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Contents

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

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(*b*) Introduction; (*c*) Methods; (*d*) Results (illustrative protocols only should be included); (*e*) Discussion (if any), and general conclusions; (*f*) Acknowledgements; (*g*) References.

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

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

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

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

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

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

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

BACTERIA. Author's 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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Electron Microscopic Observations on the Excretion of Cell-wall Material by *Vibrio cholerae*

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(*Accepted for publication* 4 *April* 1967)

SUMMARY

Thin sections of *Vibrio cholerae* harvested during the logarithmic phase of growth in alkaline peptone water or in syncase medium have revealed an excretion process of the cell wall in the form of bulging out and pinching-off of portions. An identical phenomenon has also been revealed in cells harvested after a short period of incubation (1·5 hr) in glucose saline solution at 37°. Particles closely resembling the pinched-off cell-wall structures have been detected by metal shadowing and negative staining techniques in the bacteria-free filtrates of the log phase cultures (in both media) and the glucose saline incubation medium. These particles are in the range 400–1100 Å in size with a maximum frequency in the range 600–800 Å. No similar cell-wall changes have been detected in vibrios harvested from the stationary phase of growth in any of the culture media, nor in vibrios undergoing plasmolysis. Turbidimetric tests revealed no significant lysis of the vibrios when harvested during the logarithmic growth phase and incubated for several hours in saline or in Kellenberger buffer as compared to the lysis detected in distilled water. It is suggested that the cell-wall process described represents an excretory mechanism of *V. cholerae*, and the nature of the products released by the young vibrios and their probable relation with cholera toxin is discussed.

INTRODUCTION

Vibrio cholerae, a highly pathogenic Gram-negative organism, has been extensively studied from the bacteriological, immunological, pathological and therapeutic points of view (Pollitzer, 1959; *Proc. Cholera Res. Symp.* 1965). Unfortunately the pathogenesis of the disease cholera still remains ill understood, although it has generally been supposed that some toxins or choleragenic products, which are always associated with the cholera vibrios, initiate the disease. It has been reported (Gallut, 1954) that the toxins are released when the cholera vibrios are incubated in glucose saline medium at 37° for 4–6 hr. Recent investigations (Burrows, 1965; De, Ghose & Sen, 1960; De, Ghose & Chandra, 1962; Finkelstein, Norris & Dutta, 1964; Finkelstein, 1965) have indicated that the choleragenic products are likely to be liberated by the young vibrios presumably by a secretion process; however conclusive evidence for this has not yet been obtained.

In course of our investigation of the ultrastructure of *Vibrio cholerae*, part of which has been published earlier (Das & Chatterjee, 1966), attention was drawn to an interesting differentiation in the cell-wall structure of the young vibrios. The present report is devoted to this aspect of *V. cholerae* and describes structures which suggest

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a mechanism for the excretion of substances by the vibrios. A preliminary report of our observations has already been published elsewhere (Chatterjee & Das, 1966).

METHODS

Strains. Vibrio cholerae INABA 430, INABA C84 (both obtained from the Department of Bacteriology of this Institution) and OGAWA 154 (obtained through the courtesy of Dr S. Mukherjee, Indian Institute of Experimental Medicine, Calcutta) were used throughout this investigation. The organisms were maintained by fortnightly subcultures on nutrient agar slopes.

Media. The media used for the cultivation of the vibrios included the following: (i) nutrient agar; (ii) peptone water containing 1% (w/v) bacto-peptone (Difco) and 0·5% sodium chloride in distilled water (pH 8·0); and (iii) syncase medium (Finkelstein, Atthasampunna, Chulasamaya & Charunmethee, 1966) containing 5 g. Na_2HPO_4, 5 g. K_2HPO_4, 5 g. sucrose, 1·18 g. NH_4Cl, 0·089 g. $MgCl_2.6H_2O$, 0·004 g. $MnCl_2.4H_2O$, 0·005 g. $FeCl_3.6H_2O$ and 10 g. Casamino acids (Difco) dissolved in 1 l. distilled water.

Inoculum. Vibrios were washed from the surface of nutrient agar slopes and suspended in saline solution (0·85%), and 1 ml. amounts of this suspension were then spread over the surface of nutrient agar plates and incubated at 37°. Vibrios from an 18 hr growth on the nutrient agar were resuspended in suitable volumes of 0·85% saline, and after one or two washes in the same solution, adjusted so that the optical density of the final suspension was 1·0, then 0·1 ml. of this final suspension was used as the inoculum for 20 ml. peptone water or syncase medium distributed in Erlenmeyer flasks of 100 ml. capacity (De *et al.* 1962; Finkelstein, 1965). In some cases 20 ml. of peptone water medium were seeded with a heavy loopful from an agar culture and 1 ml. of 18 hr growth in this medium at 37° was used to inoculate the final growth medium.

Growth estimation. The growth of *Vibrio cholerae* in peptone water or syncase medium at 37° was estimated by measuring the optical density at 650 mμ with a photoelectric colorimeter.

Incubation in glucose saline. Vibrios harvested from 18 hr growth on nutrient agar were washed in 0·85% saline and suspended in suitable volumes of glucose saline (NaCl 0·85 g., glucose 0·5 g., distilled water 100 ml.) (Bernard & Gallut, 1943; Gallut, 1954) distributed in Erlenmeyer flasks (100 ml. capacity) in volumes of 20 ml., the initial optical density of the suspension thus being adjusted in the range 0·8–1·0. The suspension was then incubated at 37° and its pH was recorded at regular intervals with an electronic pH meter. Incubation was continued for a maximum period of 24 hr.

Membrane filtration. The membrane filters used were type B–6, Bac-T-flex (Carl Schleicher and Schuell Co., Keene, New Hampshire, U.S.A.) obtained through the courtesy of Dr E. T. Bolton, Carnegie Institute, Washington, U.S.A. Cultures in the logarithmic growth phase or bacterial suspensions in glucose saline after different periods of incubation were filtered through these membranes; the filtrates in all cases were found free of bacteria when tested by electron microscopy. The non-inoculated culture media (controls) were also similarly filtered and the filtrates examined by electron microscopy.

Metal shadowing and negative staining. For metal shadowing, a small drop of the bacteria-free filtrate obtained from a log-phase culture or from the glucose saline medium was deposited on a carbon-coated copper grid and the excess liquid was withdrawn a few seconds later. The preparation was then washed quickly in distilled water, dried and shadowed obliquely with chromium. For negative staining 1 % (w/v) uranyl acetate solution in distilled water was deposited immediately after the residue on the carbon substrate was washed in distilled water. The excess uranyl acetate was withdrawn a few seconds later and the preparation was dried (Huxley & Zubay, 1960).

Thin sectioning. The cells were harvested after the desired periods of incubation in the growth media or in a glucose saline solution by centrifugation and fixed in 1 % (w/v) osmium tetroxide solution in Kellenberger buffer (pH 6·1) for 16–20 hr at room temperature (Kellenberger, Ryter & Séchaud, 1958). The fixed cells were washed in 0·5 % uranyl acetate in the same buffer for about 2 hr. The washed cells were placed between layers of agar, dehydrated in graded ethanol and embedded in a methacrylate mixture (methyl:butyl = 2:3) or in Epon (Luft, 1961). Methacrylate mixture was pre-polymerized (Borysko & Sapranauskas, 1954) to reduce the polymerization damage and the corresponding sections after being stained were overlaid with a very thin film of carbon (Watson, 1957) to reduce the sublimation artifact. Thin sections were cut with glass knives on a Porter Blum ultra-microtome. Sections were stained with uranyl acetate and/or lead citrate (Reynolds, 1963) or with potassium permanganate (Lawn, 1960).

Electron microscopy. All electron micrographs were taken by a Hitachi HS-6 electron microscope at instrumental magnifications ranging from ×6000 to 15,000 Measurements were made from the enlarged prints by a micrometer eyepiece (accuracy 0·1 mm).

Turbidimetric test of lysis. Vibrio cholerae were harvested from 18 hr growth on agar surface or 3 to 5 hr subcultures in peptone water or syncase medium (log phase), washed in 0·85 % saline, resuspended in (i) distilled water, (ii) saline (0·85 %), or (iii) Kellenberger buffer (pH 6·1) and incubated at 37° for different periods. To test whether the vibrios were undergoing lysis the optical density of the suspensions was measured by a photoelectric colorimeter after different periods of incubation. In some cases turbidimetric tests were checked by electron microscopic examination of the whole cells and satisfactory agreement was obtained.

RESULTS

In an earlier publication (Das & Chatterjee, 1966) the ultrastructure of the flagellum, attachment of the flagellum to the cell, the cell wall and the plasma membrane of *Vibrio cholerae* has been described. The fine structures of these organelles in *V. cholerae* were found, in general, to be similar to the corresponding organelles of *V. metchnikovii* (Glauert, Kerridge & Horne, 1963).

Ultrastructure of the protoplasm. The considerably electron-transparent central region in *Vibrio cholerae* represents its nucleus. The nuclear zone seems to be crossed over at places by bridges of cytoplasmic materials (Pl. 2, fig. 3; Pl. 3, fig. 6; Pl. 4, fig. 11). The nucleoplasm contained a random network of fibrils or strands of dimension varying between 20 and 50 Å (Pl. 1, fig. 2; Pl. 2, fig. 5). These fibrils were stained by the uranyl acetate and were presumably the chromatin strands and will be thus

denoted, although no definite evidence of their chemical nature has yet been obtained. At places the strands were observed to cross the nuclear zone transversely and merge into the peripheral cytoplasmic material (arrow, Pl. 2, fig. 5).

Numerous small granules were found in the bacterial cytoplasm. The granules mostly had a dimension between 100 and 200 Å and were presumably ribosomes. Although dense populations of these granules frequently resisted elucidation as to the nature of their disposition in the bacterial cytoplasm, evidence was obtained which indicated that the granules were frequently interconnected by fibrillar structures prevalent in the cytoplasm (Pl. 2, fig. 4; Pl. 3, figs 8, 9). Although the typical membrane structures could not be resolved by the electron microscope used, the manner in which the cytoplasmic fibrillar structures were found to be connected with the plasma membrane in many pictures (Pl. 2, fig. 4; Pl. 3, fig. 10; arrowed) suggests that some of the fibrillar structures possibly represent the infolded plasma membrane. Many of the granules were prominently stained by potassium permanganate (Pl. 3, figs 6, 8–10).

Excretion of cell-wall material. Vibrio cholerae harvested from the logarithmic growth phase was found to exhibit some interesting morphological features. The cell wall, in places, was found to bulge out, and different stages of the bulging were recorded (Pl. 2, figs 3, 5; Pl. 3, figs 6, 10; Pl. 4, figs 11, 13) in this investigation. When the wall region bulged out considerably, its neck became increasingly constricted (Pl. 2, fig. 3; Pl. 4, figs 11, 13) and the membrane sac so formed appeared to get pinched off the bacterial surface and was presumably released into the medium. It was often observed that the bulged-out cell-wall region was associated with some small granules at the surface (Pl. 2, fig. 3; Pl. 3, figs 6, 7, 10). In some cases distinct granules were not observed, but instead a greater electron density was imparted to portions of the bulged-out membrane. This was possibly due to the coalition of the individual small granules. The maximum dimension of the bulged-out membrane region, as measured from thin sections, was usually less than 1000 Å and in very few cases between 1000 and 2000 Å. Identical pictures were obtained both in methacrylate- and Epon-embedded cells of young cholera vibrios. It is of interest to note that no similar bulging process of the cell wall was detected in vibrios harvested while in the stationary phase of growth (Pl. 1, figs 1, 2). However, particles resembling pinched-off membrane sacs and apparently lying isolated in the neighbourhood of the bacterial cells have often been revealed in thin sections (Pl. 1, figs 1, 2). Thin sections of many bacteria caught in the dividing stage exhibited the phenomenon of bulging out of the cell-wall membrane regions (Pl. 3, figs 6, 7). Potassium permanganate staining revealed clearly the presence of granules in association with the bulged-out cell-wall membrane region (Pl. 3, figs 7, 10). The nature of these granules in relation to those found in the bacterial protoplasm remains unknown.

Particles resembling the pinched-off membrane sacs observed in thin sections were also detected in the bacteria-free filtrates of log-phase cultures (Pl. 5, figs 15, 16). The dimensions of these particles were found to vary between 400 and 1100 Å (Fig. 1). Identical particles have been observed when the bacteria were grown in peptone water medium or in Finkelstein's syncase medium (Finkelstein *et al.* 1964), although electron microscopic sampling indicated the greater prevalence of these particles in the latter medium than in the former. Filtrates of uninoculated culture medium did not exhibit the presence of any similar particulate structure.

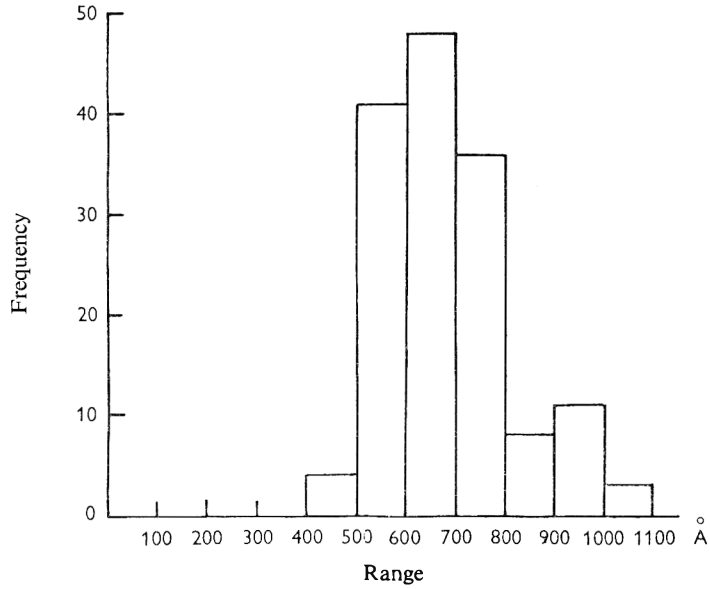

Fig. 1. Histogram showing the distribution of the diameter of particles isolated from the log-phase peptone-water-culture medium.

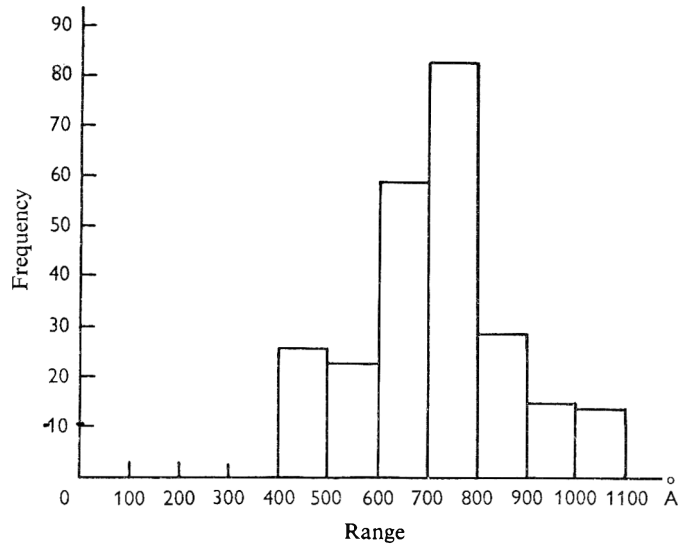

Fig. 2. Histogram showing the distribution of the diameter of particles isolated from glucose-saline filtrate.

When the vibrios harvested from 18 hr growth on agar were incubated in glucose saline and filtered after the pH had dropped to about 5·4 and all the glucose had been used up, numerous particles again resembling the membrane sacs observed in thin section were observed in the bacteria-free filtrate (Pl. 5, fig. 14). The size distribution of the particles obtained from glucose saline filtrate is shown in Fig. 2. At this stage of incubation, a considerable change in the bacterial morphology has taken place, the

details of which will be described in a separate paper. But even at the initial stages of incubation (within 1·5 hr), when metal shadowing and thin sectioning techniques revealed no significant change in the ultrastructural features of the bacterial protoplasm as compared with the resting phase (Pl. 1, figs 1, 2), numerous such particles were detected. Thin section of the vibrios at such an early stage of incubation revealed the bulging-out and pinching-off process of the cell wall (Pl. 4, fig. 13) as in the case of log-phase vibrios.

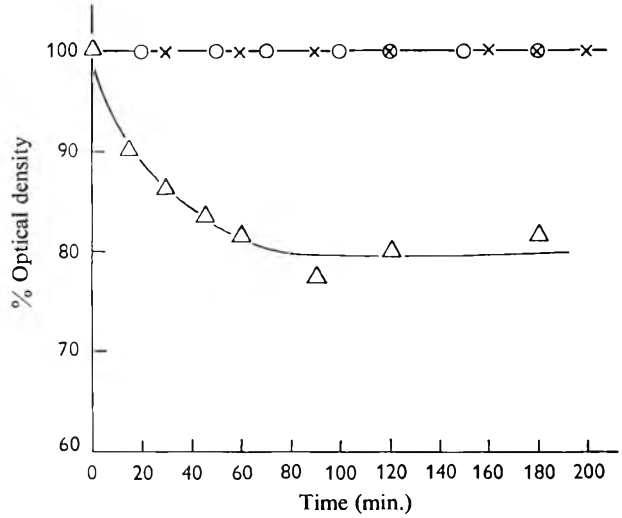

Fig. 3. Optical density against time of incubation of the vibrios at 37° in (i) normal saline –O–; (ii) Kellenberger's buffer – × – and (iii) distilled water —△—.

When vibrios were incubated in Kellenberger buffer or in normal saline at 37°, no significant fall in the turbidity of the suspension was recorded, whereas in distilled water a significant fall in turbidity was noted after as little as 20 min. incubation period, indicating that the cells were undergoing lysis (Fig. 3). At the initial stage of plasmolysis, the vibrio protoplasm was found to retract from the cell wall (Pl. 4, fig. 12) but the structure of the cell-wall membrane remained smooth.

DISCUSSION

Excretion of cell-wall material. Thin sectioning of the young cholera vibrios has revealed the process of bulging out and pinching off of the cell wall in the form of approximately spherical sacs. This phenomenon of the vibrio cell wall has been seen in methacrylate- and in Epon-embedded specimens irrespective of the electron stain used. Exactly similar observations have been made when the vibrios were harvested from 16 to 18 hr growth on agar and incubated in glucose saline solution. Neither in thin sections nor in metal-shadowed preparations of cells bearing extrusions could any significant change in the protoplasmic structure be discerned as compared with resting phase cells. Vibrios harvested from the stationary phase of growth do not show a similar turnover of the cell wall. Further, particles resembling pinched-off membrane sacs have been detected in thin sections in bacteria-free filtrates of log-phase cultures, in alkaline peptone water, Finkelstein syncase media, and in glucose saline incubation medium. On the other hand, ultra-thin section of the vibrios undergoing plasmolysis

do not show any similar membrane turnover process. In all cases where cell-wall excretion has been noted in the vibrios, no evidence has been obtained of any bulging out of the protoplasm and the plasma membrane has remained undamaged. When young vibrios were suspended in saline solution or in Kellenberger buffer only (without the osmic acid) and incubated at 37°, no significant fall in the turbidity resulted. These observations seem to rule out the possibility of any plasmolysis occurring during the preparation of the vibrios for electron microscopy. It is therefore suggested that the cell-wall changes described do represent a mechanism of excretion of cell-wall material by young cholera vibrios.

Micro-anatomical studies of secretory and other tissues have produced considerable evidence of membrane activity at the surface of certain cells (Bennett, 1956). It is by virtue of the plasticity of the cellular membrane that substances which cannot diffuse through it may be incorporated by, for example, pinocytosis (Lewis, 1931; Palade, 1953) or may be excreted from the cells. Although there may exist several mechanisms for the excretion or secretion of cellular products (Kurosumi, 1961, 1962), the mechanism of reversed pinocytosis (Palade, 1959, 1960, 1961; Lever & Peterson, 1960; Lever, 1962; Farquhar, 1961*a*, *b*) has been observed in many cells. However, a prerequisite of this method of excretion is the inclusion of secretory substances by smooth membranes within the cells, in other words the substances secreted are in some way enclosed in an intracellular vesicle, which then migrates towards, adheres to, and finally communicates with the cell membrane (Lever, 1962). The mechanism of release of the sac-like structures by *Vibrio cholerae* observed in this investigation is clearly different. However a simple pinching off of part of a cell may constitute a method of secretion (Aberchrombie, Hickman & Johnson, 1957). The extrusion of the so-called colloid bodies from the thyroid cell to the vesicle possibly occurs by the budding off of apical cytoplasm (Lever, 1961). A similar mechanism has been observed in the secretory process of the rabbit apocrine sweat glands (Kurosumi, 1962). This process has some similarity with the excretory activity of *V. cholerae*, as postulated in this study, but in detailed analysis many differences may again be detected, some of which could be due to the differences between the anatomy of a bacterial and a mammalian cell. It is only of interest to note here that a similar mechanism has also been found in the escape of certain viruses from cells (Epstein, 1962).

The excretion product. From the evidence presented above it appears that the secretion product of the vibrios possibly contains some substances of unknown chemical nature within an apparently closed sac bounded by the cell-wall membrane. It is likely that the enzymes or other chemicals of the vibrios normally located in between the cell-wall membrane and the plasma membrane are excreted in this process and are contained in the considerably electron-transparent cores, as seen in thin sections, of the spherical excretion bodies. Thin sectioning has provided some evidence of the presence of small granules in the excreted vesicles. Metal shadowing or uranyl staining has, however, failed to reveal the presence of granules. This is consistent, however, provided it is assumed that the granules are not at the outer surface of these bodies, since metal shadowing can reveal the surface details only and the negative staining with uranyl acetate or phosphotungstate is of no help in elucidating the inner structures in the case of bodies which are not penetrated by the stain. However, until more evidence is available, the presence of the granules within the excretion products, their nature and their site of formation in the bacterial cell remain to a great extent undetermined.

Probable relation of the excretion product with cholera toxin. It has been reported that the *Vibrio cholerae* cells incubated in glucose saline solution at 37° produce toxins which are lethal to mice when injected intraperitoneally (Gallut, 1954). The mode of release of the toxins by the vibrios was unknown. These toxins were detected in the bacteria-free filtrate and ultrafiltration studies indicated that they consisted of two components, one glucolipidic in nature and between 800 and 1000 Å in size while the other was of unknown chemical nature and about 40 Å in size (Gallut & Grabar, 1945). These sizes thus compare well with the dimensions (400–1100 Å) of spherical particles we have found being released by the living young vibrios while incubated under identical conditions in glucose saline solution or in culture media (peptone water or syncase medium). However, Gallut and his associates did not conceive of these toxins as being secreted by the living vibrios and the evidence put forward by them was not sufficient to decide whether these were exo- or endo-toxins. On the other hand we have not yet been able to isolate these particles, free of the higher and lower molecular weight substances that might be present, so as to determine whether they are toxic. However, it is likely that the particles we have observed being released by the vibrios correspond to the cholera toxin studied by Gallut since identical particles (identical in dimension at least) have been found under identical experimental conditions. Further similarities may be drawn since according to Gallut (1965) the major part of the choleratoxin is located in the cell wall and the residual toxicity of the vibrio protoplast is relatively weak. Thin sectioning has shown definitely in this study the presence of cell-wall material in the excreted particles.

