however typical, as it had lost all its serological activity. A water-soluble preparation of the lipopolysaccharide, when heated under neutral or mildly acid conditions (e.g. 5–30 min. at pH 4·5 and 100°) lost serological activity rapidly. This loss was paralleled by the appearance of free 3-keto-2-deoxyoctonic acid (KDO) in solution. KDO was shown to be an immunological determinant group, 2 and 10 μmoles/ml. causing 17 and 69% inhibition respectively of the precipitin reaction between lipopolysaccharide and homologous antiserum; 25 μmoles of glucose or galactose (the other side-chain sugars) caused 17 and 6% inhibition respectively. Removal of KDO caused aggregation of the lipopolysaccharide but little change in toxicity or reactivity in the Schwartzmann reaction.

Correlation between the Electrokinetic Properties and the Surface-Antigenic Structure in *Staphylococcus aureus*. By J. E. Brewer and A. M. James (*Department of Chemistry, Queen Elizabeth College, University of London*)

Electrokinetic studies were made of a number of coagulase-positive staphylococci. Strains Cowan I (NCTC no. 8530) and Wood 46 (NCTC no. 712) were studied in detail.

Curves of pH–mean mobility ($\bar{V}$) of 18 hr cells of Cowan I showed the presence of ionizable carboxyl and amino groups characteristic of protein. They were remarkable in that there was a maximum at pH 4–4·5 ($\bar{V}_{pH\,7}$ 1·36 μ/sec./V./cm.; $\bar{V}_{pH\,4\cdot2}$ 2·8 μ/sec./V./cm.). This maximum was not due to selective adsorption of any component from the suspension medium, nor to loss of any part of the cell wall.

The pH–mobility curve of Wood 46 was different from that of Cowan I. Free amino groups were absent and the low pK_a (< 2) suggested the presence of ionizable phosphate groups. There was also a maximum at pH 4–4·5 ($\bar{V}_{pH\,7}$ 2·9 μ/sec./V./cm.; $\bar{V}_{pH\,4}$ 3·2 μ/sec./V./cm.). Trypsin-treated cells of Cowan I were electrophoretically similar to untreated cells of Wood 46. The material removed by trypsin included Jensen's antigen A. Trypsinization had no effect on the mobility of Wood 46.

In whole cells of both strains, oxidation of ribitol teichoic acid with sodium metaperiodate (Garrett, A. J. (1965), *Biochem. J.* **95**, 6C) resulted in the loss of the maximum

at pH 4–4·5. Comparison of the pH–mobility curves of oxidized and of normal cells suggests that the maximum results from a change in the configuration of teichoic acid on the cell surface governed by the state of ionization of free carboxyl groups.

The ratio $\bar{V}_{max.}:\bar{V}_{pH\ 7}$ for a further sixty coagulase-positive staphylococci from human and animal sources was correlated with the presence of Jensen antigen. Ratios greater than 1·5:1 corresponded to the presence of Jensen antigen, and ratios less than 1·3:1 corresponded to its absence.

The Digestion of Bacteria by the Rumen Ciliate *Entodinium caudatum*. By G. S. Coleman and F. J. Hall (*Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*)

It has been shown, previously (Coleman, G. S. (1964), *J. gen. Microbiol.* **37**, 209) that washed suspension of *Entodinium caudatum* grown *in vitro* engulfed washed bacteria, principally *Escherichia coli*, added to the medium and that amino acids from bacterial protein were incorporated into protozoal protein. The purpose of this communication is to present evidence on the site of bacterial digestion in the protozoa.