Recent studies have indicated, as summarized by Burrows (1965), that the vibrios secrete the toxin during their early phase of growth and that it is unlikely that the toxins are leached out of the cells since they are not dialysable. Thus De *et al.* (1960, 1962) reported the liberation of cholera exo-toxin in young cultures of vibrios in peptone water medium, and Finkelstein *et al.* (1964) found the presence of choleragenic products, as tested by the infant rabbit technique (Dutta & Habbu, 1955), in young cultures of the vibrios in brain heart infusion broth and also in the syncase medium of Finkelstein *et al.* (1966). Finkelstein *et al.* (1964) further observed that the cholaragenic product could be separated by dialysis into two fractions—Procholeragen A and Procholeragen B, as they termed them, of which the factor A is non-dialysable and the factor B is dialysable. These results thus lend support to the present electron microscopic evidences in so far as the excretory activity of the young cholera vibrios and the presence of a high molecular weight component in the excretion product are concerned. It has to be admitted again that there is yet no clear relationship between the excretion product observed in this investigation and the choleragenic product described by Finkelstein *et al.* (1964). It is of interest to note in this respect that the evidence obtained so far has indicated the presence of polysaccharide and lipid components in the choleragenic product (Burrows, 1951; Dutta & Oza, 1963; Bhatia, Kaur, Bhatia & Shrivastava, 1966). Cell walls of Gram-negative bacteria are known to contain lipopolysaccharide. Knox, Vesk & Work (1966) have described the morphology of the excretion of lipolysaccharide by a lysine-requiring mutant strain of *Escherichia coli.* This is somewhat similar to the presently observed morphology of the excretion of cell-wall materal by *V. cholerae,* although the conditions of excretion in the two cases are different.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Ultrathin section of *V. cholerae* harvested from the stationary phase of growth in peptone water medium; stained with lead. × 40,000.

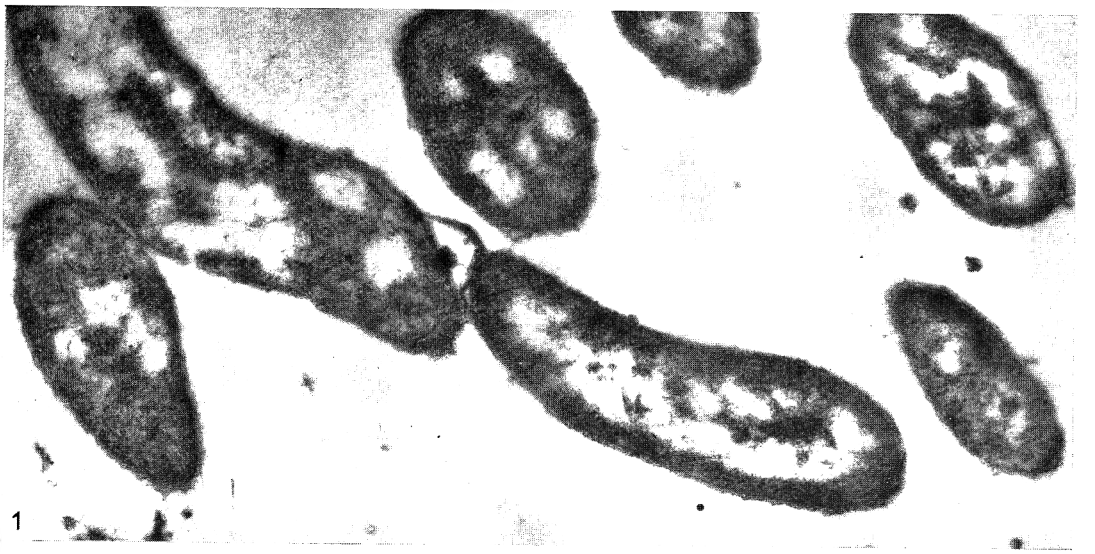
Fig. 2. *V. cholerae* cells harvested from the stationary phase of growth in Syncase medium, stained with lead. Dispersed chromatin strands (CS), 100–200 Å granules (G) and smooth cell wall (CW) and plasma membrane (PM) shown. × 100,000.

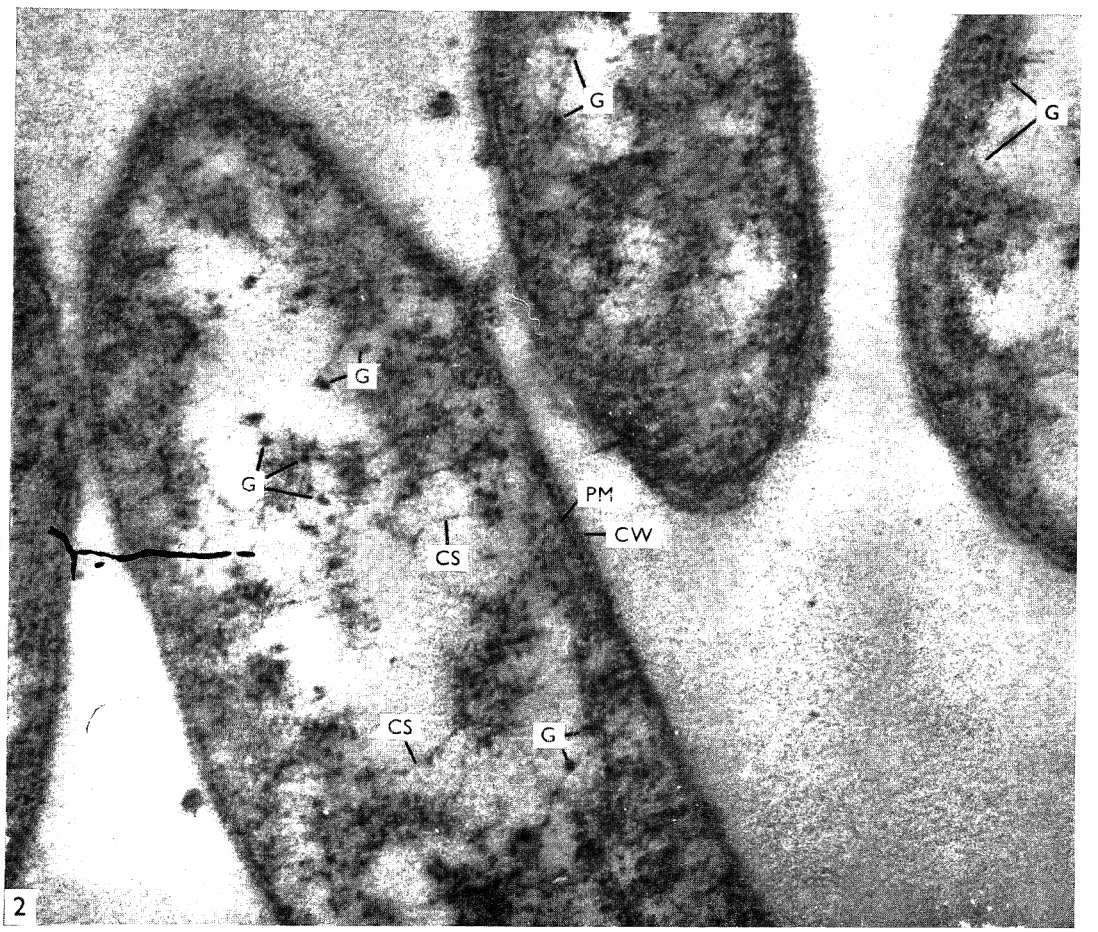
PLATE 2

Fig. 3. *V. cholerae* from the logarithmic phase of growth in peptone water; stained with lead. Many cell-wall bulges can be seen. The sequence of the process is perhaps as indicated by the sequence of the letters A, B and C. Arrows indicate the association of granules with the bulged out cell-wall membrane. × 100,000.

Fig. 4. Portion of a longitudinal section of *V. cholerae* stained with lead. Arrows indicate the regions where some of the cytoplasmic fibrillar structures may have originated by an infolding of the plasma membrane. Many 100–200 Å granules (G) can be seen interconnected by fibrils. × 110,000.

Fig. 5. *V. cholerae* harvested from logarithmic phase of growth in Syncase medium; uranyl acetate stained. Chromatin strands (CS) are distinct. Arrows show the merging of chromatin strands in the cytoplasm. Bulging of the cell wall is shown at A and B. × 93,000.





S. N. CHATTERJEE AND J. DAS

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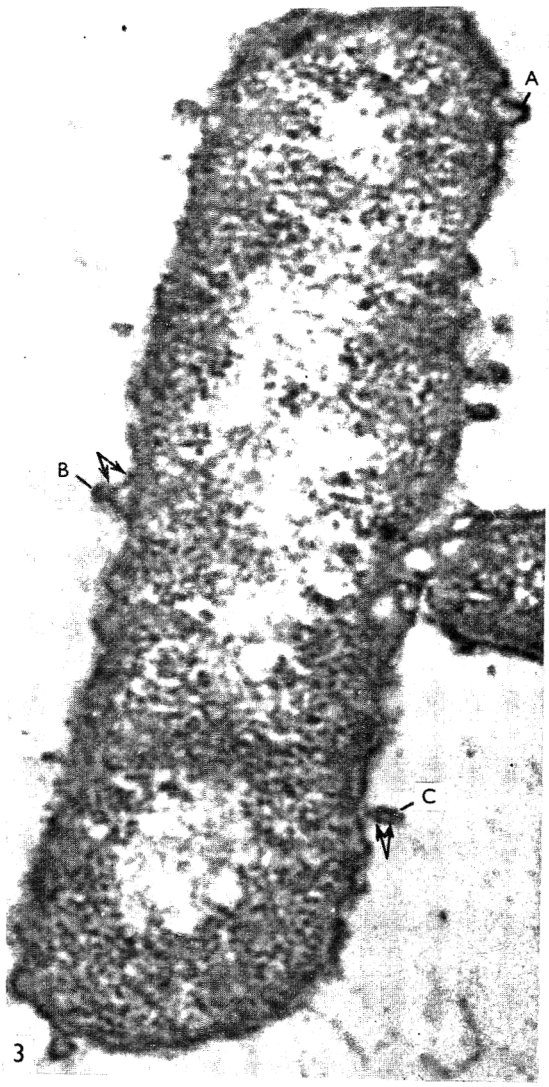

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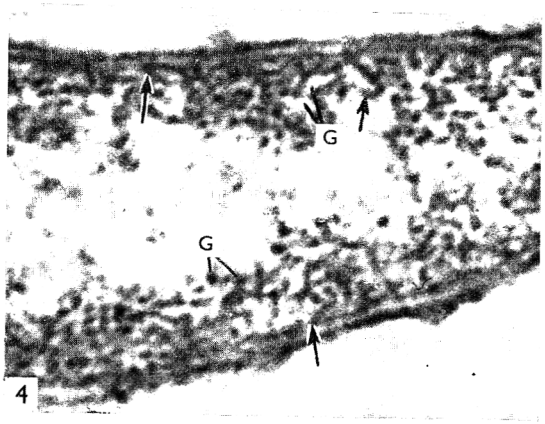

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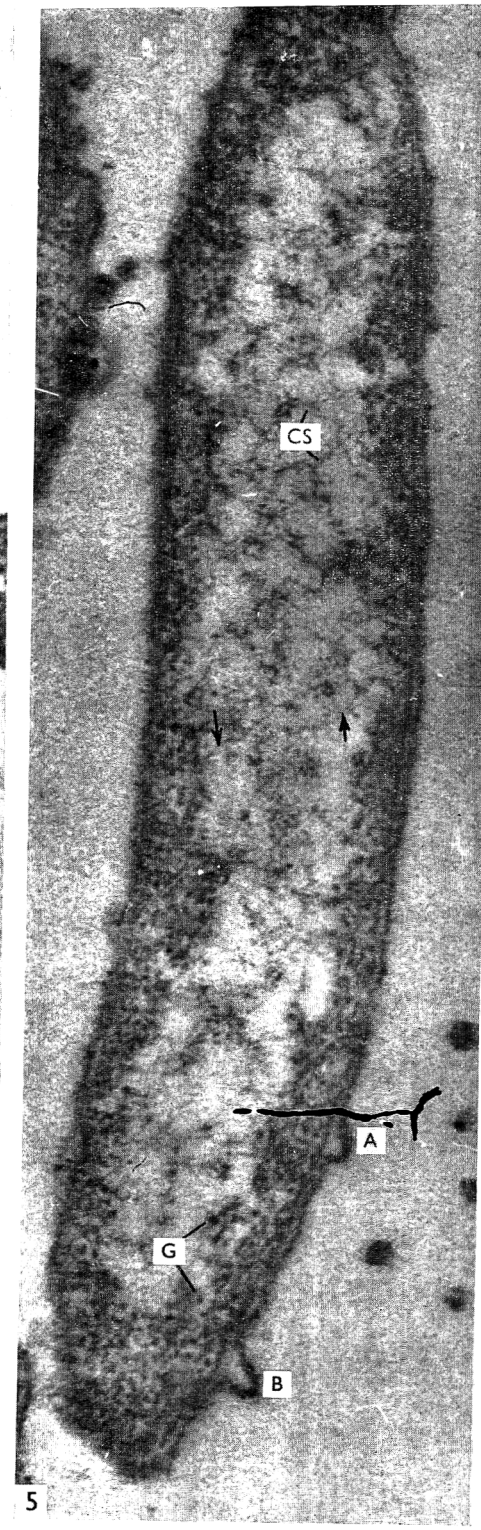

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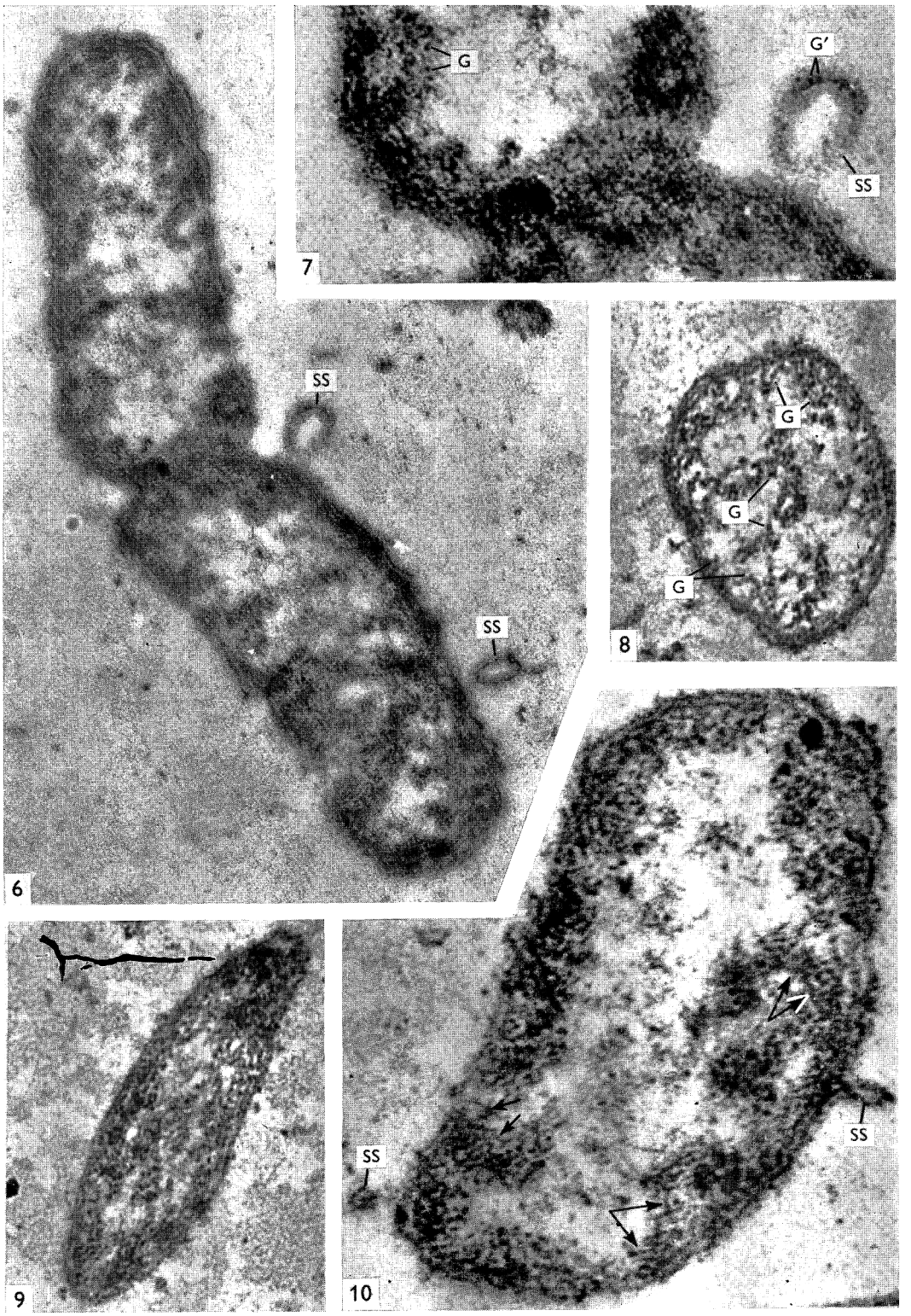

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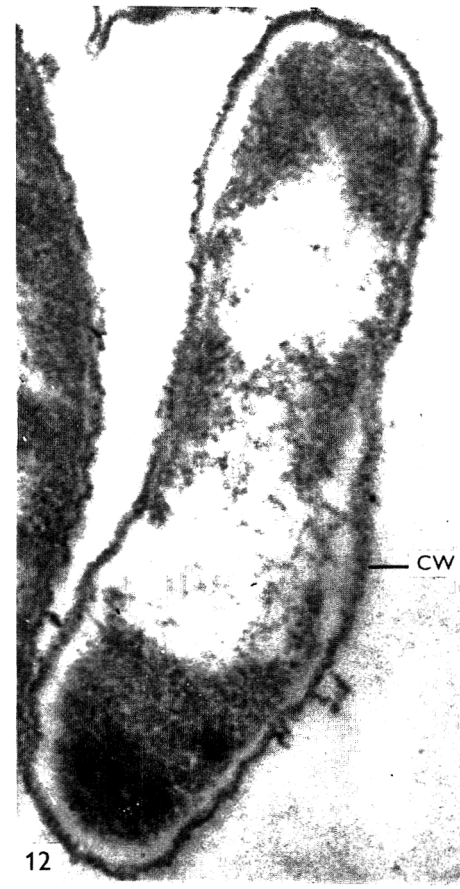

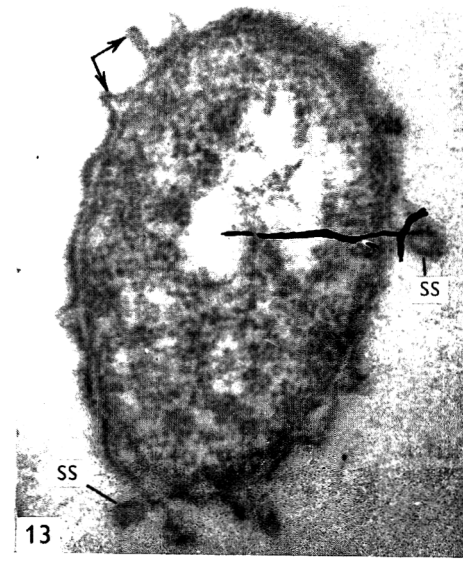

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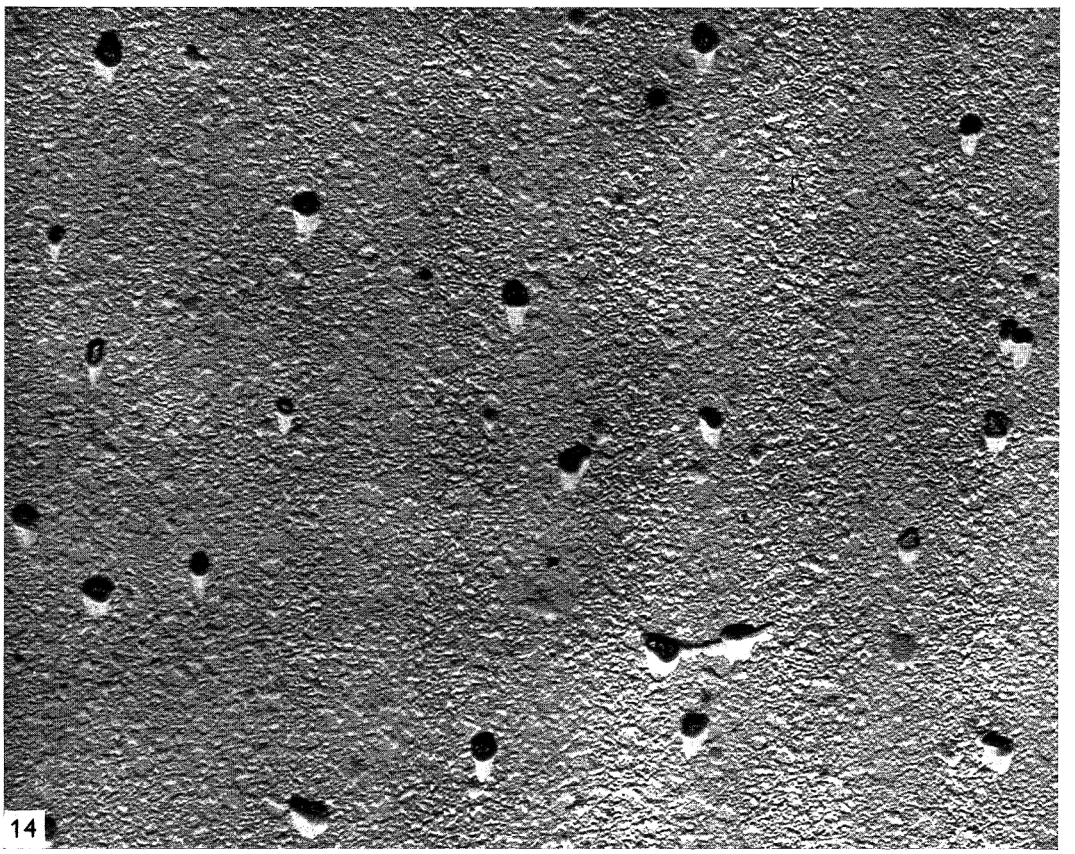
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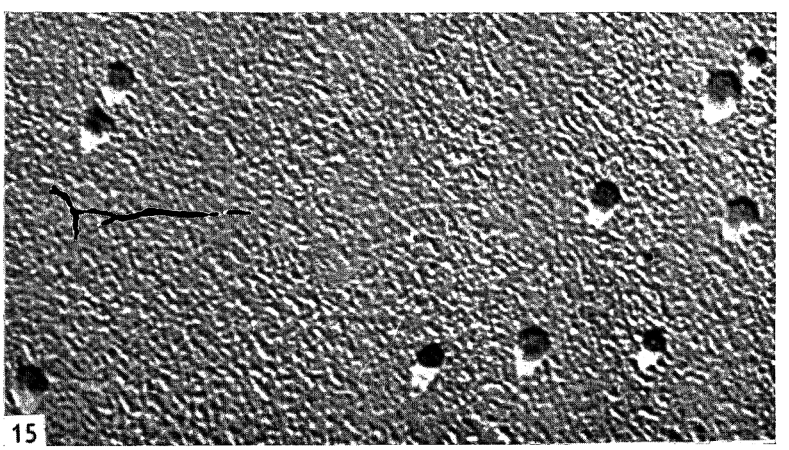
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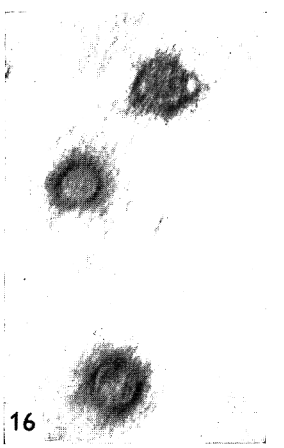
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S. N. CHATTERJEE AND J. DAS

PLATE 3

Fig. 6. A dividing cell stained with potassium permanganate. Sac-like structures (SS) formed by the bulged out cell-wall portion are presumably ready to be pinched off. × 48,000.

Fig. 7. Part of fig. 6 at a higher magnification. The granules (G') in association with the sac-like structure (SS) are distinctly seen. Many apparently similar granules (G) are seen in the bacterial cytoplasm. × 96,000.

Fig. 8. Cross-section of a cell harvested from a logarithmic phase culture in peptone water and stained with potassium permanganate. Many granules have taken up considerable stain. The granules are associated with fibrillar structures present in the cytoplasm. × 50,000.

Fig. 9. Oblique section of another cell harvested during the logarithmic phase of growth and stained with potassium permanganate. Fibrillar structures in the protoplasm and the associated granules can be seen. × 50,000.

Fig. 10. *V. cholerae* cell harvested during the logarithmic phase of growth in Syncase medium and stained with potassium permanganate. Arrows indicate the fibrillar structures which have possibly originated by infolding of plasma membrane. Sac-like structure (SS) formed by the bulging out of cell wall is presumably ready to be pinched off. An apparently pinched off sac-like structure (SS) can also be seen. × 81,000.

PLATE 4

Fig. 11. *V. cholerae* cell harvested from a logarithmic phase culture in Syncase medium with bulges (A, B) on the cell wall. Sections of apparently pinched off sac-like structures (SS) are also seen. Stained with lead. × 75,000.

Fig. 12. *V. cholerae* cell undergoing plasmolysis. The protoplast has retracted from the cell wall. Note that the cell-wall structure is smooth. Stained with lead. × 70,000.

Fig. 13. Cross-section of *V. cholerae* cell harvested after 1·5 hr incubation in glucose saline solution. Cell wall bulging and the formation of sac-like structures (SS) can be seen in several places (arrow). Stained with lead. × 87,000.

PLATE 5

Fig. 14. Particles isolated from the bacteria-free filtrate of the glucose saline solution in which the vibrios harvested from an 18 hr agar culture were incubated for 4–6 hr. Shadowed with chromium. × 60,000.

Fig. 15. Chromium-shadowed particles isolated from a logarithmic phase culture of vibrios in Syncase medium. × 60,000.

Fig. 16. Particles isolated from a log-phase peptone water culture and negatively stained by uranyl acetate. × 100,000.

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Anomalous Diffraction of Gram-positive Bacteria

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SUMMARY

Application of the anomalous diffraction theory of van de Hulst to turbidity measurements of Gram-positive bacteria is discussed. It is shown that this method is capable of detecting characteristic morphological changes during growth. Comparison with the cell mass method and direct observations of *Micrococcus freudenreichii* suggest that these changes are primarily related to cluster size. Initial application of the method in the study of osmotic change in *Bacillus megaterium* is described.

INTRODUCTION

Although it has been shown that at least some bacteria fit the homogeneous spherical models for which simple scattering theories are available (Koch, 1961; Petukhov, 1965), use of light scattering in studying bacterial growth and transport has been largely limited to empirical correlation of scattering properties with other physical measurements. The difficulties in applying scattering theory lie in the need for extrapolation of scattering data to zero concentration, determination of concentration and refractive index of particles, and the necessity to use cumbersome numerical theoretical solutions.

In this paper we wish to describe the application of the anomalous diffraction theory of van de Hulst (1946, 1947, 1962) in studying some Gram-positive bacteria. It is shown that by use of this simple approximation coupled with curve fitting by computer, kinetic studies of bacterial morphology can readily be accomplished, eliminating determination and extrapolation of concentration.

THEORY

For spherical particles with dimensions comparable to the wavelength of the measuring radiation, complete Mie theory (Mie, 1908) is generally required to account for multipole scattering and intraparticle interference. Van de Hulst (1946, 1947, 1962) has shown, however, that for particles with refractive index close to that of the medium, assumption of negligible ray deviation and back scattering can be made. The light perturbation can then be described by diffraction and phase lag in the spherical particle. The resultant efficiency factor is described by the closed expression

$$Q_{\text{ext.}} = 2 - 4/\rho \sin \rho + 4/\rho^2(1 - \cos \rho) \tag{1}$$

where

$$\rho = \frac{4\pi(n_p - n_0)a}{\lambda_0}. \tag{2}$$

n_p is the refractive index of the particle, n_0 is the refractive index of the solvent, a is the particle radius, λ_0 is the wavelength *in vacuo*. At low ρ, van de Hulst (1962) has pointed out that equation (1) is asymptotic with the Jobst equation (Jobst, 1925).

$$Q_{ext.} = \tfrac{1}{2}\rho^2. \tag{3}$$

The extent of the approximation introduced by use of equation (1) rather than Mie theory is seen in Fig. 1. Mie data (Pangonis, Heller & Jacobsen, 1957) for

$$\frac{n_p}{n_0} = 1{\cdot}05$$

is shown, as this refractive index most closely approximates that found in bacteria (see below).

The extinction efficiency factor is related to the apparent extinction coefficient γ by

$$\gamma = N\pi a^2 Q_{ext.}, \tag{4}$$

where N is the number of particles per unit volume of solution. Lewis & Lothian (1954) showed that if particles are large enough to deviate from the Jobst equation, plots of experimental turbidity with 1λ and γ with ρ (equation (4)) could be compared. By expanding the ordinate ρ of the theoretical curve so that maxima and minima were overlapped with the experimental curve, ρ values corresponding to given wavelengths were obtained. From these, values of $4\pi(n_p - n_0)a$ (we shall call this product B) were calculated using equation (2). Van de Hulst (1947) has shown that the best value of radius ($a_{obs.}$) to adopt for a heterodisperse system such as this is given by the expression

$$a_{obs.} = -o\frac{\int_0^{\rho_0} f(a)a^3\,da}{\int_0^{\infty} f(a)a^2\,da},$$

where $f(a)$ is the number of particles with size between a and $a+da$. The studies of Lewis & Lothian were made on barium sulphate suspensions and *Lycoperdon pyriforme* spores. These particles are large enough for the first maximum in Fig. 1 to be obtained with visible radiation. With the smaller size of most bacteria, this maximum is not reached with visible and near ultraviolet wavelengths, requiring non-linear curve fitting. In this work the difficulty in fitting is circumvented by the use of electronic computation. Because of the difficulty in separating refractive index and size effects in growing bacterial systems, B values will be reported as such, and corollary experiments to separate the contributions discussed.

It should be noted that the turbidity dispersion method has also been applied using numerical Mie data (see, for example, Barns & LaMer, 1946; Heller, Bhatnagar & Nakagaki, 1962; Fikhman, 1963) but refractive index must be assumed, and wavelengths of measurement are dictated by the available numerical data, making the method somewhat inflexible for biological particles.

EXPERIMENTAL

Monodisperse latexes

Monodisperse polystyrene latexes were obtained from the Bioproducts Department of The Dow Chemical Company. Sizes of the samples had been determined using

electron microscopy. The latexes were diluted with glass-distilled water and measured without further treatment.

Culture media and organisms

The principal requirement of culture media used in growth experiments is that the media be relatively transparent in the wavelength range of measurement. Chemically defined media without cofactors were used. To obtain vigorous growth it was found necessary to add small amounts of Difco-brain-heart infusion broth (BHI). The basal medium consisted of 0·3% $NH_4H_2PO_4$, 0·7% K_2HPO_4, and 5×10^{-4}% $FeSO_4$; the pH was 7·1. Organisms and media used were:

Micrococcus freudenreichii (ATCC 407). Basal medium with 0·25% Difco-Casamino acids and 0·11% BHI.

Micrococcus candidus (ATCC 8425). Basal medium with 0·5% glucose and 0·2% BHI.

Bacillus megaterium (ATCC 13632). 3·7% BHI.

Growth conditions

Two hundred and fifty ml. volumes in 500 ml. Erlenmeyer flasks were used. Flasks were vigorously shaken in a Gyrotropic shaker (New Brunswick Scientific) at 30°. *Micrococcus freudenreichii* and *M. candidus* were inoculated from 24 hr cultures at concentrations of 1×10^7 and $2{\cdot}5 \times 10^6$ organisms/ml. respectively. *Bacillus megaterium* was grown overnight, harvested and washed twice with distilled water, and gently resuspended in distilled water.

Spectrophotometer

Turbidity measurements were obtained using a Beckman DK-2A spectrophotometer. This instrument is well suited to turbidity measurements, as the distance between cell and photomultiplier is long, limiting measurements to a small solid angle around zero. Turbidity was found to follow the Beer–Lambert Law within experimental error at all wavelengths below an apparent optical density of 1·0. Errors due to slit width variation during scanning were estimated to be 1% or less. This was determined by noting the total change in slit width throughout the scan, and manually varying the slit by this amount at the extreme wavelengths by changing the sensitivity or amplifier gain. For more complete discussion of the instrumental requirements for turbidity measurements see Heller & Tabibian (1957).

Spectrophotometer measurements

The reference cell contained fresh medium. Periodic checks were made to determine that the presence of bacteria was not altering the absorption of the medium. This was done by centrifuging the culture at intervals during the growth cycle and scanning the supernatant against fresh medium. It was found in this way that *Micrococcus candidus* excretes an ultraviolet-absorbing material during growth, and measurements on this organism were confined to the 600 to 400 mμ region.

Rather than changing the environment of the organisms during growth by dilution, turbidity was reduced below 1 by using cells of 10, 5, 2, and 1 mm. path lengths. Checks were routinely made of B values upon changing path lengths during the runs.

Samples were scanned immediately after sampling to avoid temperature-induced changes in refractive index.

Refractive increment measurements

Suspensions for refractive increment measurements were made by centrifuging overnight cultures, washing twice with distilled water, followed by centrifugation of the suspension at 17,000 rev./min. in a Sorvall SS–34 head for 30 min. The supernatant was poured off and the tubes with wet organisms recentrifuged in the same manner. This centrifugation removed much of the occluded water contained in the wet mass. The organisms were then weighed and resuspended for measurement. Refractive index measurements were made in a Phoenix BS300 differential refractometer at 546 mμ. Usually five dilutions of a 1% (wet w/v) stock were measured. Although refractive index is quite temperature-dependent, differential refractive indices are much less dependent. Hence, solutions were equilibrated and run at ambient temperature (approximately 23°).

Microscopy

Direct measurements of the bacterial cluster diameters were performed using an American Optical Phase Star Microscope with Polaroid camera attachment. Samples of the growing culture were dried and stained with crystal violet after which pictures were taken at a magnification of 970. These were subsequently enlarged for diameter measurements. Both minimum and maximum dimensions were recorded for *Micrococcus freudenreichii*, as most clusters were not perfectly spherical.

Computations

Electronic computations utilized a Burroughs B5500 computer. Input consisted of turbidity measurements at 25 mμ intervals from either 500 to 350 mμ or from 600 to 400 mμ. A range of B values with specified increments in B was set for trial and error fitting. For a given spectrum the selection of best fit (and B value) was based on a minimum in

$$SS \text{ (sum of squares)} = \Sigma_i[T(\lambda_i) - F\gamma(\lambda_i)]^2,$$

where $T(\lambda_i)$ is the turbidity measured at wavelength i, $\gamma(\lambda_i)$ is the extinction calculated at wavelength i for a specified B according to equation (4), and F is the normalization factor

$$F = \frac{T(\lambda_l)}{\gamma(\lambda_l)}$$

equating theoretical and experimental turbidity at one wavelength (usually the lowest wavelength measured). This factor incorporates the $4\pi Na^2$ in equation (4), eliminating the need for concentration determination. Figure 2 shows the fit of points for the best B value with an experimental turbidity dispersion (*Micrococcus freudenreichii*). It should be noted that in theory turbidity data from only two wavelengths are required for the method, but inclusion of more data allows a better determination of the degree of fit obtained.

RESULTS

Monodisperse latexes

As a preliminary trial of the method, turbidity dispersions were taken of latexes with sizes of 1·305 and 0·796 μ. Although these particles have a uniform spherical size, the refractive index of latex relative to water (n_p/n_0 = 1·20) is somewhat greater than that desirable for use of the anomalous diffraction theory. The particle diameters for the two latexes were estimated from the turbidity measurements to be 1·310 and 0·715 μ.

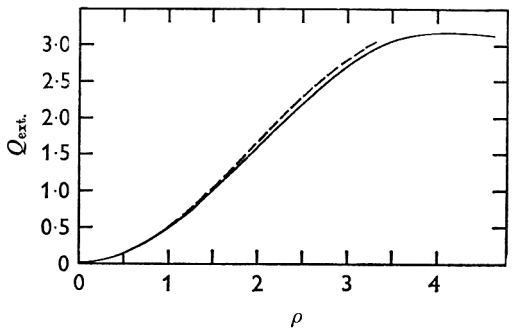

Fig. 1

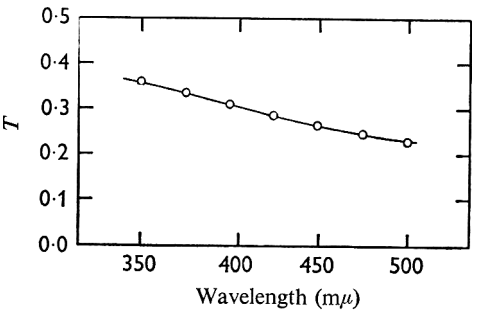

Fig. 2

Fig. 1. Comparison between the extinction efficiency factor for anomalous diffraction theory (—) and Mie theory (- - -) for n_p/n_0 = 1·05. (Mie data from Pangonis, Heller & Jacobsen, 1957.)

Fig. 2. Comparison of best fit with experimental turbidity dispersion of *Micrococcus freudenreichii*. (—), experimental; (○), best theoretical fit.

Batecrial growth

The principal question to be answered in investigating the use of this technique is to what extent is the method sensitive to bacterial morphological change during growth. Since both *Micrococcus freudenreichii* and *M. candidus* grow in cell clusters, it was of interest to determine the uniformity of cluster size in different cultures, and ask whether cluster breakup was coincident with cell division. To answer these questions, samples of the growing cultures were scanned at 20–30 min. intervals from inoculation into the start of the stationary phase. Results for two cultures each of *Micrococcus candidus* and *M. freudenreichii* are shown in Figs 3 and 4. The general growth was measured by turbidity at 550 mμ as shown in Fig. 5. It is seen that the profile of B with time is markedly different for the two bacteria. For *M. candidus* a rather rapid increase in B is seen after inoculation with subsequent drop near the centre of the log phase. In profile this curve resembles the classic cell size curve for single bacteria (Thimann, 1964). With *M. freudenreichii*, the increase is more gradual, continuing until the end of the log phase with a drop thereafter. In a series of six runs with different cultures under the same growth conditions, although the initial B values for *M. freudenreichii* varied between 910 and 1090 mμ, the maximum in B was found to be a more uniform value of 1280 ± 20 mμ.

Comparison with the cell mass method and direct observation

Because of the difficulty in making direct measurements of refractive index with cell growth (see below), independent methods of measuring cluster size were carried

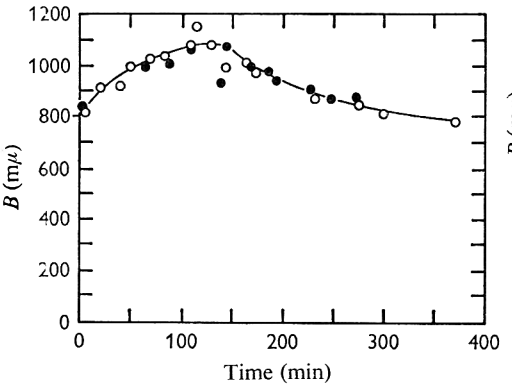

Fig. 3

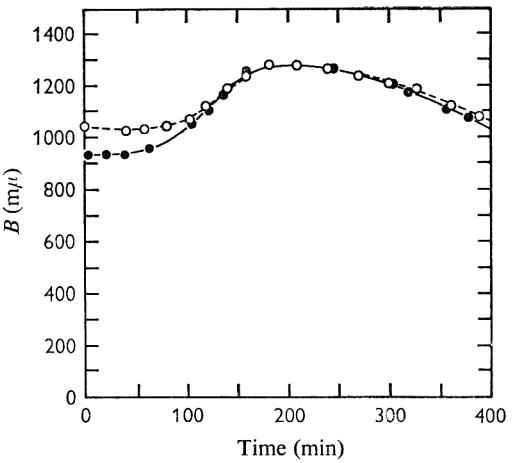

Fig. 4

Fig. 3. Profile of B change during growth cycle of *Micrococcus candidus*. (●—●), culture 1; (○—○), culture 2.

Fig. 4. Profile of B change during growth cycle of *Micrococcus freudenreichii*. (●—●), culture 1; (○—○), culture 2.

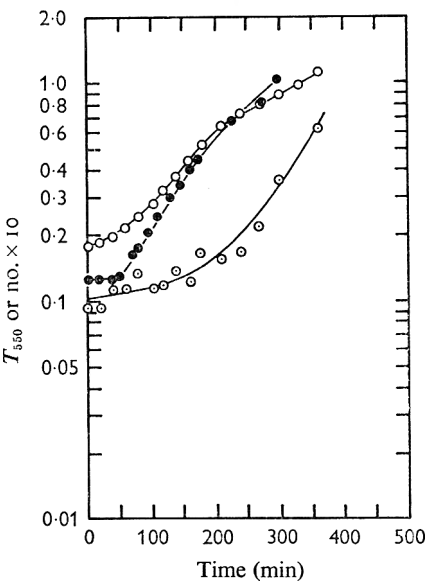

Fig. 5. T_{550} and viable count measurements during growth cycles. (○—○), T_{550} of *M. freudenreichii*; (●—●), T_{550} of *M. candidus*; (⊙—⊙), viable count of *M. freudenreichii*.

out. The standard microbiological method for measuring relative cell mass or size consists of dividing turbidity at a single wavelength by the number of viable organisms. Koch (1961) has pointed out that assuming that $n_p - n_0$ remains constant and if the bacteria follow the Jobst equation, such a measurement will actually be proportional to a^4 (for anomalous diffraction, B^4). If we assume that at high wavelengths (low ρ) the turbidity is not far removed from the Jobst equation, a direct comparison between

the anomalous diffraction and cell mass method can then be made. This is shown in Fig. 6 for *Micrococcus freudenreichii*, using B^4 values (from Fig. 4) and T_{550} and viable count data (Fig. 5). Since the cell mass method is only relative, both sets of data have been normalized with regard to the initial reading. It is seen that although the anomalous diffraction method yielded much better precision, good general agreement is obtained.

Direct measurements of cluster size were carried out for *Micrococcus freudenreichii* during a growth experiment similar to that described above. B values were observed and slides taken of the growing culture as described in the experimental section. Pictures from 3 and 62 min. were used to characterize the low B region in the lag phase and pictures taken at 180 and 210 min. were used to characterize the bacteria at the maximum B value. Measurements from anomalous diffraction yielded an observed diameter of 3·2 μ in the lag phase and 4·3 μ near 200 min., a 34% increase. Direct measurement yielded an average diameter in the lag phase of 2·3 μ, and an average diameter near 200 min. of 2·9 μ, a 26% increase. The differences between the 75 measurements from the lag region and the 44 measurements from the 200 min. region were statistically analysed using the 't' test, which showed a confidence value of $> 99\%$ for the observed difference.

Micrococcus candidus was observed to grow in chain-like clusters from 1 to 30 or more cells. Measurement of a slide taken 3 min. after inoculation revealed a cell width of approximately 0·5 μ and an average end-to-end chain length of approximately 5 μ. The value of $2a_{obs.}$ at this time was 3·2 μ.

Refractive index

Measured refractive indices of the cells in the stationary phase are shown in Table 1. Because of possible persistent error due to adsorbed or occluded water these values

Table 1. *Refractive index difference values for some Gram-positive bacteria*

Organism	$n_p - n_0$
Micrococcus freudenreichii	0·047
M. candidus	0·040
Bacillus megaterium	0·048

must be regarded as approximate. Some comparison can be made with the results of Barer & Joseph (1958) and Fikhman (1964) using phase-immersion refractometry. Although the organisms used in this work were not measured, these investigators obtained $(n_p - n_0)$ values for various bacteria from 0·045 to 0·080, and 0·037 to 0·067, respectively.

TRANSPORT EXPERIMENTS

In an attempt to determine the value of anomalous diffraction in transport experiments, experiments similar to those described by Avi-Dor, Kuczynski, Shatzberg & Magar (1956) were carried out with *Bacillus megaterium*. In this case washed bacteria were transferred from distilled water to M-NaCl and both turbidity, at 550 mμ, and anomalous diffraction observed with time. The results are shown in Fig. 7. Although the anomalous diffraction method allows the advantage that absolute B values can be determined from such kinetics, again for comparison with the single wavelength

measurements, the data is normalized with respect to the bacteria in water. It is seen that the kinetics differ, but that both methods reflect the initial increase and slow decrease reported by Avi-Dor *et al.* (1956).