Escherichia coli labelled with [U-^{14}C]L-leucine or [U-^{14}C]L-glutamic acid were fed to protozoa in the presence of absence of the corresponding ^{12}C-amino acid free in the medium. In the leucine experiment the incorporation of ^{14}C into the protozoa after 5 hr incubation was decreased by over 50 % in the presence of the ^{12}C-amino acid, whereas in the glutamic acid experiment it was unaltered. The rate of incorporation of free [^{14}C]leucine from the medium into the protozoa was seven times that for free [^{14}C]glutamic acid. These results are difficult to explain if, as had previously been thought, the bacterial protein was broken down in the gastric sac to free amino acids which were then absorbed. Under these conditions the medium amino acids would probably mix with the digestion products. However, the results are consistent with the uptake of bacteria into the protozoal cytoplasm before digestion when the effect of amino acids free in the medium would depend on their rate of penetration through the gastric sac membrane. In an attempt to provide supporting evidence for this hypothesis sections of protozoa that had engulfed bacteria were examined under the electron microscope. From these preparations it was apparent that the bacteria were engulfed by the protozoa probably by way of the mouth and then taken up into the cytoplasm before undergoing much morphological change.

Protein Synthesis in a Cell-Free System from *Crithidia oncopelti*. By G. A. M. Cross (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

There have been few investigations with cell-free protein synthesizing systems from protozoa. The only extensive studies with a flagellate have been those of Chesters, J. K. ((1966), *Biochim. Biophys. Acta*, **114**, 385) using *Crithidia oncopelti*. This communication will describe further work which has led to the development of a highly active system from *C. oncopelti*.

Washed cells of *C. oncopelti* were broken by high-speed blending with ballotini. After removing cell debris and ribosomes by centrifugation, the supernatant fluid was passed through a column of G 25 Sephadex, and the high molecular weight fraction (S) collected. A polyribosome fraction (R) was prepared from cells broken in the presence of 1 % sodium deoxycholate, using procedures similar to those described by Wettstein, F. O., Staehelin, T. & Noll, H. ((1963), *Nature, Lond.* **197**, 430).

For optimum synthesis in the cell-free system it has been found that, in addition to fraction R, a saturating amount of fraction S is required, together with ATP (1 mM), GTP (0·25 mM), CTP (0·25 mM), Mg^{2+} (4–5 mM), K^+ (40–60 mM), 2-mercaptoethanol (5–10 mM), 20 L-amino acids including [^{14}C]alanine (each at 0·025 mM), and tris buffer (50 mM, pH 7·6). K^+ can be replaced by NH^{4+} but not by Na^+. The optimum temperature is between 25° and 30°. In some experiments [^{14}C]algal protein hydrolysate has been used as a tracer, in which case five additional [^{12}C]amino acids were added to obtain optimum incorporation. Under

these conditions the rate of the reaction declined after 30–60 minutes, when the amino acid incorporation was equivalent to the addition of about thirty residues per ribosome. If at this time the system was supplemented with fraction R, synthesis was resumed at its initial rate. During the incubation there was little breakdown of polyribosomes, and no release of nascent polypeptide chains from the ribosomes. Thus it appears that the failure of ribosomes to be released from messenger RNA may limit the reaction.

The system is inhibited by puromycin, tetracyclines, and actidione, but not by chloramphenicol. In this and other respects it resembles systems derived from mammalian rather than bacterial cells.

Evidence for a Diaminopimelic Acid Decarboxylase in *Crithidia fasciculata*. By W. E. Cutteridge (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

All bacteria, blue-green and green algae, vascular plants and phycomycete fungi with biflagellated or anteriorly uniflagellated spores so far examined utilise the diaminopimelic acid (DAP) pathway for lysine biosynthesis. In contrast, *Euglena*, and most fungi examined make use of the α-aminoadipic acid pathway (Vogel, H. J. (1965), in *Evolving Genes and Proteins*, p. 25, ed. by Bryson, V. and Vogel, H. J., Academic Press, New York). Lysine appears to be an essential amino acid for animals and most Protozoa. The flagellate *Crithidia oncopelti* is an exception and was found to utilize the DAP pathway (Gill, J. W. & Vogel, H. J. (1962), *Biochem. biophys. Acta*, 56, 200). This result was considered to be unexpected in view of possible phylogenetic relationships between flagellates, euglenids and higher fungi. It was stated therefore that the 'bipolar bodies' in the organism (Newton, B. A. & Horne, R. W. (1957), *Exptl. Cell Res.* 13, 563) are endosymbiotic bacteria which synthesize lysine for the host by the bacterial (DAP) pathway.