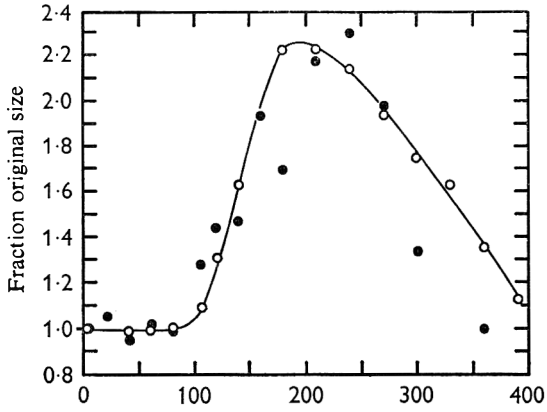

Fig. 6

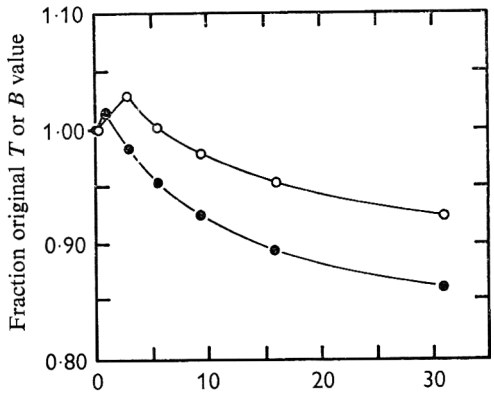

Fig. 7

Fig. 6. Comparison of cell mass and anomalous diffraction methods for *Micrococcus freudenreichii*. (○—○), B^4; (●—●), T_{550}/no. Both curves are normalized with respect to the first point.

Fig. 7. Transport kinetics of *Bacillus megaterium* following immersion in M-NaCl. (○—○), B values from anomalous diffraction; (●—●), T_{550} values. Both curves normalized with respect to zero time.

DISCUSSION

Neither the latexes nor the bacteria tested completely meet the theoretical requirements of the anomalous diffraction theory. The requirement for low particle refractive index relative to that of the solvent is only approximated with the former, and the requirement for spherical shape is only approximated with the latter. None the less, good agreement is attained with the latexes, especially with the 1·305 μ particle diameter. This size falls in the ρ region where deviation between the Mie and anomalous diffraction theories for $n_p/n_0 = 1{\cdot}20$ is not as large as with smaller sizes.

Because the roughly spherical clusters of *Micrococcus freudenreichii* most closely meet the shape requirement, this bacterium was chosen for principal emphasis. The changes in cell mass and directly observed diameter with growth are in good agreement with the changes in B^4 and $2a_{obs.}$, respectively. This indicates that the profile of B with growth cycle primarily reflects size changes. This is reasonable, since Barer & Joseph (1958) have pointed out that the macromolecules in bacteria have similar refractive indices. As a result, large changes in $n_p - n_0$ would reflect large changes in water content of the cytoplasm which would not be expected during growth. The data also indicate that cell division and cluster break-up are independent phenomena for this bacterium. It is interesting to speculate that the more uniform $a_{obs.}$ of different cultures at the end of the log phase may be related to a control mechanism for cluster breakup.

The agreement in absolute size is not as good with *Micrococcus freudenreichii* as with the latexes, probably due to either errors in refractive increment determination due to adsorbed water or the high weighting of the size distribution noted above.

Since the experimental fits are good throughout the wavelength range, we conclude that the refractive index dispersion and bacterial light absorption do not contribute significant errors to the size determination.

In spite of the complicated geometry of *Micrococcus candidus* clusters, good fits of turbidity dispersions were obtained using anomalous diffraction theory. The $2a_{obs.}$ value lies between the cluster diameter and end-to-end distance. It will be interesting to see if this holds true for other rod-shaped clusters, for which simple light-scattering theories are not available.

Studies of bacteria exposed to a new osmotic environment have proved important in studies of active transport (see, for example, Packer & Perry, 1961; Rogers & Yu, 1963). The difficulty in interpreting such measurements is that in this case, refractive index changes from solvent transport are expected to be important, making it difficult to separate the effect of solute and solvent. We feel that since anomalous diffraction weights refractive index and size differently than single wavelength turbidity, use of both methods simultaneously may be useful in separating solute and solvent transport. Due to complications in interpreting $a_{obs.}$ with rods (van de Hulst, 1962), an attempt to perform separations of this sort will better await more detailed experiments utilizing spherical bacteria.

In conclusion, although more work needs to be done to clearly define the potential of the method, the experiments above show that the anomalous diffraction technique can readily be used for studies of bacterial morphology. It is possible that the method can also be utilized in studies of mitochondria and red blood cells as well.

The expert technical assistance of Miss Janice Wilder is gratefully acknowledged.

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Electron Microscopic Anatomy of Motile-phase and Germinating Cells of *Dermatophilus congolensis*

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SUMMARY

'Whole' and autolysed motile-phase cells of *Dermatophilus congolensis* were examined after negative staining with phosphotungstate. Motile-phase cells and cells in other developmental stages were examined in thin sections. The integument of motile-phase cells was homogeneous and about 30 mμ thick and was associated with an outer diffuse capsule-like matrix. Three types of intracytoplasmic organelles and occasionally a large fluid-filled vesicle were present. Flagella varied from a few to more than 50 per cell; their diameter was estimated to be approximately 8–9 mμ. In autolysed cells, the flagella were seen to be attached to differentiated regions of the cytoplasmic membrane. The peripheral zone of the integument of cells in all developmental stages appeared to undergo sloughing. In 'young hyphal' and 'packet' cells, stratification of the integument was frequently observed. Germinating cells of *D. congolensis* were larger than motile-phase cells and contained characteristic translucent membraneless cytoplasmic inclusions. The general anatomical features of *D. congolensis*, especially of germinating cells, closely resembled those of *Streptomyces violaceoruber*.

INTRODUCTION

Dermatophilus congolensis is the causative agent of an exudative dermatitis of several animal species including man. Its distinctive developmental cycle with hyphal, motile and germinating phases has been thoroughly described by Thompson (1954) and Roberts (1961). Briefly, this cycle consists of the motile-phase cell becoming non-motile and germinating by formation of a germ tube. The germ tube grows in length and concurrently septations develop in both transverse and longitudinal planes. Through differentiation each hyphal cell becomes transformed into a motile-phase and through dissolution of the hyphal cell wall these motile-phase cells escape. Often the hypha breaks apart into packets of cells and the motile-phase cells escape from these packets. Its biochemical, morphological and colonial characteristics have suggested a taxonomic relationship to the order Actinomycetales (Austwick, 1958; Gordon, 1964). Recently, Gordon & Edwards (1963) have described its fine structural details, establishing a morphological similarity to Gram-positive bacteria and members of the Actinomycetales; i.e. in thin section the cells exhibit a membraneless nucleoid, thick integument, electron dense cytoplasm and conspicuous (often lamellated) invaginations of the cytoplasmic membrane. They noted that the peripheral substance of the integument of hyphal cells underwent a progressive dissolution before the coccoid

motile-phase cells were released. The inner zone of the hyphal integument displayed a 'fine network of fibrils enmeshing the coccal forms.' Although their electron micrographs were of high quality, Gordon & Edwards (1963) were unable to obtain information on the locomotory apparatus of motile-phase cells.

Electron microscopic evidence of flagella-like appendages on coccoid cells of *Dermatophilus congolensis* was first obtained by Thompson (1954) on shadow-cast specimens. Their presence was confirmed by Edwards using negative-staining (Gordon, 1964). In neither of these studies was the mode of attachment of the flagella to the cell discerned.

Anatomical details reflecting differentiation of motile-phase cells into germinating cells are lacking. The present study was undertaken to obtain a more detailed understanding of the anatomical features of both motile and germinating cells of *Dermatophilus congolensis* since they have been implicated as responsible for the spread and development of disease (Roberts, 1961).

METHODS

Dermatophilus congolensis, isolate B (Pier, Richard & Farrell, 1964) was used. The organism had been maintained for about 1 year in 10 % (v/v) serum broth with semi-monthly transfers. Colonial growth was established in 72 hr at 37° on brain-heart infusion agar containing 5 % (v/v) defibrinated bovine blood. Plates were then incubated at room temperature (23–25°) for periods up to 1 week. Lowering the incubation temperature enhanced the development of mucoid colonies predominantly populated with organisms in the motile spore stage.

For negative staining, organisms were generally harvested in a minimum volume of distilled water so as to yield a turbid suspension. Portions were then transferred by loop (with thorough mixing) through 4 successive distilled water dilutions in a spot-test plate to separate the cells from detritus. Equal volumes of the final suspension of washed cells were then mixed with 2 % (w/v) potassium phosphotungstate (PTA) prepared according to the method of Brenner & Horne (1959). The organisms were mounted by the 'loop-film' method of Murray (1963) on carbon-coated collodion-filmed grids and examined immediately in a Philips EM 200 electron microscope using double-condenser illumination.

For thin sectioning, organisms were fixed by the method of Kellenberger, Ryter & Séchaud (1958) and embedded in divinylbenzene (DVB) cross-linked methacrylates (Kushida, 1961). Sections were cut on an LKB Ultrotome with a diamond knife and post-stained with one of several alkaline lead solutions (Karnovsky, 1961; Millonig, 1961; Reynolds, 1963).

RESULTS

Negative-stained preparations

'Whole' cells. Intact *Dermatophilus congolensis* cells harvested in any stage of growth were generally opaque to the electron beam because of their turgor, dense intracellular contents, and a diffuse capsule-like envelope. Septa, flagella, and the integument were most readily observed in cells washed free of their capsular substances (Pl. 1, fig. 1).

Occasionally, especially in 7- to 10-day-old cultures, motile-phase cells were encountered in which intracellular details could be discerned. These cells contained

large numbers of cytoplasmic inclusions distributed randomly within an otherwise homogeneous matrix (Pl. 2, fig. 3). The inclusions represented at least three distinct types: (*a*) small weakly electron scattering oval bodies measuring less than 25 mμ; (*b*) irregularly shaped membranous organelles of moderate electron scattering power and measuring less than 150 mμ in maximum diameter, presumably 'plasmalemmasomes' (Gordon & Edwards, 1963); and (*c*) smooth-contoured opaque bodies measuring about 100 mμ. Occasionally, one or more peripherally located fluid-filled vesicles could be recognized by their relative transparency to the electron beam. When their fluid was evaporated by beam energy, reticulation developed within them (Pl. 2, fig. 3). Membranous organelles, type *b* above, did not always appear to be contiguous with the cytoplasmic membrane.

When adequately resolved, the integument appeared homogeneous and measured approximately 30 mμ thick (Pl. 2, fig. 3). The cytoplasmic membrane could not be recognized in intact cells, perhaps due to close apposition to the cell wall. Flagella generally emerged in tufts from restricted areas of the cell surface (Pl. 1. fig. 1). The number of flagella per cell was highly variable, ranging from a few to more than 50. Flagellar diameters, measured in the negative stain, were about 8–9 mμ. Frequently, cocci possessed flagella while still within the hyphae or packets (Pl. 1, fig. 1).

Autolysed cells. Motile-phase cells harvested one week or more after obtaining stationary growth phase were collapsed and displayed a wide range of degenerative states (Pl. 1, fig. 2). The cytoplasm of cells exhibiting minimum autolytic degeneration appeared amorphous or compartmentalized by a complex system of membranes (Pl. 2, fig. 4). Both conditions could exist in the same organism. Detachment of the cytoplasmic membrane from the cell wall was common though not extensive. Occasionally, a flagellar basal granule was suspected. Adjacent to the sites of flagellar insertion a large homogeneous centrally located vesicle was frequently noted. Its homogeneity may be due to fluid contents.

More extensively autolysed cells generally displayed a highly contracted cytoplasmic membrane and several smaller membranous sacs within the cell wall (Pl. 1, fig. 2; Pl. 2, fig. 5). Flagella were still attached to the cytoplasmic membrane over a limited area but basal granules could not be recognized nor could ultrastructural details of the flagellar attachment site be discerned. It seemed likely, however, that the flagella arose from the cytoplasmic membrane since their insertion through the integument frequently resulted in a characteristic 'shepherd's hook' configuration (Pl. 2, figs. 4, 5). When large numbers of flagella remained with the cell residue they often entwined it like a skein of yarn (Pl. 1, fig. 2).

Thin-sectioned preparations

Cells of *Dermatophilus congolensis* isolate B as seen in thin sections after embedding in DVB cross-linked methacrylates appeared similar to those of the four strains examined in non-crosslinked methacrylates by Gordon & Edwards (1963). The cytoplasm of all developmental stages was structurally complex and of generally high contrast. It contained a variety of membranous formations and inclusions and often was separated from the integument by a translucent zone (Pl. 2, fig. 6). Cocci ghosts were also encountered (Pl. 4, fig. 12).

The integument was generally of lower contrast than the cytoplasm and its thickness varied from 25 to more than 100 mμ. This wide range was due principally

to the angle of sectioning, although the wall of germinating and hyphal cells was usually thicker than that of the motile-phase cells. The integument frequently appeared stratified into zones of different depth and density, most clearly seen in 'packet' cells (Pl. 3, fig. 7) and young unsegmented hyphae (Pl. 3, fig. 8). This differentiation was less obvious in free cocci (Pl. 2, fig. 6), septating hyphal cells (Pl. 3, fig. 9) and germinating cells (Pl. 4, figs. 10, 11). In 'packet' cells, this stratification frequently appeared as smooth-contoured edges at the septa like a 'bourrelet' configuration (Thompson, 1942) (Pl. 3, fig. 7). The peripheral zone of the integument of most cells was amorphous and appeared to slough as described by Gordon & Edwards (1963). The presence of this material could account for the encapsulated appearance of motile cocci examined in the negative stain although sloughing was most frequently noted with germinating cells.

Germinating cells were larger than motile-phase cells. In their cytoplasm could usually be seen numerous membraneless translucent inclusions (Pl. 4, fig. 10), reminiscent of the storage vacuoles of *Streptomyces violaceoruber* (Glauert & Hopwood, 1961). Some germinating cells contained such inclusions in the periphery of their cytoplasm (Pl. 4, fig. 11). Similar translucent inclusions were less frequently observed in hyphal cells (Pl. 2, fig. 6). A large chondrioid body was observed in a cell presumed to be in the initial stage of germination (Pl. 4, fig. 12).

The flagellar apparatus could not be seen in thin sections, although numerous variations in fixing and embedding were tried. Only on one occasion was a tuft of fibrils clearly discerned, but no information of their mode of attachment to the cytoplasmic membrane could be obtained (Pl. 4, fig. 13).

DISCUSSION

Negative-staining of 'whole' or partially autolysed cells served as a useful and complementary technique to thin-sectioning. Anatomical details of the cell including flagella and the number and types of its intracellular inclusions were readily observable in such preparations. Negative stained preparations provided evidence for an attachment of the flagella of *Dermatophilus congolensis* to the cytoplasmic membrane similar to that found in other Schizomycetes. Cytoplasmic fine structure and stratification of the integument could, however, be revealed only in sectioned specimens.

The cells of *Dermatophilus congolensis* were marked by an integument of variable thickness (often stratified and subject to sloughing) and a variety of intracellular inclusions. These characteristics further support a taxonomic relationship to the Actinomycetales. The cytoplasmic compartmentalization and numerous inclusions of *D. congolensis* might allow a diversity of functionally interdependent metabolic facilities in keeping with its phylogenetic complexity. Its general anatomical features, especially those of germinating-phase cells, more closely resemble *Streptomyces violaceoruber* (Glauert & Hopwood, 1961) than either mycobacteria (Koike & Takeya, 1961) or *Nocardia asteroides* (Kawata & Inoue, 1965). Germinating cells of *D. congolensis* appear to differ from motile-phase cells by being larger and containing numerous translucent membraneless inclusions. The functional significance of these inclusions and the large chondrioid also observed in a germinating cell was not assessed.

The origin of capsular substance associated with most motile-phase cells is unknown. It may represent adhering hyphal integument (Gordon & Edwards, 1963) or

it may arise from the coccal wall proper by biosynthetic processes. The presence of 'bourrelet' configurations in the stratified integument suggests that its layers have different physiochemical properties. It would be interesting to know if this inhomogeneity is related to the morphogenesis of the motile-phase cell. Fluid-filled cytoplasmic vesicles in close proximity to the site of flagellar attachment in *Dermatophilus congolensis* may provide a pool of precursors for flagellar synthesis. A similar consideration has been applied to regions of rarefied cytoplasm observed in the flagellated pole of *Spirillum serpens* (Murray & Birch-Andersen, 1963).

Lechevalier & Holbert (1965) have investigated the flagella of another motile actinomycete, *Actinoplanes* P 128. From their electron micrographs of shadowed preparations we have estimated the flagellar diameter of this organism as approximately 20 mμ. For *Dermatophilus congolensis* the flagellar diameter is 8–9 mμ, a much smaller figure. Most bacterial flagella are about 12 mμ in diameter (Glauert, 1962; Rhoades 1965). The 8–9 mμ diameter of *D. congolensis* flagella more closely approaches that of the fimbriae (pili) of *Escherichia coli* (Duguid, 1959; Brinton, 1965) and *Klebsiella* sp. NCTC 9644 (Thornley & Horne, 1962), estimated to be about 7 mμ.

It was our experience, like that of Gordon & Edwards (1963), that the flagellar apparatus of *D. congolensis* was not revealed in thin sections. A similar circumstance was noted by Lechevalier & Holbert (1965) with the flagella of *Actinoplanes* sp. P 128. Information is lacking on the chemical nature of the flagella of both *D. congolensis* and *Actinoplanes* sp. P 128. Perhaps this information would be of value for developing suitable fixing and embedding procedures for these appendages and their attachment apparatus.

Our observation that cocci often possess flagella before being released from the hyphae could explain the mycelial movement frequently observed in phase contrast microscopy.

The authors wish to thank Mr W. H. Knaack for his skilful technical assistance.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. PTA negative stain of portion of 'whole' metamorphosed hypha of *Dermatophilus congolensis*. Cells contain some intracellular fluid and exhibit septa (S) and flagella (F). Note the two cells at the left in which the protoplast is eccentrically located. × 100,000.

Fig. 2. PTA negative stain of autolysed motile-phase cells from aged colonies. Note persistence of cell wall and flagella (F) associated with one cell. × 50,000.

PLATE 2

Fig. 3. PTA negative stain of intact motile-phase cell with 30 mμ cell wall (CW), large reticulated peripheral vacuole (Vac.), and three types of intracellular inclusions (I). Note superposition of images of some inclusions. × 125,000.

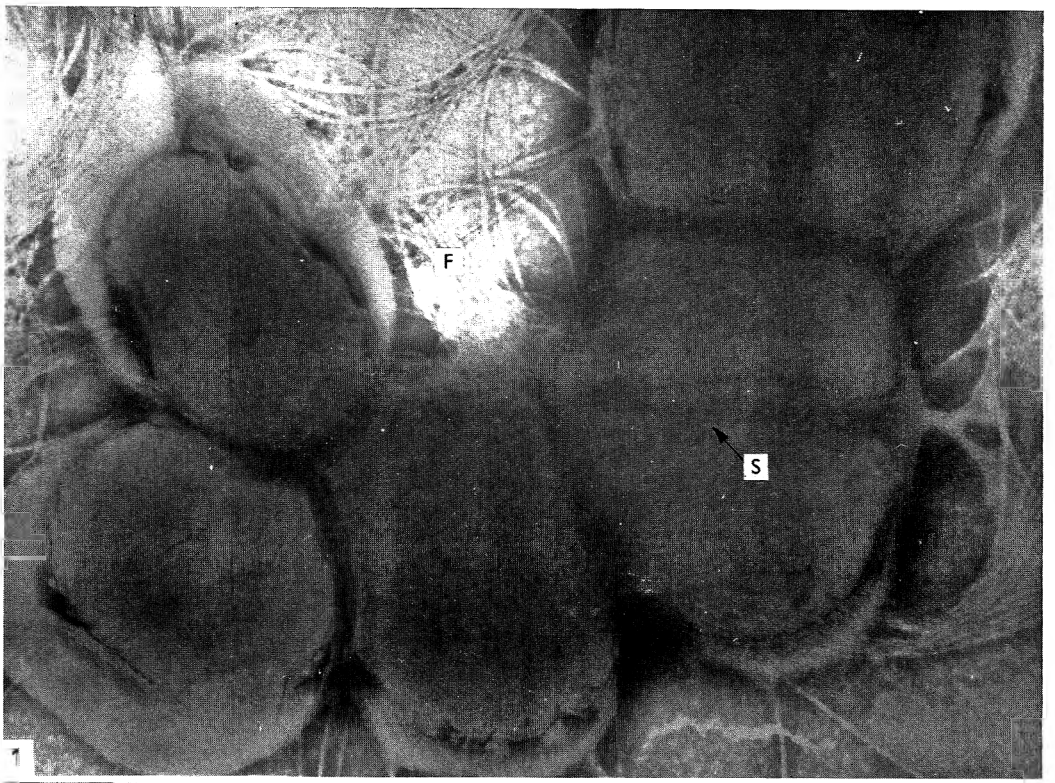
Fig. 4. PTA negative stain of partially autolysed motile-phase cells with division of cytoplasm into compartments, large fluid-filled vesicle (Ves.) adjacent to flagellar attachment sites, and detachment of the involuted cytoplasmic membrane (CM) at arrows. Note 'shepherd's crook' configuration of flagella at lower centre. × 78,000.