The flagellate *Crithidia fasciculata* can be grown in a completely defined, lysine containing medium (Kidder, G. W. & Dutta, B. N. (1958), *J. gen. Microbiol.* 18, 621). It has now been found that the lysine can be completely replaced by α-ϵ-diaminopimelic acid (a mixture of the L and *meso* isomers) but not by α-aminoadipic acid. No lysine could be detected in the sample of DAP used and the growth response of the organism to equimolar amounts of the DAP and lysine were identical. Tritiated DAP is incorporated into the TCA precipitable fraction of the organism as lysine. The amount of the incorporation is markedly reduced if lysine is present in the incubation medium. These results indicate that *C. fasciculata*, which has no 'bipolar bodies', contains a DAP decarboxylase and possibly a DAP racemase, enzymes which form part of the DAP pathway for lysine biosynthesis. The occurrence of the DAP pathway in *C. oncopelti*, therefore, cannot be used as evidence for the bacterial nature of the 'bipolar bodies' that it contains.

Physiological Ecology of *Leucothrix mucor*. By T. D. Brock and M. Louise Brock (*Department of Bacteriology, Indiana University, Bloomington, Indiana, U.S.A.*)

Leucothrix mucor is a widespread micro-organism in the marine environment, growing primarily as an epiphyte on benthic algae. Because of its distinct filamentous morphology, the organism can be recognized microscopically directly in nature, and its ease of cultivation makes it possible to relate laboratory to field observations. Radioautographic studies with tritiated thymidine have revealed that filamentous growth is not confined to the tip, but occurs throughout the length of a filament, both in the laboratory and in nature. However, statistical analysis of grain distributions on radioautographs revealed a surprising difference between the growth habit of pure cultures and natural material: in pure cultures cell division is completely random along a filament, whereas in nature cell division occurs in clustered areas, while other parts of the filament seem completely dormant. These clustered areas of growth are distributed randomly along a filament. When pure cultures of *L. mucor* are grown epiphytically on pure cultures of the red alga *Antithamnion sarniense* this growth clustering is not seen, so that it is not a result of the epiphytic habit *per se*, but of some aspect of growth in the natural environment. The same growth clustering was seen in incubations performed at Narragansett Bay, Rhode Island and in the Flaxafoi

fjord, Iceland, so that it is of general occurrence. When growth in culture is slowed either by reduction in temperature or by nutrient depletion, the cells in a filament round up and form gonidia which are released and have gliding motility. Presumably gonidial formation in nature also occurs whenever environmental conditions result in a slowing of growth. The holdfast can be visualized by fluorochrome staining with primuline, and it is found that the gonidia do not have preformed holdfast material, but apparently synthesize it in response to surface contact. Studies on a number of strains isolated from a variety of temperate sea coasts have revealed that these strains are remarkably uniform in thermal optima, nutritional requirements and DNA base composition, and differences exist mainly in the degree of gonidial formation. By use of radioautography it has been shown that two nutrients which are used well by pure cultures, glucose and glutamate, are also used well by natural material. The ecological implications of morphogenesis in *L. mucor* will be discussed.

Endogenous factor in Differentiation of *Streptomyces griseus*. By G. Szabó, S. Vitális and I. Békési (*Biological Institute, Medical University, Debrecen* 12, *Hungary*)

Two *Streptomyces griseus* strains were isolated. One of them no. 52–1 growing only in mycelial form in submerged culture, while the other no. 45 produces conidia. From the fermentation liquid of the conidia-producing strain an extract was made which, when added to the growth medium of strain no. 52–1, brought about conidia production.

The substance acting on the differentiation process was purified. It turned out to be of polypeptide nature and has been called factor C. Studying its mode of action, a change in nucleic acid metabolism was demonstrated. We have obtained some evidence that factor C increased the messenger RNA content of *Streptomyces griseus*.

Some Structural Features of Bacterial Flagella. By D. Abram* H. Koffler and A. E. Vatter (*Department of Biological Sciences, Purdue University, Lafayette, Indiana, and Department of Pathology, The University of Colorado Medical Centre, Denver, Colorado, U.S.A.*)

Bacterial flagella consist of the following three morphologically distinct parts: a basal region that is closely associated with the cytoplasmic membrane, a proximal hook, and the main portion of the organelle, a spiral filament. In the case of flagella from cells of *Proteus vulgaris* the basal structure is nearly spherical, 110–140 A in diameter, and is connected to the hook by a constriction. The nature of the material that is observable at the base of flagella from cells of various strains of *Bacillus*, if it represents a real structure at all, is not as clearly defined. In part it consists of cytoplasmic membrane, and its varied shape may be caused by the folding of the membrane around the proximal end of the flagellum at the site of attachment. The hook can be distinguished from the main filament not only by its morphology, but also by differences in fine structure and lesser solubility in acid and/or alcohol. A specialized structure is associated with the hooks of flagella from cells of *B. brevis* and *B. circulans*. Intact flagellar filaments from cells of the various strains studied differ in fine structure. The filament of *B. pumilus* appears to consist of six fibres composed of nearly spherical subunits coiled around an empty core. The filament of flagella of *B. stearo-thermophilus* 2184 has two regions that show marked differences in the manner in which the subunits appear to be organized.

Cell-free Synthesis of Flagellin. By H. Koffler and F. H. Gaertner (*Department of Biological Sciences, Purdue University, Lafayette, Indiana, U.S.A.*)

The main spiral filamentous portion of the flagellum is constructed of subunits composed of the globular protein flagellin. In the case of flagella from cells of *Bacillus pumilus* the subunits are arranged in the form of six fibres that are coiled around an empty centre.

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Flagellin from this organism has a molecular weight of *c.* 25,000–35,000, does not contain cystein, tryptophan and tyrosine, and is capable of polymerizing *in vitro* by self-assembly to form flagella-like filaments.

To better understand the morphogenesis of this organelle, especially the regulation of the process, an effort was made to obtain cell-free synthesis of flagellin. Lysates prepared by the osmotic disruption of lysozyme-treated cells were centrifuged at 15,000 g, and the supernatant liquid was used in a 10 ml. reaction mixture of the following composition: pH 7·8 tris buffer, magnesium acetate, 2-mercaptoethanol, ammonium chloride, adenosine triphosphate, guanosine triphosphate phospho-enolpyruvate, phospho-enolpyruvate kinase, ^{12}C-L-amino acids, ^{14}C-L-lysine, ^{14}C-L-arginine, and ^{14}C-L-leucine. After incubation of the reaction mixture at 37° for 30 min. carrier flagellin was added, and the reaction was terminated by adjustment of the pH to 2, a hydrogen concentration at which flagellin is soluble; acid-insoluble materials were removed. Increase of the pH to 5·4 resulted in the coprecipitation of the pH 2 soluble radioactive material and carrier flagellin. This precipitate was washed with TCA, dialysed, and purified further electrophoretically. Coincident carrier protein and radioactive materials were pooled, digested with trypsin, and chromatographed on the short column of the Beckman Spinco amino acid analyzer. Almost all of the peptides in the digest were radioactive. Resolution of the system is being attempted with the hope of learning about the role, if any, that the materials at the base of the flagellum play in the synthesis of flagellin.