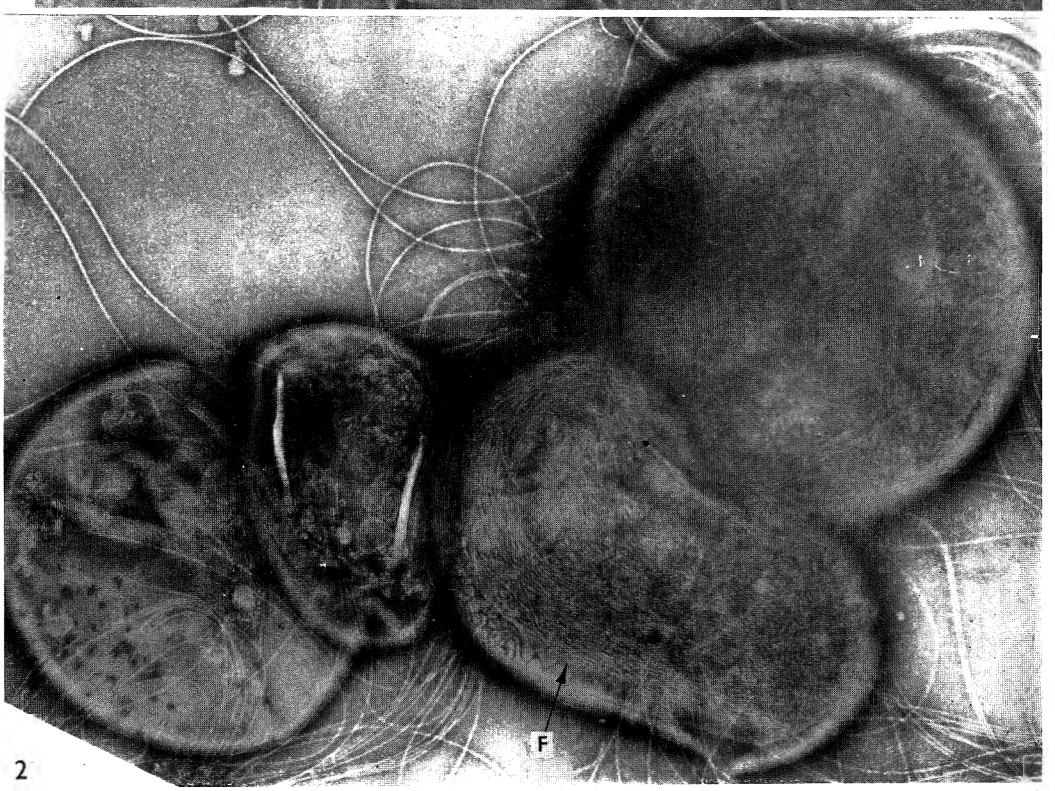
Fig. 5. PTA negative stain of an extensively autolysed motile-phase cell with collapsed cell wall (CW) and contracted cytoplasmic membrane (CM) with attached flagella. Note diminutive residual inclusions (I). × 100,000.

Fig. 6. Survey field of thin-sectioned preparation of *Dermatophilus congolensis*. Note variability of cell-wall thickness, reticular nature of the cytoplasm and variety of inclusions. Cytoplasmic retraction (arrow) was frequently seen in cells of low density. × 62,000.

PLATE 3

Fig. 7. Thin-section of 'packet' cells of *Dermatophilus congolensis* with cell wall stratification and a 'bourrelet' configuration (B). × 73,000.





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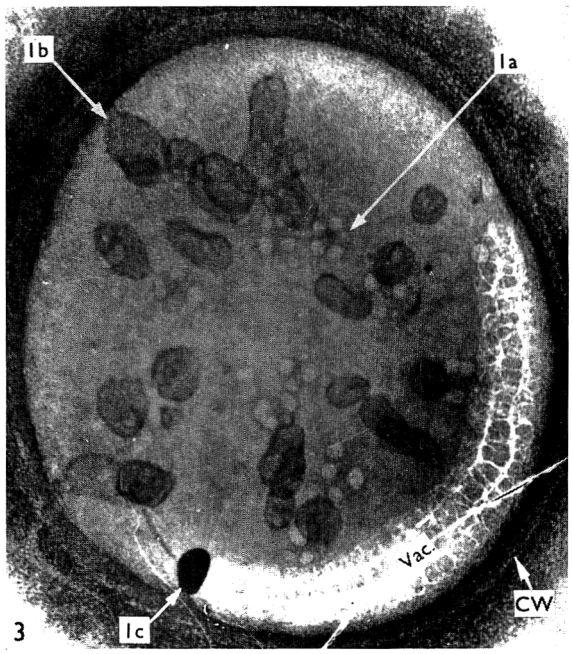
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1a
Vac.
CW
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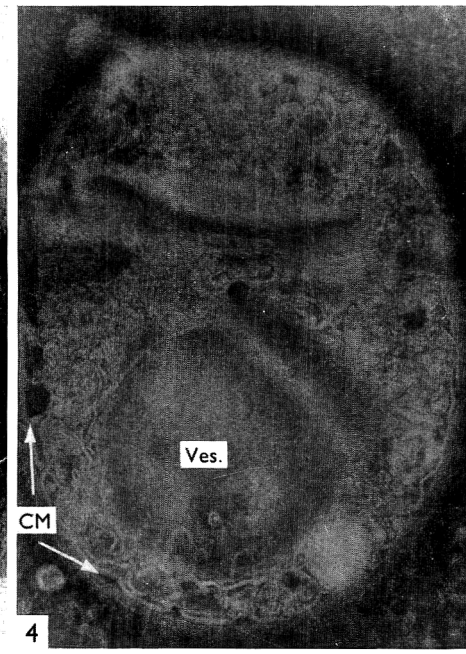
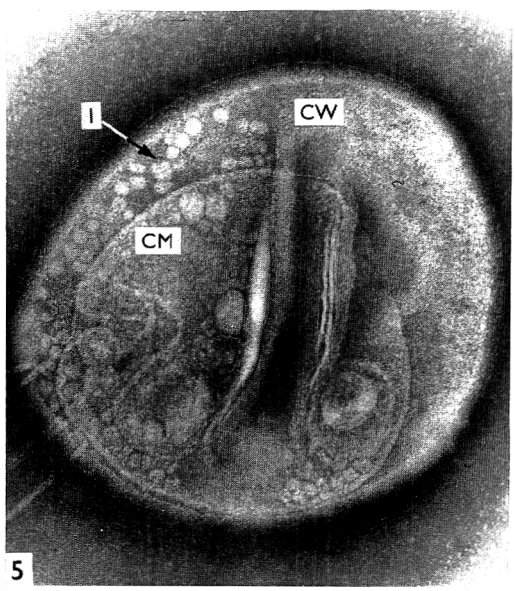
3


Ves.
CM
4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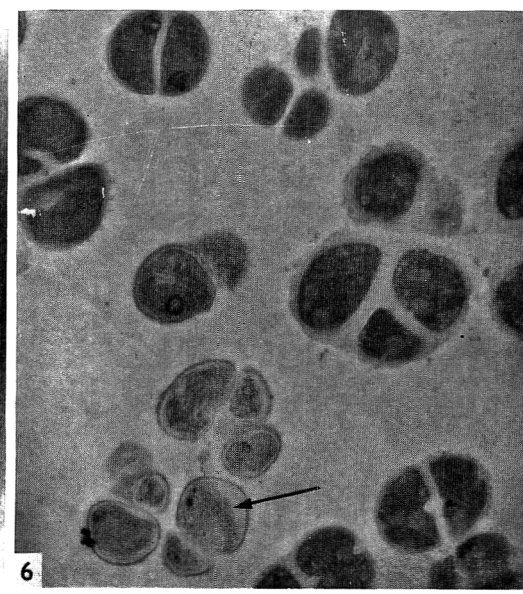
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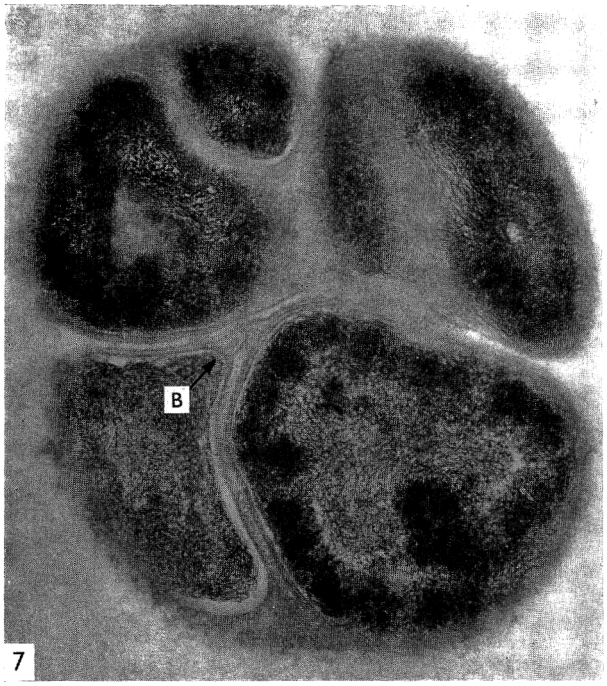
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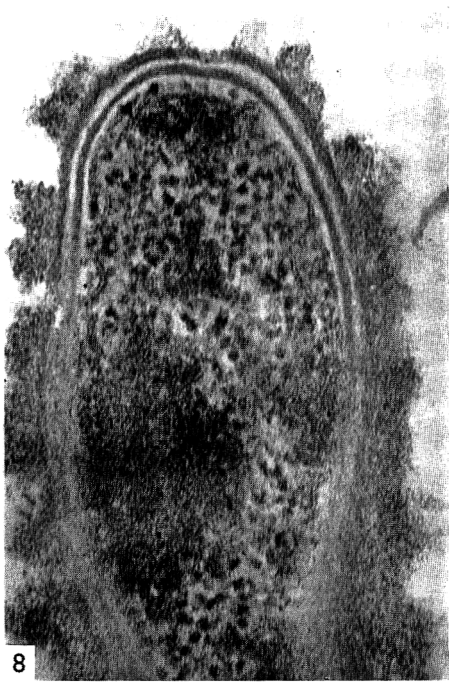
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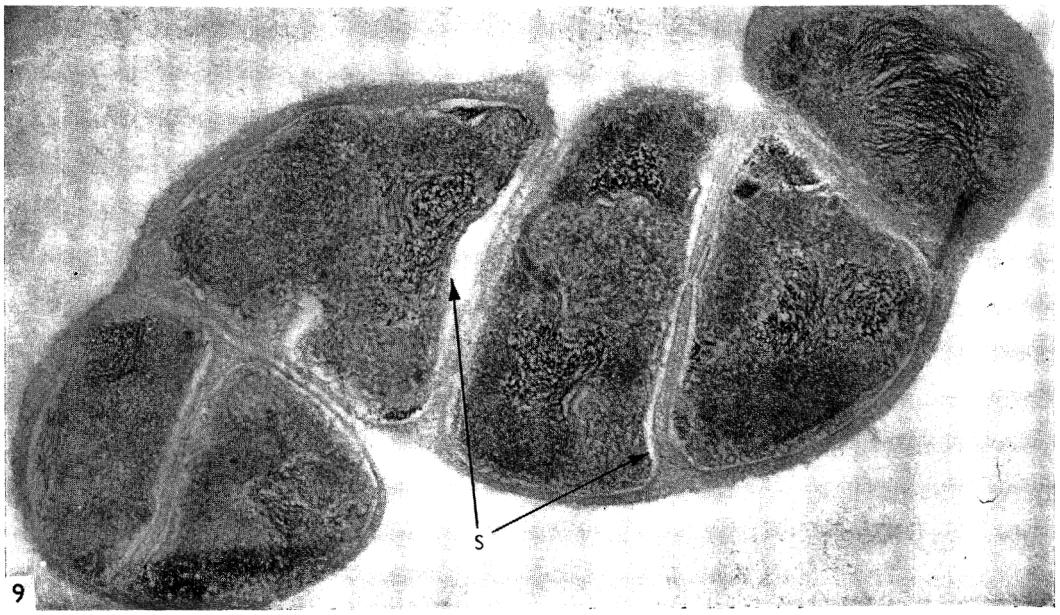
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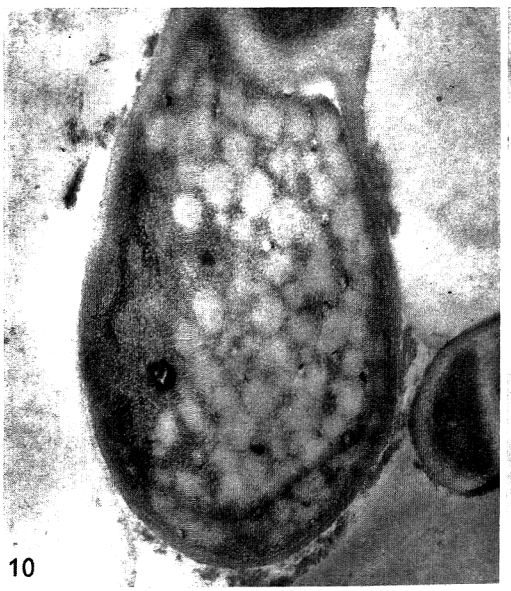
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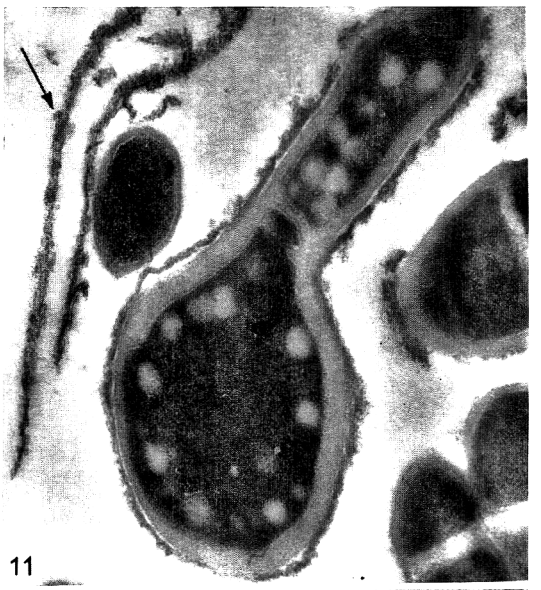
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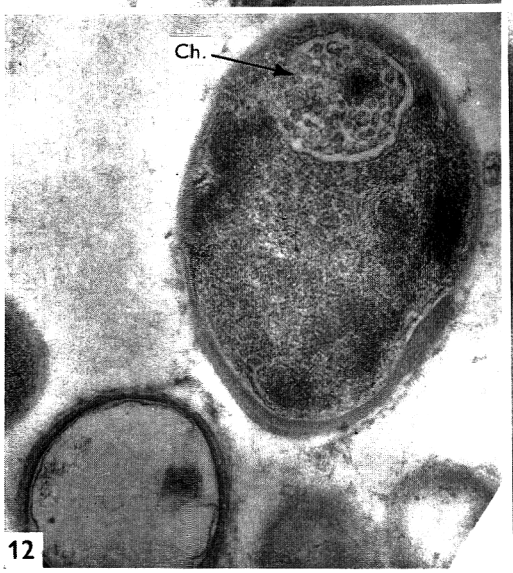

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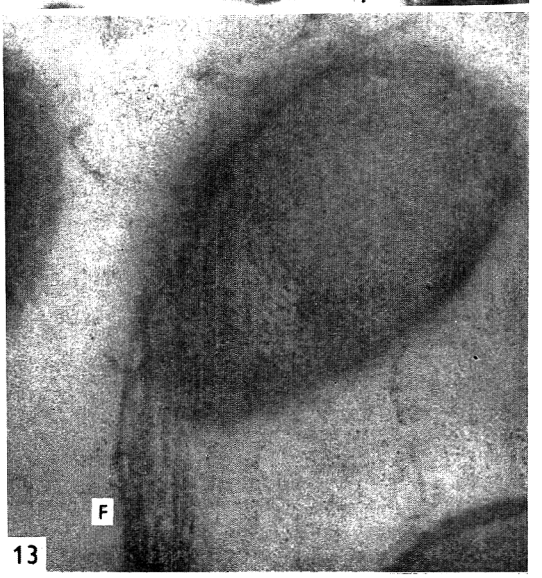

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Fig. 8. Thin-section of terminal portion of young unsegmented hyphal cell with amorphous peripheral cell wall mass and underlying stratification. Note small, dense cytoplasmic granules, presumably ribosomes. × 125,000.

Fig. 9. Thin-section of a portion of a segmenting hypha with complex reticulated cytoplasm. There is little evidence of sloughing of the amorphous cell wall. Note cytoplasmic retraction at the septa (S). × 110,000.

PLATE 4

Fig. 10. Thin-section of germinating spore of *Dermatophilus congolensis* with numerous membraneless translucent inclusions. Note relative size of germinating cell and hyphal cell at right. × 71,000.

Fig. 11. Thin-section of germinating spore with cell wall sloughing and distribution of translucent cytoplasmic inclusions into the initial hyphal cell. Note cast-off integument material at upper left (arrow). × 73,000.

Fig. 12. Thin-section of germinating spore with large chondrioid (Ch). Note small cell ghost at lower left. × 54,000.

Fig. 13. Thick-section of motile-phase cell with flagellar tuft. × 145,000.

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Isolation and Some Characteristics of Haemin Dependent Mutants of *Bacillus subtilis*

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SUMMARY

Haemin dependent mutants were isolated from *Bacillus subtilis* after (*a*) ultraviolet irradiation and selection by streptomycin, (*b*) exposure of the organisms to copper ions and (*c*) treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The isolates were grown in a synthetic medium containing haemin when substances able to neutralize inhibitory factors and to stabilize the medium were included. Two of the mutants were capable of growth in media supplemented only with δ-aminolaevulinic acid, the first intermediate of porphyrin synthesis. Transformation of the mutants to haemin independence was accomplished using deoxyribonucleic acid.

INTRODUCTION

Genetic recombination in *Bacillus subtilis* can be effectively studied by means of deoxyribonucleic acid (DNA)-mediated transformation and transduction. These methods have already been used to provide a detailed understanding of the genetic control of drug resistance and of the metabolic pathways leading to the synthesis of amino acids and nucleic acid bases. The present paper describes the isolation of porphyrin auxotrophs of *B. subtilis*. A defined chemical medium for the growth of these auxotrophs has been found and use of this medium has been made in preliminary experiments to investigate genetic recombination between these auxotrophs.

METHODS

Bacterial strains. The origin and characteristics of each *Bacillus subtilis* strain used in these experiments are given in Table 1.

Phages: SP 50, SP 71, SP 91, SP 100 were isolated and described by Földes & Molnár (1964). The characteristics of phage 3NT and its method of assay have been previously described by Csiszár & Ivánovics (1965).

Media. Hartley's meat digest medium (MD) and yeast extract medium (TYE), containing in g./l.: tryptone (Oxoid), 5; yeast extract (Oxoid), 3; di-sodium hydrogen orthophosphate (anhydrous), 1; at pH 7·6, were used for culture under non-defined conditions. Eight per cent (v/v) horse blood was added to MD to make blood medium (BM). The chemically defined medium (GGM) of Csiszár & Ivánovics (1965) was slightly modified and used to establish the essential requirements of strains. The composition was in g./l.: sodium citrate, 3; di-potassium hydrogen orthophosphate, 2; ammonium sulphate, 1; L-glutamic acid, 0·2, magnesium sulphate (hydrated), 0·75; ferric ammonium citrate, 0·05; glycerol, 15; distilled water to 1 l. Glutamic acid was

converted tc its monosodium salt before adding it to the medium. The pH of the medium was finally adjusted to 7·4. For cultivation of strain RI 2 and all derivative strains the medium was first supplemented with 50 μg. tryptophan/ml. Additional supplements were introduced as required from sterile stock solutions immediately prior to use of the medium. Solid media contained 1·2 % agar. The minimal medium of Spizizen (1958) was used, with supplements, for transformation experiments.

Table 1. *Descriptive table of bacterial strains*

Strain	Source	Relevant phenotype				Genotype*	Former designation
		Trp	His	Hem	Str		
RI 1	a	+	+	+	S	Prototrophic	Marburg Yale
RI 2	a	−	+	+	S	*trp*	168 ind⁻
RI 3	b	−	−	+	S	*trp*, *his*	SB 25 ind⁻his⁻
RI 4	c (RI 2)	−	+	+	R	*trp*, *str-1*	.
RI 5	c (RI 2)	−	+	−	r	*trp*, *str-2*, *hem-1*	.
RI 6	c (RI 4)	−	+	−	R	*trp*, *str-1*, *hem-2*	.
RI 7	c (RI 4)	−	+	−	R	*trp*, *str-1*, *hem-3*	.
RI 8	c (RI 4)	−	+	−	R	*trp*, *str-1*, *hem-4*	.
RI 9	c (RI 4)	−	+	−	R	*trp*, *str-1*, *hem-5*	.
RI 10	c (RI 4)	−	+	−	R	*trp*, *str-1*, *hem-6*	.
RI 11	c (RI 4)	−	+	−	R	*trp*, *str-1*, *hem-7*	.
RI 12†	c (RI 3)	−	+	−	S	*trp*, *hem-3*	.

Trp = tryptophan, His = histidine, Hem = haemin, Str = streptomycin
*According to the recommendations of Demerec, Adelberg, Clark & Hartman (1966).
(a) P. Schaeffer, Institute Pasteur, Paris.
(b) S. Zamenhof, University of California, Los Angeles.
(c) This laboratory, with parent strain in parentheses.
†By transformation with DNA from RI 7.
(+) = synthesized, (−) = required, (S) = sensitive, (r) = resistant to 50 μg./ml., (R) = resistant to 500 μg./ml.

Cultivation techniques. The incubation temperature was 37° unless stated otherwise. Liquid cultures of bacteria in 10 ml. medium in 100 ml. Erlenmeyer flasks were aerated in a Gyrotory Shaker (New Brunswick Scient. Co., New Brunswick, U.S.A.) at 260 cyc./min. Growth of cultures in flasks with side-arms was followed by measuring optical density of suspensions at 640 mμ with a Bausch Lomb Spectronic 20 photometer. Optical density values were then converted either to the number of viable colony forming units, or to dry weight of bacteria per ml. Minimal requirements for cultivation of bacterial strains were first established on supplemented GGM agar and confirmed in liquid media. Anaerobic incubation of bacteria was made in Fildes jars.

Chemicals Haemin (British Drug Houses Ltd., Poole, England) was prepared as a 0·1 % (w/v) stock solution (Spencer & Herriot, 1965) under aseptic conditions and stored in the refrigerator at 4°. A solution of Protoporphyrin IX (B grade, Calbiochem Ltd., Los Angeles, U.S.A.) was made in the same way. Solutions of δ-aminolaevulinic acid hydrochloride (ALA) (A grade, Calbiochem Ltd.) dissolved 0·1 % (w/v) in water; cytochrome *c* (A grade, Calbiochem Ltd.) dissolved 0·1 % (w/v) in physiological saline; catalase (British Drug Houses Ltd., 150,000 e.u./ml.) diluted 1/10 (v/v) in physiological saline; and bovine albumin fraction V (AFV) (Armour Pharmaceutical Co. Ltd., Eastbourne, England) dissolved 10 % (w/v) in water, were each sterilized by Seitz filtration. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Aldrich Chemical Co. Inc., Milwaukee, Wisconsin, U.S.A.) was dissolved 0·1 % (w/v) in sterile

water. Deoxyribonuclease (DNAse) (British Drug Houses) was dissolved 0·1 % (w/v) in sterile physiological saline.

Preparation of DNA. Bacteria were harvested in the late exponential phase from 450 ml. aerated cultures grown in TYE medium supplemented adequately for haemin auxotrophs contained in 2 l. flasks and DNA extracted according to the method of Marmur (1961). Both purified DNA and deproteinized crude extracts were used in transformation studies. The concentration of DNA was estimated in purified preparations by measuring optical density at 260 mμ and in crude extracts chemically according to the method of Burton (1956).

Transformation. The method recommended by Anagnostopoulos & Spizizen (1961) was employed after suitable modification—see text.

Cytochrome estimation. Absorption spectra of bacterial suspensions were measured in the reduced state at temperatures of liquid nitrogen (−190°) in a Cary 15 recording spectrophotometer.

RESULTS

Isolation of haemin auxotrophs

Selection by streptomycin after irradiation with u.v. light. Samples were taken from a culture of RI 2 during the exponential phase of growth in MD broth, centrifuged, resuspended in buffered saline and irradiated with u.v. Fluorescence Lamp (Hanovia, Slough, Bucks., England). The suspension was then diluted 1/10 (v/v) with MD broth and re-incubated for 2 hr before 0·1 ml. samples were plated on MD agar containing 200 μg. streptomycin sulphate/ml.; pre- and post-irradiation dilutions were plated on MD agar to determine the survival rate. At 5 % survival the colonies after 48 hr incubation on the streptomycin plates were of two types; (*a*) creamy colonies more than 3 mm. in diam. and (*b*) round, transparent colonies less than 0·5 mm. in diam. Strain RI 4 was taken from a colony of the first type; it was resistant to 500 μg. streptoymcin/ml. but was auxotrophic for tryptophan alone. Strain RI 5 was taken from a colony of the second type which showed an increase in size only on subculture to media containing blood; it was resistant to 50 μg. streptomycin/ml. Although a similar increase in colony size was observed on MD agar supplemented with 2·5 μg. haemin/ml., identity as a haemin auxotroph was not established until a minimal medium had been defined. In two further experiments, in which the survival rate was less than 1 %, small colony formers were encountered but none showed enhanced growth on haemin supplemented MD agar.

Exposure to copper ions. Mutants of *Bacillus subtilis* have been isolated by Weed (1963) after exposure of organisms to copper sulphate. We modified his method to include 2·5 μg. haemin/ml. in the copper medium and inoculated 10 ml. medium in 100 ml. screw-capped bottles with 1 ml. of an exponentially growing culture of strain RI 4 in MD broth, containing 1×10^8 colony formers per ml. Bottles were incubated at either 37° or 43°. At intervals from the second to the seventh day loopfuls of suspension were plated out on MD agar containing 1 μg. haemin/ml. Inspection with a hand lens in the region of individual colonies after 48 to 72 hr incubation revealed a low proportion of transparent colonies, 0·1–1 mm. in diam., amongst the creamy, 5 mm. diam. colonies of the parent strain; these transparent colonies were subcultured on to MD agar, both plain and supplemented with 2·5 μg. haemin/ml. Five strains, which showed improved growth on haemin media, were later confirmed as haemin auxotrophs

and labelled RI 6 (from 37° incubation), RI 7, RI 8, RI 9 and RI 10 (from 43° incubation).

Exposure to N-methyl-N'-nitro-N-nitrosoguanidine. The technique independently described by Davis (1948) and Lederberg & Zinder (1948), in which penicillin was used to enrich the yield of auxotrophs, has since been adapted successfully by many workers. The fact that a minimal medium was defined (see below) in which haemin auxotrophs grew well permitted the use of the following modification to isolate one further strain, RI 11. Bacteria of strain RI 4, growing exponentially in MD broth, were washed with buffered saline at pH 7 and resuspended in the same volume of buffered saline containing 50 μg. MNNG/ml. Exposure for 30 min. at 37° was terminated by immersion in melting ice before the tubes were centrifuged. The washed sediment was resuspended in 10 ml. MD broth containing 5 μg. haemin/ml. plus 500 μg. AFV/ml. and incubated overnight. Samples of 2 ml. each were added to 50 ml. GGM in 250 ml. flasks containing 200 μg./ml. vitamin-free casamino acids (Difco Labs, Detroit, Michigan, U.S.A.) and incubated for 4 hr in the shaker before Methicillin to a final concentration of 1000 μg./ml. was added. After further overnight incubation, 0·1 ml. samples from a suspension in GGM of repeatedly washed bacteria were spread on GGM suitably supplemented for haemin auxotrophs. 160 colonies were replica plated but only one strain, RI 11, was haemin dependent.

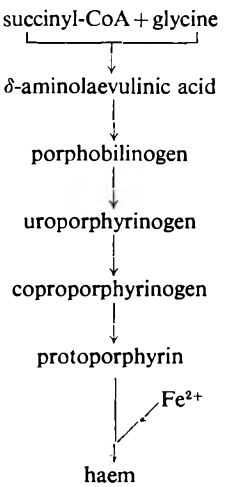
Cultural characteristics of haemin auxotrophs

The results of growth on MD supplemented with haemin were inconsistent and always poorer than those obtained on BM. The absence of colony formation on minimal medium supplemented with haemin alone or in combination with casamino acids, vitamins or nucleic acid bases indicated the importance of other factors in the promotion of growth. It was observed that the presence of non-specific substances, e.g. charcoal or DEAE cellulose, in a minimal medium containing casamino acids and haemin permitted a limited degree of colony formation; when AFV was used instead of charcoal the growth was further improved. Finally it was found that for routine cultures of haemin auxotrophs of *Bacillus subtilis* (directly or indirectly derived from RI 2) a minimal medium supplemented with tryptophan (50 μg./ml.), cysteine or cystine (10–25 μg./ml.), AFV (500 μg./ml.) and haemin (5 μg./ml.) promoted colony formation, after 48 hr incubation, almost equivalent to that obtained on BM after 24 hr. The requirements in fluid media were identical. Neither methionine nor thioglycollic acid was capable of replacing cysteine or cystine in the minimal medium.

The mutants (RI 5 to RI 11) were maintained by weekly transfer on to fresh BM and were stored in the refrigerator at 4° after initial incubation for 24 hr. The requirement for haemin was not seen to be diminished or lost in any strain; spontaneous reversion to haemin independence occurred at a frequency less than 10^{-7}. Cellular morphology and reaction to Gram stain did not suffer from those of the parent and the biochemical markers, tryptophan dependence and streptomycin resistance, were present. Sensitivity to a set of *Bacillus subtilis* phages was identical in parent and mutant strains. The ability of the mutants to sporulate was impaired. The colony formed by haemin auxotrophs on supplemented minimal medium after 48 hr incubation was small, 2–3 mm. in diam., moist and more regular in comparison to the 5 mm. diam., dry, irregular colony of strain RI 2, from which they were all derived; this difference was not further examined. Neither parent nor mutant strains could be cultured anaerobically.

Porphyrin utilization and synthesis by auxotrophs

The range of haemin concentrations necessary to support growth showed an upper and a lower limit. Whilst 2·5–5 μg./ml. promoted adequate colony formation on solid, defined media, concentrations between 1 and 2 μg. haemin/ml. were satisfactory in fluid media, although a uniform bacterial density was not reached in every experiment. Quantities in excess of 5 μg./ml. proved inhibitory to growth in both solid and fluid media.



(from Lascelles, 1964).

Fig. 1. Pathway of porphyrin synthesis.

None of the mutants grew when haemin was substituted by protoporphyrin IX or the porphyrin containing compounds, cytochrome *c* and catalase. Coproporphyrin, uroporphyrin and porphobilinogen were not tested for their ability to replace haemin. However, two isolates, RI 5 and RI 6, were capable of utilizing δ-aminolaevulinic acid, the first specific intermediate in the pathway to porphyrin synthesis (see Fig. 1), and grew well in minimal medium supplemented only with tryptophan and 2·5 μg. ALA/ml.; no growth was obtained when ALA was replaced by pyridoxal phosphate. The growth response of RI 5 to ALA in minimal medium is illustrated in Fig. 2.

While catalase itself did not satisfy the porphyrin requirements of the mutants it allowed a considerable reduction in the quantity of haemin required for optimal growth of the bacteria. This 'sparing' effect was observed in solid and fluid media; with 40 e.u./ml. catalase a concentration of 0·1 μg. haemin/ml. was sufficient for optimal growth.

The cytochrome absorption spectrum of three mutants, RI 5, RI 6 and RI 7, was not found to vary significantly from RI 2, whether the organisms were grown in non-defined medium or supplemented minimal medium. Absorption maxima representing bands of cytochrome $a-a_3$ (598–602 mμ) and cytochrome c_1 (553–555 mμ), with their β (525–527 mμ) and γ or Soret bands (440 and 420 mμ), were identified in all samples using references taken from Smith (1954) and Sherman (1963).

Transformation efficiency of mutants

The haemin auxotrophs required longer incubation times than RI 2 to reach comparable cell densities in supplemented minimal medium. Tomasz (1966) and Thorne & Stull (1966) have stressed the importance of transferring bacteria at an optimum cell density from the first growth medium (GM) into fresh transformation medium (TM) to obtain a high yield of transformants. Bacteria were grown to a cell density of 5×10^8 colony formers/ml. in GM, then centrifuged, resuspended in 10 vol. TM and incubated in the shaker for 90 min. before DNA was added to a final concentration of 10 μg./ml.

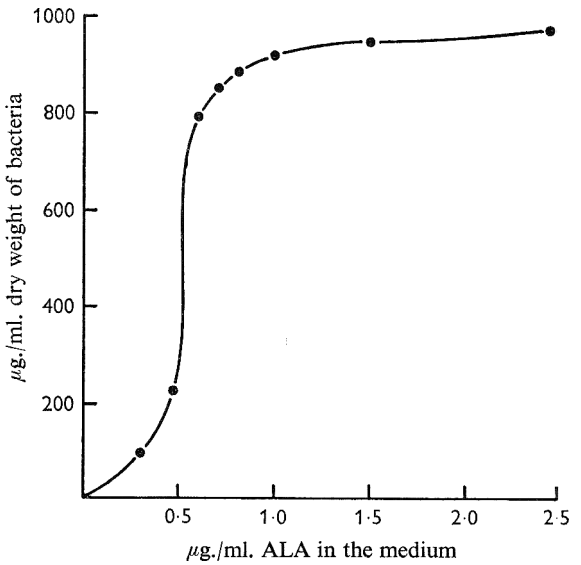


Fig. 2. Growth responses of strain RI 5 to δ-aminolaevulinic acid. 10 ml. supplemented minimal medium in 100 ml. flasks were inoculated with 5×10^4 washed colony formers from an overnight culture of the strain in supplemented minimal medium containing 2·5 μg. ALA/ml. The optical density value after 20 hr incubation was converted to dry weight of bacteria per ml. The bacterial yield is plotted as a function of the concentration of ALA.

Exposure for 30 min. was terminated by the addition of DNAse to a final concentration of 100 μg./ml. and incubation was continued for 15 min. before 0·1 ml. samples of dilutions in minimal medium were spread on appropriately supplemented minimal agar. Colony counts were made after 48 hr incubation. The results of transformation experiments are summarized in Table 2.

Transformation occurred with acceptable efficiency in the strains RI 5 and RI 6 when the experiments were performed in media supplemented with ALA. The yield of transformants was one log. unit less in haemin-supplemented minimal media even with reduced concentrations of haemin in the presence of catalase. When RI 5 was used as recipient with diminishing concentrations of prototrophic (RI 1) DNA a marked reduction in transformants occurred at concentrations below 0·1 μg./ml., although transformants were still recovered at a concentration of 0·005 μg./ml.

A low rate of transformation was found in RI 7 and RI 8 both of which could only be grown in media supplemented with haemin. When DNA from RI 7 (carrying

hem-3) was used to transform RI 3 to histidine independence the resulting colony formers were replica plated on to supplemented minimal medium with and without haemin to give a rate for double transformants (genotype *trp*, *hem-3*) of 4×10^2 per 10^8 recipients. The haemin solution used was freshly made and did not contain histidine. One of these double transformants, RI 12, was transformed to haemin independence in haemin media at a higher rate than RI 7 (see Table 2) under identical conditions, although both strains carried the same defective allele (*hem-3*) for haem synthesis.

Table 2. *Transformation of Bacillus subtilis strains*

Recipient strain	Donor strain (DNA conc. 10 μg./ml.)	Transformation to independence in 10^8 recipients				
		Trp	Hem	Trp+Hem	His	Trp+His
RI 2	RI 1	$1{\cdot}5 \times 10^5$	.	.	.	.
RI 4	RI 1	$1{\cdot}0 \times 10^5$	.	.	.	.
RI 5*	RI 1	$1{\cdot}4 \times 10^5$	$1{\cdot}3 \times 10^5$	$4{\cdot}0 \times 10^3$	.	.
RI 5*	RI 6*	< 10	< 10	< 10	.	.
RI 5*	RI 9	< 10	$1{\cdot}0 \times 10^5$	< 10	.	.
RI 6*	RI 1	.	$1{\cdot}0 \times 10^4$	.	.	.
RI 7	RI 1	.	$5{\cdot}0 \times 10^2$	.	.	.
RI 8	RI 1	.	$1{\cdot}5 \times 10^2$	.	.	.
RI 3	RI 1	$3{\cdot}0 \times 10^4$	.	.	$2{\cdot}4 \times 10^4$	$2{\cdot}4 \times 10^4$
RI 3	RI 7	.	.	.	$1{\cdot}0 \times 10^4$	.
RI 12	RI 1	$1{\cdot}2 \times 10^5$	$0{\cdot}8 \times 10^5$	$3{\cdot}0 \times 10^3$	.	.

Trp = tryptophan, Hem = haemin, His = histidine, (*) = utilizes ALA

Transformation experiments were performed as described in the text. Samples of 0·1 ml. from appropriate dilutions were plated on suitably supplemented minimal medium. Averages of colony counts on three plates after 48 hr incubation are expressed as transformants per 10^8 recipients.

DISCUSSION

A streptomycin resistant mutant of *Staphylococcus aureus* was reported to show a requirement for haemin by Jensen & Thofern (1953). Beljanski & Beljanski (1957) isolated a similar mutant from *Escherichia coli* by a process of selection using streptomycin. The isolation of a haemin auxotroph of *Bacillus subtilis* on a medium containing streptomycin provided a further example of the intriguing relationship between these two characters.

A greater number of haemin dependent mutants was obtained when colonies were selected on the basis of diminished colony size after growth in copper-containing medium. In these experiments a small quantity of haemin was added to the selection medium in order to supplement the variable, intrinsic concentration of porphyrins in MD. The 'small colony' strain, SC 22, isolated by Weed (1963) after exposure to copper, was studied in this laboratory due to the kindness of Dr Weed. His strain was not found to have an improved growth on media supplemented with haemin and was refractory to a group of subtilis phages to which RI 2 and haemin auxotrophs were sensitive.

Jensen & Thofern (1953) and Beljanksi & Beljanski (1957) used a peptone-glucose medium supplemented with haemin to cultivate their mutants under conditions of diminished oxygen concentration and were unable to define a 'synthetic' medium.

The haemin auxotrophs of *Bacillus subtilis* attained full aerobic growth in a chemically defined minimal medium (GGM or Spizizen) supplemented with cysteine or cystine and AFV in addition to haemin. Under these conditions a normal cytochrome electron transport mechanism was formed as judged by the cytochrome absorption spectrum. The minimal concentration of haemin (0·1 μg./ml.) to which the response of the strains was optimal compared favourably with the minimal porphyrin requirement of *Haemophilus influenzae* strains (Granick & Gilder, 1946) and confirmed the specific nature of the dependence.

That the strains utilizing ALA were capable of optimal growth in media supplemented with ALA alone suggested that AFV and cysteine or cystine were important only in haemin media. Pollock (1947) demonstrated the ability of charcoal, albumin and starch to neutralize the inhibitory effects of unsaturated fatty acids in the medium. Davis & Dubos (1947) found bovine albumin, fraction V, to be most effective in promoting growth in the presence of inhibitors (e.g. unsaturated fatty acids) and attributed this property to the capacity of the molecule to bind, and slowly release, compounds inhibitory to growth at higher concentrations. We did not characterize the substances inhibiting the growth of haemin auxotrophs. The supportive effect given by charcoal, albumin, or cellulose, of a cationic character, to growth of the haemin auxotrophs in the presence of haemin indicated the acid properties of the inhibitors. Haemin was growth-limiting above certain concentrations; it was also possible that AFV, by virtue of its capacity to bind and release compounds, maintained the haemin concentration at growth-promoting levels.

Cysteine or cystine was indispensable for growth in haemin minimal medium already containing substances able to neutralize fatty acids and peroxides. The mechanism necessitating the inclusion, specifically, of either of these amino acids was not determined. The activity of catalase in sparing haemin was explained by the known destructive effect of H_2O_2 on haem compounds (Gilder & Granick, 1947).

The inability of the strain of Jensen & Thofern (1953) to utilize protoporphyrin was attributed to a deficiency of the enzyme 'ferrochelatase', which catalyses the insertion of iron into protoporphyrin. Lack of this enzyme did not account for the failure of strains RI 5 and RI 6 to grow in media supplemented with protoporphyrin since growth and synthesis of porphyrins by these strains in minimal media supplemented with ALA indicated the possession of all enzymes except ALA synthetase. It is possible that protoporphyrin did not penetrate into these organisms. It is also possible that, in *Bacillus subtilis* species, iron is inserted into the porphyrin ring at an earlier stage.

The transformation experiments were only preliminary. Their purpose was to demonstrate how a detailed study of a larger number of similar mutants could contribute to an understanding of the genetic control of iron-porphyrin synthesis in *Bacillus subtilis*. The high percentage of wild-type transformants recovered from RI 3 exposed to prototrophic (RI 1) DNA confirmed the proximity of the defective loci for histidine and tryptophan synthesis in that strain (Nester, Schafer & Lederberg, 1963). By comparison the low recovery of double transformants from RI 5 even with saturating levels of prototrophic (RI 1) DNA was interpreted to mean that the haem and tryptophan loci were distant from each other. An intrinsic, low level of competence, i.e. ability to accept exogenous DNA, was thought to be the reason for the poor efficiency of transformation in some strains.

In the nomenclature of bacterial genetics it has been recommended (Demerec,

Adelberg, Clark & Hartman, 1966) that a capital letter should follow the abbreviation of the mutated metabolic pathway in order to indicate which enzyme step is involved, where this is known. We propose, therefore, that *hemA* should be used to denote that region of the chromosome of *Bacillus subtilis* which determines ALA synthetase activity since the reaction catalysed by this enzyme is well established (Lascelles, 1964) as the initial step on porphyrin synthesis. Strain RI 5 then carries *hemA*1 and RI 6 carries *hemA*2; it is hoped that subsequent work will establish where the block is in the porphyrin metabolic pathway of the other mutants.

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Loss of Type Antigen in a Type III Streptococcus and Identification of the Determinant Disaccharide of the Remaining Antigen

By J. M. N. WILLERS AND GEERTRUIDA H. J. ALDERKAMP

WITH

A Note on the Nomenclature of Certain Polysaccharides Resembling the Group Antigens of Streptococci

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SUMMARY

By subcultivating a streptococcal z3 III strain in medium containing anti-III serum a strain lacking the type antigen was isolated. Evidence is given that this strain possesses only z3 antigen. From partial acid hydrolysates of formamide extracts of both z3 and z3 III bacteria the same disaccharide was isolated. The most probable structure of the disaccharide is 3-*O*-α-*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine. It was 250 times more active than α-methyl-*N*-acetylglucosamine in the inhibition of the z3/anti-z3 system. This denotes that it is an important part of the determinant group of the z3 antigen. By ethanol fractionation of a formamide extract of z3 III bacteria two distinct fractions were isolated. The first fraction reacted only with type III antiserum and consisted of glucose, galactose and rhamnose in the ratio 5:3:1. The second polymer was composed of rhamnose, glucosamine and galactosamine in relative amounts of about 2:1:1. Chemical and serological evidence suggests very strongly that this is the z3 antigen. The similarities between z and true group antigens are discussed.

INTRODUCTION

Ottens & Winkler (1962) described several strains of haemolytic and non-haemolytic streptococci carrying, apart from the group antigen F, one of five type antigens of polysaccharide nature. These type antigens have repeatedly been found in strains belonging to other serological groups, for instance in groups C, G and T (Ottens & Winkler, 1962), A (Jablon, Brust & Saslaw, 1965), and L (Willers, Ottens & Michel, 1964*b*). The availability of type-specific antisera led to the discovery of other strains in which no known group antigen could be detected. These strains were designated by Ottens & Winkler (1962) as OI, OII, OIII and OIV, where O stands for zero. Whether zero denoted the absence of a group antigen or the presence of a hitherto unknown antigen was not known. Since it has been shown in several cases that zero

stands for an unknown carbohydrate antigen it seems practical to discontinue the use of O for zero so as to avoid confusion between zero-antigens and group O antigens. In this paper we will therefore allude to strains containing type and no group as zI, zII etc. The lower case z was deliberately chosen to avoid any suggestion of group or type status for these antigens. A numerical indicator is used to differentiate between different z antigens. For example, the symbol OIII is replaced by z3 III (see Addendum)

In group B streptococci four polysaccharide type antigens have also been described. Lancefield (1934) separated the type antigens in group B from the group antigen by fractional precipitation with ethanol. Lancefield (Curtis & Krause, 1964) isolated a group B strain lacking type antigen by subcultivating a BI strain in the presence of homologous type-specific antiserum.

We have used both methods for obtaining type antigen and a z antigen in a pure form from a z3III strain; the results are reported here.

METHODS

Streptococcal strains. A non-haemolytic streptococcal strain z3III was isolated by Dr C. E. de Moor (National Institute of Public Health, Utrecht, the Netherlands) and tentatively designated as Streptococcus MG 216.

Culture conditions. Organisms were grown for 36 hr at 37° in Todd–Hewitt broth containing g./l.: 17 Todd–Hewitt (Difco) medium, 6·4 glucose, 3 sodium bicarbonate. The bacteria were collected by centrifugation in a continuous flow centrifuge MSE-17 at 17,000 rev./min. and washed once with distilled water.

Formamide extraction. The antigenic polysaccharides were extracted from the bacteria with formamide according to Fuller (1938). After centrifugation the extraction was repeated and the extracts combined. The extract was further treated as described by Willers, Michel, Sijsma & Winkler (1964*a*). In one experiment purification and fractionation as described below was done.

Purification and fractionation. The crude formamide extract was dialysed against several changes of distilled water; insoluble material in the dialysis residue was discarded after centrifugation. Nucleic acids were removed with streptomycin sulphate according to Hu, Wolfe & Reithel (1959). After treatment with trypsin, the extract was dialysed against distilled water and finally purified on DEAE-cellulose (Willers *et al.* 1964*a*).

To 1 vol. of purified formamide extract 2 vol. of ethanol 96% (v/v) in water were added. After standing at 4° for at least 2 hr the precipitate was removed by centrifugation. Two additional volumes of ethanol were added to the supernatant fluid and after removal of the precipitate formed the volume of the last supernatant fluid was concentrated in a flash evaporator to the original volume of the formamide extract. Addition of 5 vol. of acetone gave a precipitate. All precipitates were dissolved separately in 30–50 ml. distilled water and refractionated with ethanol and acetone. Finally the fractions were freeze-dried.