Studies on the Biosynthesis of Polymyxin B. By M. J. Daniels (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Polymyxin B is a branched cyclic lipopeptide antiobiotic containing six residues of L-2,4 diaminobutyric acid (DAB), two of L-threonine, and one each of L-leucine, D-phenylalanine and (+) 6-methyloctanoic acid (MOA). The structure of the antibiotic has been determined by Suzuki *et al.* ((1963), *J. Biochem., Tokyo*, **54**, 555). Polymyxin has been purified from culture fluids of *Bacillus polymyxa* by salting into isopropanol with ammonium sulphate. The extract was then desalted by a cation-exchange technique (Dixon H. B. F. (1959) *Biochim. biophys. Acta*, **34**, 251) and other basic peptides separated from the antiobiotic by paper electrophoresis. Polymyxin has been assayed by an agar diffusion method, using *Escherichia coli* K12 as the test organism. A synthetic medium has been devised which permits a rapid growth of *Bacillus polymyxa*, together with satisfactory antibiotic production and minimal gum formation. This medium is an improvement on that described by Paulus & Gray ((1964), *J. biol. Chem.* **239**, 865.) Polymyxin is first detected in the medium towards the end of exponential growth. Chloramphenicol and chlortetracycline at concentrations ten times those required to inhibit growth and protein synthesis do not inhibit the synthesis of polymyxin whether measured by the increase in antibiotic titres or by the incorporation into the peptide of [^{14}C]abelled DAB (prepared by the Schmidt degradation of [^{14}C]L-glutamic acid).

Cell-free extracts of *B. polymyxa* have been obtained which will incorporate [^{14}C]DAB into basic peptides when incubated in a medium containing tris-HCl buffer 7·5, β-mercaptoethanol, magnesium and potassium ions, ATP, creatine phosphate and creatine phosphokinase, L-threonine, L-leucine, L-phenylalanine and MOA. Some characteristics of this cell-free system will be described.

Synthesis of the Antibiotic Nisin and Other Basic Proteins by *Streptococcus lactis* Grown in Batch Culture. By A. Hurst (*Unilever Research Laboratory, Sharnbrook, Bedford*)

Many bacteria make basic polypeptide antibiotics but their function to the producing organism is unknown. This question is being studied using a nisin producing strain of *Streptococcus lactis* grown in a batch culture which was examined at intervals as follows. Bacteria were removed by centrifugation; the nisin and basic proteins were extracted by hot 0·05 N-HCl and concentrated by use of *n*-propanol and salt (Cheeseman, G. C. & Berridge, N. J. (1957), *Biochem. J.* 65, 603); the supernatant and interface from this

procedure were freeze-dried. This material was redissolved in an equal volume of 5% acetic acid, desalted and fractionated on columns of Amberlite resin IRC-50 (Dixon, H.F. B. (1959), *Biochim. Biophys. Acta*, **34**, 251). Nisin was eluted with 50% acetic acid, freeze-dried and redissolved in a minimal amount of 0·05 N-HCl. This material contained 10–20% (w/w) nisin and was further examined by electrophoresis in polyacrilamide gels (Tombs, M. P. (1965), *Anal. Biochem.* **13**, 121).

The following changes were observed. At the beginning of logarithmic growth the nisin ($< 10^{-10}$ g./ml.) introduced with the inoculum could not be recovered from the cells. At 7 hr the nisin and basic proteins are recognizable by electrophoresis but in lower concentrations and in different ratios than in a 24 hr culture. The main nisin component from the 7 hr culture is slow moving and appears to be labile because with increasing age of culture the intensity of this band diminishes. The main component of the fully grown culture is faster moving and is stable.

Analogue Computer Studies of the Growth Characteristics of Survivors and Non-survivors in Dihydrostreptomycin-treated Cultures of *Escherichia coli*: the Concept of 'Lethality'. By B. Hammond, M. Kogut and J. W. Lightbown (*National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W.7*)

It was recently reported (Kogut, M., Lightbown, J. W. & Isaacson, P. (1965), *J. gen. Microbiol.* **39**, 165) that addition of dihydrostreptomycin to aerobically growing cultures of *Escherichia coli B*, followed by removal of extracellular antibiotic before growth had ceased, resulted in populations which grew at reduced, though apparently exponential rates for several hours and then gradually reverted to normal growth rates. Viable counts showed that such populations contained proportions of non-viable organisms. These proportions, as well as the extent of decrease in growth rate, were functions of the dihydrostreptomycin treatment. Microscopic examinations and the apparent constancy of growth rates before the onset of recovery suggested that during this phase the majority of individuals in such populations must continue to grow.