Hydrolysis conditions. For the qualitative and quantitative sugar analysis, 1 ml. of a solution containing 50 mg. polysaccharide was hydrolysed in 2 N-HCl at 100° for 5 hr. After cooling the hydrolysed material was neutralized with Dowex 1 in the carbonate form.

Paper chromatography was done as described by Willers *et al.* (1964*a*). The following solvents were used: solvent A, *n*-butanol+acetic acid+water (60+10+20, by vol.); solvent B, 2·4–2·5 lutidine+water (65+35, by vol.); solvent C, *n*-butanol+pyridine+water (60+40+30, by vol.).

Determination of sugars and aminosugars. Glucose was determined with glucose oxidase (Hugett & Nixon, 1957), galactose with the galactose oxidase reaction (Avigad, Amaral, Asensio & Horecker, 1962) and rhamnose with the thioglycollic acid+sulphuric acid reaction according to Gibbons (1955). Total hexosamines were estimated with the modified Elson & Morgan reaction (Rondle & Morgan, 1955). Glucosamine and galactosamine determinations were kindly done by Dr J. A. F. Op den Kamp (Laboratory of Biochemistry, State University of Utrecht) by gas chromatography by a method described by Perry (1964) and modified by Op den Kamp & van Deenen (1966). *N*-acetyl-hexosamine was determined by the modified Morgan & Elson reaction (Reissig, Strominger & Leloir, 1956).

Isolation of a streptococcal strain which lacked the type antigen. The z3III strain was subcultivated 30 times in a medium containing 0·25 ml. Todd–Hewitt broth and 0·25 ml. type III antiserum. After the last passage the streptococci were inoculated into Todd–Hewitt broth without antiserum. After incubation for 16 hr a slide was made and tested with fluorescent type III antiserum. None of the bacteria showed fluorescence, proving that streptococci carrying the type III antigen had disappeared from the culture. This culture of bacteria lacking in type III antigen was stable for this property after subcultivation several times in Todd–Hewitt broth.

Biochemical tests. Biochemical reactions of the z3III and z3 strain were kindly made by Dr C. E. de Moor (National Institute of Public Health, Utrecht, the Netherlands). The streptococci were grown in trypticase+yeast extract+cystine broth containing 1% of one of the following compounds: glucose, lactose, saccharose, maltose, salicin, trehalose, raffinose, starch, inulin, mannitol, glycerol, sorbitol, aesculin, arginine, sodium hippurate. Reactions were read after 1 and 5 days.

Preparation of antisera and quantitative precipitin inhibition technique. These were as described by Willers *et al.* (1964*a*).

Capillary precipitin reactions with all streptococcal grouping sera were kindly done by Dr C. E. de Moor.

Partial hydrolysis and isolation of two oligosaccharides. Controlled hydrolysis of formamide extracts of z3III and z3 streptococci prepared as described by Willers *et al.* (1964*a*) was done by hydrolysis at pH 3, 2·5, 2, 1·5 and 1, and at temperatures of 60°, 70°, 80° and 90° for each pH value, every hydrolysis step taking 30 min. After each step the mixture was dialysed against distilled water with stirring for at least 4 hr. All diffusates were combined and neutralized with N-sodium hydroxide. The volume of the diffusate was reduced to about 50 ml. by evaporation *in vacuo*.

Separation of monosaccharides and salts from oligosaccharides was done on a charcoal column prepared as described by Schiffman, Howe & Kabat (1958). The diffusate obtained after partial hydrolysis was slowly adsorbed on a column (50 × 2·5 cm) containing 40 g. Darco G60+40 g. Celite 535. Elution was started with water (3 l.) to remove the salts and monosaccharides, followed by 3 l. of 5% (v/v) ethanol in water. The 5% (v/v) ethanol fraction was evaporated to dryness and redissolved in 5 ml. distilled water. This fraction was tested for inhibitions in the quantitative precipitin reactions of the z3III and z3 systems. The 5% (v/v) ethanol fraction was

further fractionated on washed Whatman paper no. 3 MM, with solvent C as eluent.

Borohydride reduction and periodate oxidation was done as described by Michel & Willers (1964).

RESULTS

Properties of the strain lacking type III antigen. The strain which was isolated after subcultivation in Todd–Hewitt broth containing type III antiserum, and which did not react any longer with type III antiserum, was tentatively called z3.

Table 1. *Qualitative sugar analysis of formamide extracts of z3 III and z3 streptococci*

	Formamide extract of	
Sugars	z3 III	z3
Glucose	+	+
Galactose	+	−
Rhamnose	+	+
Glucosamine	+	+
Galactosamine	+	+

Table 2. *Inhibition by sugars of the quantitative precipitin reactions of z3 III/anti-type III and z3/anti-z3.*

Sugars	z3 III/ anti-type III	z3/anti-z3
Glucose	90*	.†
Galactose	90	.
N-acetyl-glucosamine	.	3

* The number of μmoles of sugar necessary to obtain 50 % inhibition is given.
† Inhibition with 90 μmoles less than 10 %.

A formamide extract of z3 bacteria did not react with any known group antiserum. An antiserum against this strain could be prepared. Both strains gave identical reactions in the biochemical tests; they did not ferment trehalose, a reaction which is nearly always positive with group F streptococci. A comparison of the sugar composition of the formamide extracts of z3 III and z3 bacteria showed that both had the same composition except that galactose was absent in the z3 formamide extract (Table 1). As previously described, the determinant group of the type III antigen contains probably glucose and galactose as the most important sugars (Willers *et al.* 1964*a*). In the z3/anti-z3 system however glucose and galactose did not give any inhibition of the quantitative precipitin reaction, while *N*-acetylglucosamine was a good inhibitor (Table 2).

Partial acid hydrolysis of formamide extracts of z3 III and z3 formamide extracts resulted in both cases in the production of inhibitory material. From the 5% (v/v) ethanol effluent an oligosaccharide, called compound A2, was isolated in both cases. This material had a R_g value in solvent A of 0·55 and in solvent B of 1·15. Further purification was obtained through chromatography on Whatman paper no. 3 MM with solvent C as eluent, and on a small charcoal column.

Complete hydrolysis of compound A 2 showed it to be composed of equal amounts of glucosamine and galactosamine. When the substance was first reduced with sodium borohydride and then hydrolysed, the glucosamine content remained unchanged and the galactosamine content became negligible. From this it was inferred that compound A 2 is a disaccharide containing *N*-acetylgalactosamine at its reducing end.

Evidence of the presence of the hexosamines in the acetylated form in the disaccharide was obtained as follows. When 0·5 mg. of the disaccharide was put on a Dowex 50 H^+ column and eluted with water, all the material was recovered from the water eluate. De-acetylated hexosamines are not eluted from a Dowex 50 H^+ column with water (Gardell, 1953). The amount of colour produced by this disaccharide in

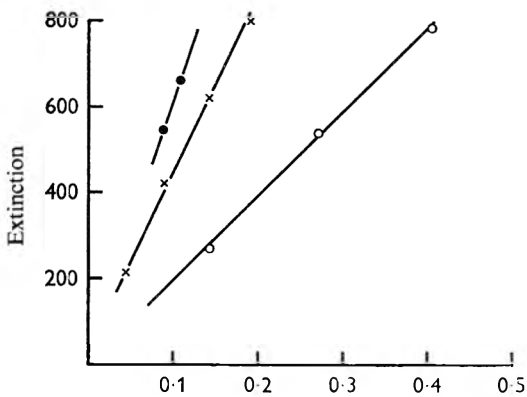

Fig. 1

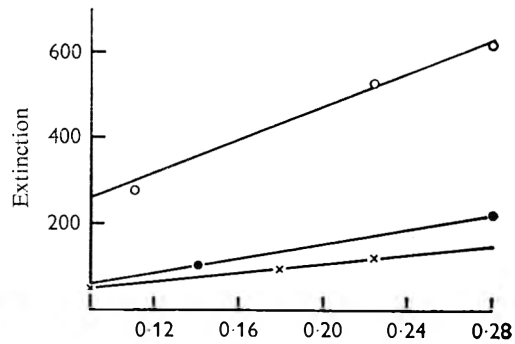

Fig. 2

Fig. 1. The Morgan & Elson reaction of : × — ×, *N*-acetyl-glucosamine; ○—○, *N*-acetyl-galactosamine; and ●—●, compound A 2. Compound A 2 was isolated from partial acid hydrolysates of formamide extracts of z3 III and z3 bacteria.

Fig. 2. The Elson & Morgan reaction of: × — ×, *N*-acetyl-glucosamine; ○—○, glucosamine; and ●—● compound A 2. Compound A 2 was isolated from partial acid hydrolysates of formamide extracts of z3 III and z3 bacteria.

the Morgan & Elson reaction was about the sum of that obtained by equimolar concentrations of *N*-acetylglucosamine and *N*-acetylgalactosamine; glucosamine and galactosamine are negative in this reaction (Fig. 1). Equimolar concentrations of glucosamine, *N*-acetylglucosamine and the disaccharide were tested by the Elson & Morgan reaction. Figure 2 shows that the acetylated hexosamine and the disaccharide behaved similarly in this reaction, while glucosamine produced a higher amount of colour.

To obtain information about the linkage between the two *N*-acetylhexosamines periodate oxidation was done. The disaccharide consumed 2·7 mole periodate/mole after 7·5 hr and a total of 3·7 mole in 24 hr. After 24 hr the formaldehyde liberated from the sample was 1 mole/mole. These results indicate a 1–3 linkage as the most probable structure. The α_D^{20} in water was +80°. In accordance with this was the good inhibition given by α-methyl-*N*-acetylglucosamine. Assuming that both hexosamines are in the D-configuration, the most probable structure of compound A 2 is 3-*O*-α-*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine (Fig. 3).

From the partial acid hydrolysate of the formamide extract of z3 bacteria a second oligosaccharide, consisting of rhamnose and glucosamine in the relative amounts of

2:1 was isolated. The elution of the oligosaccharide from the charcoal column with 5% (v/v) ethanol in water and a R_g value in solvent C of 1·25 point to a trisaccharide.

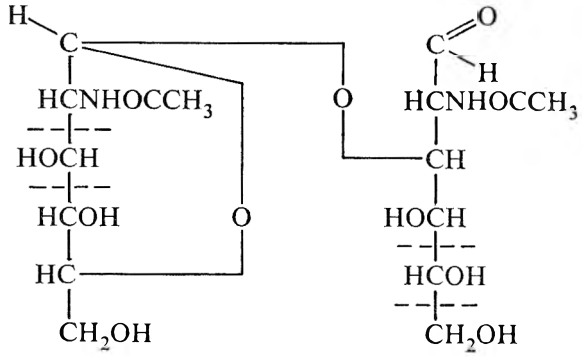

Fig. 3. 3-*O*-α-*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine isolated from partial acid hydrolysates of formamide extracts of z3III and z3 bacteria. The dotted lines indicate the diol linkages which are broken on periodate oxidation (theoretical 4 mole/mole, found 3·7 mole). On breakage of these linkages formic acid is formed and formaldehyde (theoretical 1 mole/mole, found 1 mole) is released.

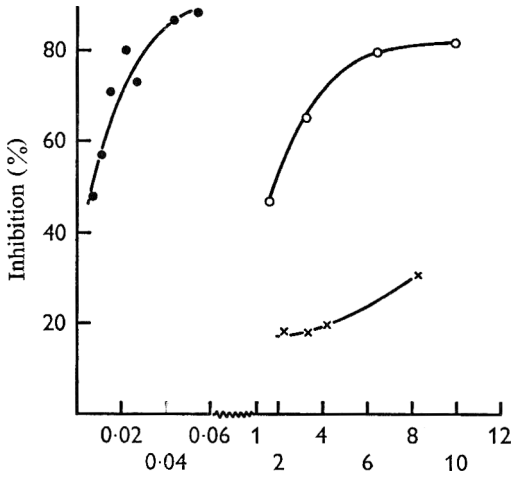

Fig. 4

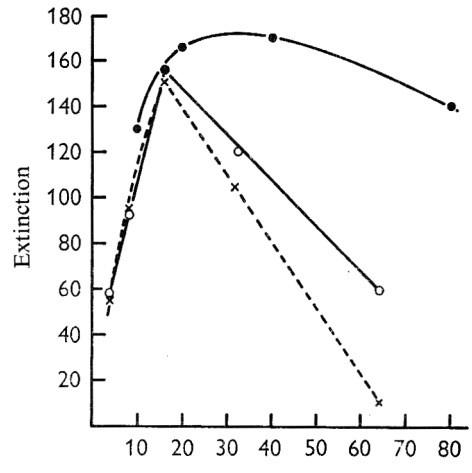

Fig. 5

Fig. 4. Inhibitions of the antigen-antibody reaction of the z3/anti-z3 system by: ×—×, β-methyl-*N*-acetyl-glucosamine; ○—○, α-methyl-*N*-acetylglucosamine; and ●—●, 3-*O*-α-*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine.

Fig. 5. Quantitative precipitin reactions of: ×—×, acetone fraction of z3/anti-z3 serum; ○—○, acetone fraction of z3III/anti-z3 serum; and ●—●, alcohol fraction of z3III/anti-type III serum.

Serological activity of the disaccharide. The inhibitory activity of 3-*O*-α-*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine is shown in Fig. 4 and compared with the inhibitions given by α-methyl-*N*-acetyl-glucosamine and β-methyl-*N*-acetyl-glucosamine. From Fig. 4 it can be seen that with 0·007 μmole the disaccharide gave 50% inhibition of the antigen/antibody reaction, whereas 1·7 μmole of α-methyl-*N*-acetyl-

glucosamine was necessary to obtain 50% inhibition. β-methyl-*N*-acetyl-glucosamine did not reach the 50% inhibition.

Fractionation of a formamide extract of z3 III bacteria. A purified formamide extract was fractionated with ethanol and acetone. On qualitative analysis by paper chromatography no galactose was found in the acetone fraction. The ethanol fractions contained rhamnose, glucose, galactose and small amounts of hexosamines. Refractionation with ethanol gave two main fractions. The first fraction was precipitable with 2 vol. of ethanol and contained glucose, galactose and rhamnose. The second fraction was precipitated with acetone and contained rhamnose and both hexosamines. Both final fractions were freeze-dried. By applying the same fractionation technique to a formamide extract of z3 bacteria, the main fraction precipitated with acetone. The quantitative sugar analysis of these fractions is presented in Table 3. In the first (2 vol. ethanol) fraction of z3 III glucose, galactose and rhamnose were present in the ratio of about 10:7:2. The composition of the acetone fractions of z3 III and z3 bacteria were much alike; the relative amounts of rhamnose, glucosamine and galactosamine were 2:1:1, with slightly more glucosamine than galactosamine (Table 3).

Table 3. *Quantitative sugar analysis of the ethanol and acetone fractions of a formamide extract of z3 III streptococci and the acetone fraction of a formamide extract of z3 III streptococci*

Fraction	z3 III ethanol fraction	z3 III acetone fraction	z3 acetone fraction
Glucose	44	1	3·3
Galactose	31	·	·
Rhamnose	9	35·5	34
Glucosamine	0·5	19	17
Galactosamine			15

The figures give the percentages of the sugars calculated on the dry weight of the polysaccharides before hydrolysis.

The serological activity of the different fractions was measured in quantitative precipitin reactions. In the reaction between the ethanol fraction of z3 III and type III antiserum a peak was obtained with 40 μg. antigen (Fig. 5). The acetone fractions of z3 III and z3 did not react with type III antiserum but gave almost identical precipitin curves with z3 antiserum; in both curves a peak was obtained with 16 μg. antigen. The quantitative precipitin reaction of the acetone fraction of z3 III with z3 antiserum was for 50% inhibited by 0·009 μmole of 3-*O*-α-*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine.

DISCUSSION

To study a possible z antigen (Ottens & Winkler, 1962) without hindrance of the type antigens two techniques also used for the isolation of the group B antigen were used. Subcultivation of z3 III bacteria in medium, containing type III antiserum, resulted in a strain which was no longer reactive with type III antiserum. The formamide extract of these z3 streptococci did not contain galactose, in contrast to the extract of z3 III streptococci. The serological differences are more striking. The z3 III/anti-III reaction was inhibited by glucose and galactose, whereas *N*-acetyl-glucosamine was completely inactive (Table 2). In the z3/anti-z3 system *N*-acetyl-glucosamine was a very good inhibitor, whereas other sugars including glucose and galactose were inactive.

Partial acid hydrolysis of formamide extracts of both z3III and z3 streptococci yielded after purification and isolation a disaccharide composed of two *N*-acetylated hexosamines. The most probable formula of the disaccharide is 3-*O*-α-*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine (Fig. 3). In the inhibition reaction of the quantitative precipitation of the z3/anti-z3 system, the disaccharide was a very potent inhibitor. An amount as small as 0·007 μmole gave 50% inhibition in a volume of 0·6 ml. containing 16 μg. of antigen and 0·1 ml. of antiserum, giving strong evidence for the disaccharide being the determinant group of the z3 antigen.

Lancefield (1934) used ethanol fractionation as a means of separating group antigen from type antigens in group B streptococci. Michel & Krause (1967) were able to separate the group and the type antigens from an FII strain by ethanol fractionation on a cellulose column; the group antigen appeared in the acetone fraction. When the same technique was applied to an extract of a z2II strain pure type II and z2 antigen were obtained. The solubility in ethanol of the z2 antigen was similar to those found for regular group antigens.

In our case application of the ethanol fractionation to a formamide extract of z3III bacteria resulted in a separation in two chemically and serologically distinct fractions. The 2 vol. ethanol fraction was reactive only with type III antiserum and must therefore be considered to be the type antigen. It contains besides rhamnose, both the sugars inhibitory in the type III system, namely glucose and galactose. The relative amounts of glucose, galactose and rhamnose are about 5:3:1 which was unexpected. Since rhamnose is serologically not active it was expected to be the backbone of the molecule and to be present in higher amounts. A possible destruction of rhamnose during formamide extraction has to be considered.

The acetone fraction was not reactive with type III antiserum but only with z3 antiserum, and gave a peak in the quantitative precipitin reaction with only 16 μg., indicating a high degree of purification. Fractionation of a formamide extract of z3 bacteria yielded mainly acetone-precipitable material; 16 μg. of this material gave with z3 antiserum an amount of precipitate equal to that obtained with the acetone fraction of z3III. Further evidence for the identity of the acetone fractions of formamide extracts of z3III and z3 bacteria is given by the inhibition reactions of the quantitative precipitation with z3 antiserum. The disaccharide 3-*O*-α-*N*-acetyl-D-glucosaminoyl-*N*-acetyl-D-galactosamine gave 50% inhibition with the acetone fraction of z3III with c·009 μmole and with the acetone fraction of z3 with 0·007 μmole. The sugar analysis shows the same sugars for the acetone fractions of formamide extracts of z3III and z3 bacteria. The relative amounts of rhamnose, glucosamine and galactosamine of the acetone fraction of extracts of z3III and z3 bacteria are about 2:1:1, with slightly more glucosamine than galactosamine. This suggests that the z3 antigen consists of a rhamnose backbone, with on every second rhamnose a determinant group, while possibly on some rhamnoses only a *N*-acetyl-glucosamin e isattached. This is also suggested by the isolation of a trisaccharide consisting of rhamnose and glucosamine in the ratio of 2:1.

The following results show the similarity in properties between the z antigen and the other group antigens. A strain, z3, lacking type III antigen could be isolated. The sole antigen from strain z3 seems to be identical with the acetone-precipitable non-type antigen from strain z3III. This antigen has the solubility also found in other group antigens of streptococci. By injecting z3III streptococci in rabbits no anti-z antibodies

are formed. This is in accordance with the results obtained with strains carrying a group F and a type antigen (Ottens & Winkler, 1962), which give by injection in rabbits only anti-type antibodies. It is to be expected that more z antigens will be isolated and identified.

The authors are indebted to Professor Dr K. C. Winkler for constant advice.

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A Note on the Nomenclature of Certain Polysaccharides Resembling the Group Antigens of Streptococci

By M. F. MICHEL, C. E. DE MOOR, H. OTTENS, J. M. N. WILLERS AND K. C. WINKLER

On the basis of serological reactions, Ottens & Winkler (1962) showed that the majority of streptococcal strains carrying the group F antigen possess one out of five additional type antigens. Accordingly these strains have been designated either FO or FI to FV. The antigens I and II presumably are identical with the type antigens described by Bliss (1937). All these type antigens are polysaccharides and are probably located on the surface of the cell wall. Antisera prepared in rabbits with vaccines of strains carrying both group and type antigen usually contain type-specific antibodies but no group-specific antibodies. Using type-specific antisera several type antigens have repeatedly been found in strains of other serological groups, FI in groups C, G and T (Ottens & Winkler, 1962), L (Willers *et al.* 1964*b*) and A (Jablon *et al.* 1965). The availability of type-specific antisera further led to the discovery of a substantial number of streptococcal strains carrying a type antigen but no known group antigen. Such strains have been designated OI, OII, OIII or OIV where O stands for zero. When these strains were described it was not clear whether zero denoted the absence of a group antigen or the presence of a hitherto unknown antigen.

Michel & Krause (1967) separated the group and type antigen present in an FII strain on the basis of a difference in alcohol solubility of the F and the II antigen. When the same technique was applied to extracts of an OII strain (Michel & Krause, 1967) and an OIII strain (Willers & Alderkamp, this paper) two different polymers were isolated in each instance. The polymers with the lowest solubility in alcohol were found to be serologically and chemically identical in both cases with known type antigens. The second set of polymers had the same physico-chemical properties as the group antigen F (Michel & Krause, 1967). It is therefore believed that the so-called 'O' (zero) polysaccharides have the same location in the cell wall as regular group antigens. They have not been recognized before because the 'O' polymers are exclusively found in strains carrying a type antigen. As indicated before the presence of type antigens generally suppresses the formation of group-specific antibodies. The masking effect of the presence of the type antigen on the antigenicity of an 'O' polymer was however clearly demonstrated by Willers *et al.* (this paper), with a mutant derived from strain OIII and lacking type antigen III and containing only the original 'O' antigen.

From this it appears that the symbol 'O' did not stand for the absence of a group

antigen but indicated the presence of an unknown group-like material. The zero polymers which have been isolated from three single strains (OI, OII and OIII) can clearly be differentiated from each other by their chemical composition. Moreover, the designation of several group-like materials denoted by capital O might give rise to confusion with the existing group O streptococcal polysaccharide. It is therefore suggested that a new provisional nomenclature for the 'O' antigens of streptococcal strains which can be found in association with a type antigen be introduced. It is proposed to replace the 'O' by a lower case 'z' followed by a numerical indicator. The lower case has been chosen to avoid any suggestion that these antigens are true group antigens, before they have been studied more elaborately. The first three indicators will be assigned to well investigated strains so that strain IS 8 formerly defined as O I will now be designated z1I, strain HS 189 (OII) z2II and strain MG 216 (OIII) z3III Unpublished observations indicate that different combinations of z and type polymers can be found and that the number of z polymers is larger than the number used here.