In further experiments, growth of dihydrostreptomycin-treated cultures, after removal of extracellular antibiotic, was followed for 4–6 hr by viable counts and extinction measurements. These determinations divide each culture into two constituent populations: (*a*) the 'survivors' which continue to grow and produce visible colonies 48 hr later, and (*b*) the 'non-survivors' which do not produce colonies; hence must stop growing at some stage. By use of an Analogue Computer, we have tried to answer the following questions: (i) How would the growth rates of each of these constituent populations have to vary during the course of the experiment to fit the observed data? (ii) Could both constituent populations grow at similar rates for a period, and for how long? (iii) How does the extent of dihydrostreptomycin treatment affect the growth rates of each constituent population? (iv) Is there any interchange from 'survivors' to 'non-survivors' or vice versa? What constitutes a 'lethal event'?

Inability to Demonstrate that Streptomycin causes Mis-reading of the Amino acid Code *in vivo*. By K. McQuillen and F. T. McClure (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Brock (*Symp. Soc. Gen. Microbiol.* **16**, 1966), in reviewing the mode of action of streptomycin, mentions that in an *in vitro* system the antibiotic 'effects the attachment of the wrong amino acyl-s-RNA in response to the presence of a specific amino acid codon'. The consequence of this is that artificial messenger-RNA's give rise to abnormal polypeptides.

Attempts have been made to see if this occurs *in vivo*. Cultures of *Escherichia coli* were grown for varying lengths of time in the absence and in the presence of concentrations of streptomycin adequate to cause considerable inhibition. Mixtures of radioactive amino acids or algal hydrolysates were added. The protein was hydrolysed and separated into

amino acids which were estimated and counted. The total protein had the same amino acid composition within experimental error whether or not the drug had been present. The newly formed proteins (indicated by the relative specific radioactivities of two, or most, of the amino acids) were also indistinguishable. Further experiments were carried out to see if the protein of the bacterial membrane fraction was specifically effected. For these *Bacillus megaterium* was used and the membrane fraction was obtained by lysis of protoplasts formed by lysozyme treatment of the organisms after growth in radioactive amino acids with and without streptomycin. Again it was not possible to detect differences in composition of the protein formed in the presence and absence of streptomycin.

These results do not demolish the hypothesis that this antibiotic owes its effectiveness to an ability to cause 'ambiguity' in translation of mRNA into polypeptide. Such alterations might be self-compensating so that overall composition was relatively unaffected. This seems improbable. However, it is entirely possible that some proteins are mis-made while most continue to be made normally. The progressive effects of this could be lethal even though gross changes in composition were not apparent. A necessary approach seems now to be to fractionate individual proteins or groups of proteins and to investigate their amino acid make-up.

Studies of the Mode of Action of Tetracycline. By I. H. MAXWELL (*Subdepartment of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Gale & Folkes ((1953), *Biochem. J.* **53**, 493) found that the primary action of tetracycline was to inhibit protein synthesis. Franklin ((1963), *Biochem. J.* **87**, 449) showed that tetracycline inhibited transfer of amino acids from amino-acyl transfer RNA to polypeptide in cell-free systems from rat liver and *Escherichia coli*.

In the present work, investigations have been carried out to discover whether the antibiotic affects attachment of either messenger RNA or transfer RNA to ribosomes.

(1) Cultures of *Bacillus megaterium* have been pulse labelled with ^{3}H-uridine in presence and absence of tetracycline. In both cases label was found in polysomes in lysates prepared from the cells.

(2) Polysomes have been prepared from cells of *Bacillus megaterium*, pulse labelled with ^{3}H-uridine, and have been dissociated by dialysis against low Mg^{2+} buffer. The labelled component, isolated by sucrose gradient centrifugation, was found to bind to ribosomes in presence or absence of tetracycline. It is concluded that the antibiotic does not inhibit the binding of messenger-RNA to ribosomes *in vivo* or *in vitro*.

Two recent reports indicate that tetracycline partially inhibits the poly U directed attachment of phenylalanyl transfer RNA to ribosomes in the Nirenberg & Leder system (Nirenberg, M. & Leder, P. (1964), *Science*, **145**, 1399). Effects of tetracycline on the attachment oflabelled transfer RNA to polysomes in cell-free amino-acid incorporating systems will be described. Preliminary results suggest that tetracycline may partly inhibit this process.

Reversible Antibacterial Mechanisms of 3,5,3′,4′-Tetrachlorsalicylanilide. By R. C. S. WOODROFFE and B. E. WILKINSON (*Unilever Research Laboratory, Isleworth, Middlesex*)

Tetrachlorsalicylanilide (TCS) is a germicide which accumulates on skin and is active against Gram-positive skin flora, although its use on skin is clearly undesirable because of its photosensitizing properties (Wilkinson, D. A. (1961), *Br. J. Derm.* **73**, 213). The present investigations are aimed at elucidating the mechanism of action of TCS against bacterial cells. The results show that TCS is bacteriostatic at low concentration and bactericidial at high concentration against *Staphylococcus aureus*; these activities vary with the strain used, are controlled by the number of bacteria originally present in the contact tube and are also related to alterations in cell membrane permeability which varies with TCS concentration. Release of ninhydrin-positive compounds from the bacteria after addition of TCS does not lead to cell death but only to bacteriostasis. Subsequent addition of

serum to remove TCS from the cells leads to complete recovery of viability, at least up to 2 hr after initial contact with the germicide. After longer periods there is a fall in viability. It is therefore apparent that this test organism can lose a proportion of its amino acid 'pool' without suffering immediate lethal damage provided the germicide is removed.

Glucose oxidation by *S. aureus* is inhibited by TCS at 1 μg./ml. and its succinoxidase activity at 0·2 μg./ml. However, uptake of ^{14}C-L-glutamic acid, L-lysine or L-aspartic acid is inhibited by TCS, as is sodium succinate and glucose uptake; this explains the apparent inhibition of succinoxidase and glucose oxidation. All these inhibited processes are restored after the addition of serum.

^{14}C-TCS taken up by *Bacillus megaterium* becomes localized on the cytoplasmic membrane. It is not released from other cell components during cell fractionation and isolated cell membranes and whole cells absorb similar concentrations of TCS. However, the association between TCS and the membrane is impermanent since TCS is recoverable from membranes by treatment with serum. Normal, phase-dark protoplasts from this organism become phase-bright and distorted on addition of TCS; osmotically-resistant 'protoplasts' in buffer can also be obtained from TCS-treated *B. megaterium* by subsequent treatment with lysozyme. Both these processes are inhibited or reversed by horse serum.

SYMPOSIUM ON BIOCHEMICAL STUDIES OF ANTIMICROBIAL DRUGS

The contributions to this symposium have been published as a book by the Cambridge University Press. The titles of the articles, the names of the contributors and their place of work are as follows:

The Object of the Exercise. By E. F. GALE (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Physiology of Antibiotic Production: the Role of the Producing Organism. By H. BOYD WOODRUFF (*Merck Sharp and Dohme Research Laboratories, Division of Merck and Co., Inc., Rahway, New Jersey, U.S.A.*)

Antibiotics Affecting Cell-Wall Synthesis. By P. E. REYNOLDS (*Sub-Department of Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Some Observations on Murein Synthesis and the Action of Penicillin. By JAMES T. PARK (*Department of Microbiology, Tufts University School of Medicine, Boston, Massachusetts*)

The Mode of Action of Griseofulvin. By K. J. BENT and R. H. MOORE (*Imperial Chemical Industries Limited, Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire*)

The Interference by Antibiotics (especially Nystatin and other Polyenes) with Specific Membrane Functions. By J. OLIVER LAMPEN (*Institute of Microbiology, Rutgers, The State University, New Brunswick, New Jersey, U.S.A.*)

Streptomycin. By THOMAS D. BROCK* (*Department of Bacteriology, Indiana University, Bloomington, Indiana*)