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Analysis and Comparison of the Carotenoids of *Sarcina flava* and *S. lutea*

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SUMMARY

The carotenoid pigments of *Sarcina flava* (*Staphylococcus afermentans*) and *S. lutea* were extracted, purified and separated by thin-layer chromatography into seven fractions. These fractions were hydrocarbon or polar materials. The two bacteria appeared to synthesize identical pigments which could be complexed *in vivo* to different proteins or to different amounts of a protein, which would explain the apparent differences in the colour of colonies of the two bacteria when grown on nutrient agar.

INTRODUCTION

A number of non-photosynthetic bacteria synthesize carotenoids which are usually of a highly polar nature (Goodwin, 1954). Although the pigments of *Sarcina lutea* have been investigated by several workers, those of *S. flava* (*Staphylococcus afermentans*) have received little attention. Chargaff & Dieryck (1932) proposed that *S. lutea* had two carotenoids present, from the evidence of partition between 90% methanol and light petroleum (b.p. 60–80°). They assumed that the epiphase consisted of one compound which they considered to be a hydrocarbon (sarcinene). They inferred the presence of a more polar carotenoid (probably a xanthophyll) which was hypophasic in this system. Further work by Chargaff (1933) seemed to confirm this. Nakamura (1936) suggested that a single esterified pigment was present in *S. lutea* whereas Takeda & Ōta (1941), from the same bacterium, obtained a crystalline compound which they considered was a xanthophyll (sarcinaxanthin). Sobin & Stahly (1942) examined *S. lutea* and *S. flava* and reported that the former contained two carotenoids (carotenols), whereas the latter had only one carotenol, identical with one of those found in *S. lutea*. They stated that no esters or carotenoid acids were present in either of these bacteria. Since these results seem inconclusive, a new investigation into the pigments of *S. lutea* and *S. flava* was undertaken.

METHODS

Bacteria. Sarcina lutea (strain NCTC 196) and *S. flava* (strain NCTC 7503) were obtained from the National Collection of Type Cultures, London, and stock cultures were maintained by cultivation alternately on nutrient agar and in nutrient broth (Oxoid Ltd.) in the dark at 30°. Bulk cultures were grown on nutrient agar containing 5% glucose, in oblong aluminium dishes sealed and sterilized inside nylon tubing.

Chemicals. The solvents were methanol (Analar), hexane (reagent grade redistilled before use) and diethyl ether, made peroxide- and moisture-free.

Pigment extraction procedures. Two extraction methods were used; the results from each were identical although the extraction of pigment by method (*b*) was not so complete.

Method (*a*). The bacteria were harvested into 10 ml. volumes of 95% methanol in water in 50 ml. centrifuge tubes and subjected to 5 min. ultrasonic disintegration at 0°. The combined suspensions were transferred to a 500 ml. round-bottomed flask and the volume made to 100 ml. A porous chip was added, the flask made light-proof with black material and the suspension refluxed at 80° for 3 min. After cooling and centrifugation, the bacterial sediments were re-extracted with absolute methanol which left them white. The pigment-containing supernatant fluids were combined, concentrated and stored under nitrogen at 0° in the dark.

Method (*b*). This method was used as a check since Hughes & Cunningham (1963) stated that ultrasonic disintegration can produce very high temperatures and pressures around the cone. We wished to see whether ultrasonic treatment and heating as used in method (*a*) were not having any deleterious effects on the pigments. Unfired porcelain was ground to a fine powder in a ball mill, and the harvested bacteria, suspended in methanol, were mixed with an equal volume of this powder, homogenized in a Potter homogenizer, centrifuged, and the supernatant fluid decanted. After four such extractions, the bacterial remnants were virtually white; the combined supernatant fluids were concentrated and stored under nitrogen at 0° in the dark.

Purification of the total extract. The lipid precipitation technique of Blessin (1962) was followed and the ether of the resultant supernatant fluid was removed on a rotary evaporator under reduced pressure at 34°. A pigment solution was saponified in 10% (w/v) KOH in methanol overnight in the dark at room temperature and in an atmosphere of nitrogen. The unsaponifiable material was extracted into ether in the usual way.

Separation of the pigments. Thin-layer chromatography on silica gel G (Merck) washed with chloroform was used. To obtain reproducible R_F values, the conditions of Dallas (1965) were used. Layers 0·25 mm. thick on 200 × 200 mm. plates were used and the plates developed in an S-chamber (according to the design of Davies, 1963) in a constant temperature room at 15°. The plate and cover were clipped firmly together; this enabled development to be done in a normal thin-layer chromatography tank previously equilibrated with solvent. The solvent quoted by Rothblat, Ellis & Kritchevsky (1964) was used and was benzene + methanol + acetic acid (87 + 11 + 2 by vol.). In all, twelve plates were developed to ensure reproducibility. After development, the individual spots were identified by: (i) visibility; (ii) behaviour under ultraviolet radiation; (iii) spraying with a saturated solution of antimony trichloride in chloroform (Morton, 1942); (iv) treatment with iodine vapour (Truter, 1963); (v) spraying with 2·5 M-H_2SO_4. The last two detected not only carotenoids, but also their colourless precursors.

For preparative thin-layer chromatography, solutions of the carotenoids in ether were applied to a 0·5 mm. layer on 200 × 200 mm. plates by using the mechanical applicator produced by Desaga (Camlab (Glass) Ltd.). For optimum resolution only 200–300 μg. material could be applied to each plate. The plates were developed in the same solvent as before; the S-chamber was not used. After development of the plates, they were dried in a stream of nitrogen. The individual fractions were removed from the plates, eluted first into methanol and then into ether which was thoroughly washed

with water to remove any traces of acetic acid. The pigments were again taken to dryness and redissolved in methanol for storage as before.

Comparison of the pigments. (i). Spectra were determined in spectroquality hexane (British Drug Houses Ltd.) and in methanol (Analar) with a Unicam SP.800 spectrophotometer. The wavelength calibration was checked with a holmium filter and was correct to within $\pm$ 1·4 mμ.

(ii). Each fraction was partitioned between 95 % (v/v) methanol in water and hexane according to Petracek & Zechmeister (1956).

(iii). The *cis* or *trans* configuration of each pigment was determined by the iodine isomerization test (Zechmeister & Polgár, 1943).

(iv). The following method was used to test for 5–6 epoxides (Curl & Bailey, 1954; Jungalwala & Cama, 1962). The pigment was dissolved in methanol; to 3 ml. in a 1 cm. Unicam glass cuvette, one drop of a solution of 0·05 N-HCl in methanol was added. The $\lambda_{max.}$ were read before and after addition of the acid methanol and again after 10 min. A spectral shift of 20–25 mμ is characteristic of a 5–6 epoxide group.

(v). An HCl test outlined by Karrer & Jucker (1950) was done; a blue colour is given by certain structural features, e.g. epoxides, aldehydes and possibly by carotenoids with several hydroxyl groups.

RESULTS

Chromatographic comparison of the total pigment before and after saponification suggest that before saponification, some carotenoids tended to associate, probably by occlusion, with polar lipids which were saponifiable. There was no evidence for any simple carotenoid esters. Chromatography of the purified pigments from the two bacteria showed that both synthesized identical pigments. The results of the tests carried out on the individual fractions are shown in Table 1.

Table 1. *Characteristics of the pigments of Sarcina flava and S. lutea*

Pigment	R_F (S-chamber)	$\lambda_{max.}$ methanol (mμ)	$\lambda_{max.}$ hexane (mμ)	Partition coeff.	*Cis/trans*
1	0·96	—, 415, 439, 469	—, 415, 437, 466	0/100	All *trans*
2	0·60	—, 415, 439, 469	—, 415, 437, 466	27/73	All *trans*
3	0·42	—, 415, 439, 469	—, 415, 437, 466	71/29	All *trans*
4	0·41	331, 413, 436, 466	331, 412, 435, 464	71/29	*cis* isomer
5	0·26	331, 413, 436, 466	Insoluble	100/0	*cis* isomer
6	0·19	—, 415, 439, 469	Insoluble	100/0	All *trans*
7	0·06	331, 413, 436, 466	Insoluble	100/0	*cis* isomer

Pigment	HCl spectral shift	Colour with HCl	Colour with $SbCl_3$	Type
1	—	Nil	Brown	Carotene
2	—	Nil	Blue	Monohydroxy
3	—	Nil	Blue	Dihydroxy
4	—	Nil	Blue	Dihydroxy
5	—	Nil	Blue	Poly -OH
6	—	Nil	Blue	Poly -OH
7	—	Nil	Blue	Poly -OH

DISCUSSION

The extreme polarity of many of the compounds meant that their extraction was only possible with polar solvents such as methanol. The effectiveness of methanol may be aided by its denaturing effect on the bacterial proteins with which at least some of the pigments are complexed. Such pigment-protein complexes in *Sarcina lutea* were suggested by Matthews & Sistrom (1959), and it has been suggested that in *Micrococcus lysodeikticus* the pigments are bound to the cell membrane (Gilby, Few & McQuillen, 1958). That no deleterious effect on the carotenoid as a result of using ultrasonic treatment on organic solutions was found, agrees with the findings of Zolotoba (1965).

Thin-layer chromatography provided an excellent method with the solvent system used for the preparative isolation of seven pigment fractions in a chromatographically pure state. The advantage of the S-chamber in the early stages of this work meant that the spots were very compact and that R_F values were reproducible. The use of the S-chamber in an ordinary thin-layer chromatography tank overcame the problem of solvent evaporation from the solvent trough which can occur when using commercially available S-chambers.

Table 1 gives an indication of the type of carotenoids which these two bacteria synthesized. It would appear that the pigments of these two bacteria are similar. They have the same chromophoric group and, from the positions of the $\lambda_{max.}$, a system of nine conjugated double bonds is suggested. Colonies of the two bacteria do not look alike when grown on nutrient agar and this difference may be due to differences in the concentration of the individual pigments or to the way in which they exist *in vivo*. If the pigments are bound to protein, different proteins or different protein:pigment ratios may be involved, which might cause a shift in the $\lambda_{max.}$ in the visible region.

Rothblat *et al.* (1964) published details of the carotenoids of *Micrococcus lysodeikticus*; its pigments have a similar $\lambda_{max.}$ value to those of the sarcinas examined here, but the R_F values and partition values quoted mostly differ from those found here. *Micrococcus lysodeikticus* and *Sarcina flava* are possibly two of the many different strains of *Staphylococcus afermentans* (Dr S. P. Lapage, private communication). The characteristic of strains of *S. afermentans* is that they do not produce acid in peptone water+glucose or in the medium of Hugh & Leifson (1953). Thus it is not surprising that similarities were found; as Sobin & Stahly (1942) remarked, different strains of some species of bacterium may well be found to produce the same pigments.

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Studies on Filamentous Forms of *Bacillus cereus* strain T

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SUMMARY

A mutant growing as long filaments was regularly observed in continuous cultures of *Bacillus cereus* strain T. The filaments had approximately the same diameter as the parent bacilli and septa were observed at regular intervals, corresponding to the length of single bacilli of the parental type. The links between the individual organisms in the filaments appeared to possess high mechanical strength, as shown by their resistance to ultrasonic treatment. Lysozyme treatment resulted in a complete fragmentation of the filaments into bacilli of the same size as single normal organisms. Electron microscopy showed that the septa of the filaments were thinner than those displayed by dividing normal organisms. A zone of lower electron density, which developed in the contact zones between dividing normal bacilli in the early stages of division, was not observed in the links between the filamentous bacilli.

INTRODUCTION

Several observations have been reported on the formation of filaments from many different bacteria under the influence of, for example, penicillin, magnesium starvation and growth at elevated temperatures. As pointed out by Hughes (1956) these filaments are formed in response to adverse growth conditions and they give rise to organisms of normal appearance when ordinary conditions for growth are restored. The occurrence of a stable filamentous variant of *Bacillus megaterium* was, however, reported by Shaforostova (1962). This variant was obtained in continuous culture and could be maintained through several transfers on nutrient agar. It replaced the normal bacilli at high dilution rates and created a flocculent growth in the culture vessel. It was asporogenous, and no septa were detected in the filaments by using phase-contrast microscopy.

In the present investigation a stable filamentous variant was produced by continuous cultivation of *Bacillus cereus* T and a comparative study of some characteristics of normal and filamentous organisms was made.

METHODS

Cultivation methods. Nutrient agar (Difco) was used for maintenance of the bacterial strains and for the study of colonial morphology. Liquid cultures were grown in indented Erlenmeyer flasks on a rotary shaker. The incubation temperature was 30°. The media used were nutrient broth (Difco) and a minimal medium of the following

composition (g./l.): glucose, 10; $(NH_4)_2SO_4$, 1; KH_2PO_4, 3; K_2HPO_4, 7; $MgSO_4.7H_2O$ 0·2; 0·5 ml. of a trace elements solution of the following composition (g./l.): $CaCl_2$, 1; $FeCl_3.6H_2O$, 33; $ZnSO_4.7H_2O$, 0·36; $CuSO_4.5H_2O$, 0·32; $CoCl_2.6H_2O$, 0·37. Turbidity measurements were made in a Bausch and Lomb colorimeter. Dry weights were determined in duplicate on 20 ml. samples by centrifuging the organisms at 5000 *g* for 20 min., washing in 0·01 M-phosphate buffer (pH 7) and drying to constant weight at 100°. The morphology of the organisms was examined by phase-contrast microscopy.

Chemical methods. Glucose was determined enzymically (Levin & Linde, 1962). Pyruvate analysis was done according to Friedemann & Haugen (1943) and lactate analysis according to Barker & Summerson (1941). Poly-β-hydroxybutyrate was determined gravimetrically as follows. The organisms were treated with sodium hypochlorite (commercial solution, approximately 12% active chlorine) followed by washings of the liberated granules in distilled water, acetone, ethanol and diethylether and extraction in boiling chloroform (Williamson & Wilkinson, 1958, Law & Slepecky, 1961). The solution was filtered, the chloroform evaporated and the remaining material was weighed.

Cell walls were prepared by disintegration of the organisms in a freeze press (Edebo, 1960) followed by differential centrifugation (Salton, 1964). The cell-wall preparations were washed three times in 0·1 M-citrate phosphate buffer (pH 7) and three times in M-sodium chloride. Poly-β-hydroxybutyrate was removed from the cell walls by treatment with chloroform. Analysis of amino acids and amino sugars was done by thin layer chromatography according to Stahl (1962). Extracellular polysaccharides were extracted (Wilkinson, 1958) and analysed according to Snell & Snell (1953).

Electron microscopy. Preparations for electron microscopy were made according to Ryter & Kellenberger (1958) and Murray, Steed & Elson (1965). The bacteria used were grown on nutrient agar for 10 hr. Dehydration and embedding in epon was done according to Lundquist (1965). All specimens were stained with uranyl acetate (Watson, 1958) and lead acetate (Karnovsky, 1961).

RESULTS

Morphology

The colonies of the filamentous forms did not show any detectable difference from those of the normal bacilli. Under the microscope, however, the filamentous forms displayed a mycelial appearance. The filaments usually had a length corresponding to more than 100 normal bacilli. The filaments had approximately the same diameter as the normal bacilli and septa were observed at regular intervals, corresponding roughly to the length of normal cells (Pl. 1, figs 1, 2; Pl. 3, fig. 7).

Cultivation experiments

The appearance of the filamentous variant in the microscope was very characteristic. To ascertain its purity serial transfers were made from the original isolate by picking one colony onto nutrient agar medium. During a continued serial transfer with 2-day intervals for 2 months no difference in the morphology of the organisms was observed. Normal bacilli were grown in parallel under the same conditions and they also retained their microscopical appearance.

No differences in nutrient requirements or growth rate in liquid media were detected

during experiments with minimal medium and nutrient broth. The filamentous forms sporulated readily after incubation for 2 days at 30° on nutrient agar. A spore suspension prepared from the outgrowth of filamentous forms was heated to 80° for 30 min. and then plated on nutrient agar. The resulting colonies consisted of filamentous forms. Normal bacilli subjected to the same treatment also retained their morphology.

Metabolism

Normal and filamentous organisms were grown in the minimal medium with the addition of Casamino Acids (Difco) 5 g. and sodium acetate 2 g./l.; glucose concentration 2 g./l. The cultures were inoculated with organisms from a culture which had

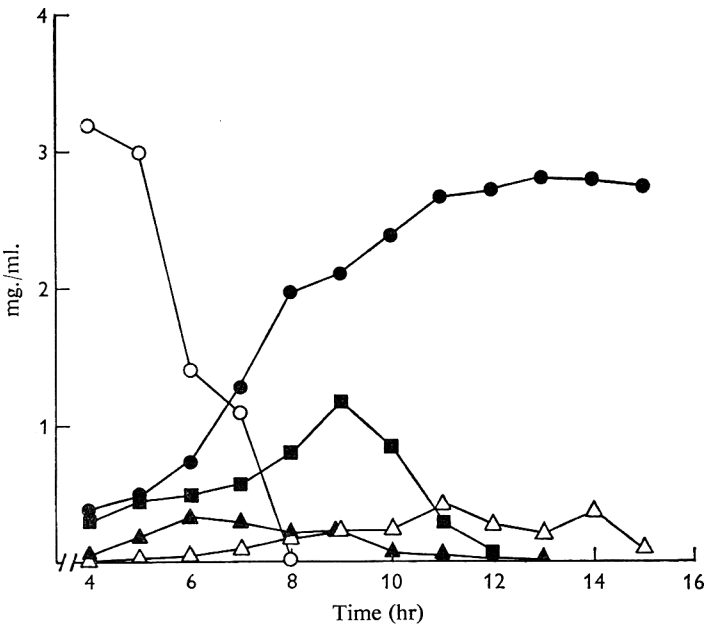

Fig. 1. Some metabolic properties of normal bacilli of *Bacillus cereus* T. The curves illustrate glucose utilization, lactate and pyruvate formation and poly-β-hydroxybutyrate accumulation during growth. Dry weight, ●—●; glucose, ○—○; lactate, ■—■; pyruvate, ▲—▲; poly-β-hydroxybutyrate, △—△.

grown for 10 hr. The growth curves, the analytical data on poly-β-hydroxybutyric acid accumulation, the formation of lactate and pyruvate and the utilization of glucose did not show any significant differences between the two morphological forms (Figs 1, 2).

Lysozyme treatment

Normal organisms of *Bacillus cereus* T are highly resistant to lysozyme, no protoplasts appearing under appropriate conditions even after prolonged incubation with the enzyme (Salton & Pavlik, 1960). However, organisms in the early logarithmic phase, showing short chains, were rapidly fragmented into separate rods when lysozyme was added. In one experiment with normal and filamentous forms, organisms were grown in minimal medium and harvested in the late log phase. Here normal bacilli occurred separately. Both forms were suspended in 0·01 M-phosphate buffer

(pH 7) containing 0·5 M-sucrose. The suspensions were divided in two portions: to one portion of each suspension lysozyme was added to 2 mg./ml.; the other portion served as a control. After treatment for 30 min. at room temperature the filaments began to fragment into rods corresponding in length to the normal bacilli and after 2 hr no filaments were seen. No effect of the lysozyme treatment on the normal bacilli was noted and no morphological change occurred in the controls during the experimental period. The bacilli resulting from the lysozyme-treated filaments were viable, as shown by spreading them on nutrient agar; after incubation for 36 hr at 30° colonies were formed which showed only filamentous forms when examined microscopically.

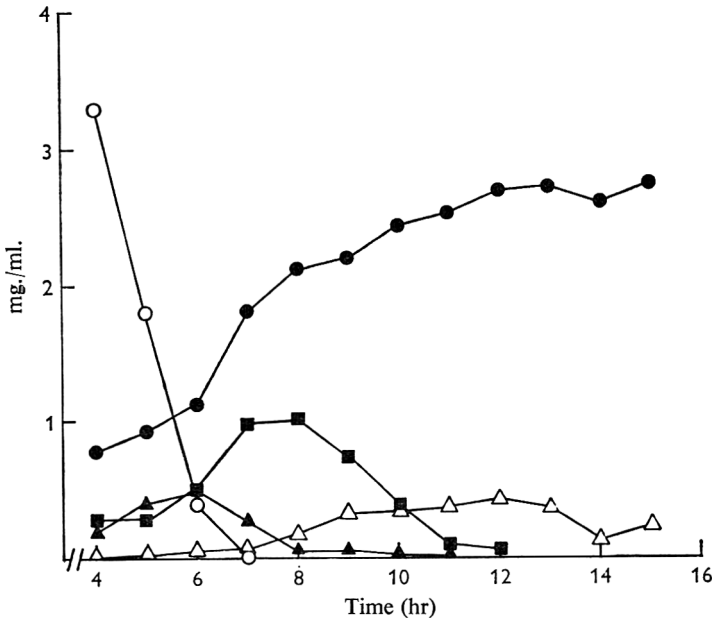

Fig. 2. Some metabolic properties of the filamentous forms of *Bacillus cereus* T. For symbols, see fig. 1.

Ultrasonic treatment

Ultrasonic disintegration was applied to preparations of both morphological forms in order to compare cell-wall fragments under the microscope. Normal bacilli and filamentous forms grown on nutrient agar for 10 hr were suspended in 0·01 M-phosphate buffer (pH 7). The suspensions were treated in a MSE sonic disintegrator at maximum efficiency for 30 sec. and then examined by phase-contrast microscopy. In Pl. 1, fig. 3, the appearance of disintegrated normal bacilli is shown; most of the fragments consist of halved cell walls. In Pl. 1, fig. 4, disintegrated filamentous forms are shown; most of the fragments consist of two cell-wall halves connected by a septum showing the mechanical strength of the links between the cells.

Chemical composition of cell walls

Cell-wall preparations from normal bacilli and from filamentous forms were hydrolysed in 6 M-hydrochloric acid for 12 hr at 105° in sealed tubes. Thin-layer chromatography on the hydrolysate showed the presence of the following substances: alanine,

α-ε-diaminopimelic acid, glutamic acid, glucosamine, galactosamine. One unidentified rapidly migrating substance was also detected. No difference in the cell-wall composition with regard to amino acids and amino sugars was found by this method. Since the size and colour intensity of the spots from chromatograms from the two morphological forms did not indicate any major quantitative differences no attempt to elaborate a quantitative analysis was made.

Electron microscopy

The normal bacilli were characterized by rounded ends and contact zones of varying breadth (Pl. 2, fig. 5). Sometimes the bacilli occurred in short chains; these chains regularly consisted of pairs of rods with a septum, each pair separated from the next by a small contact zone. Each bacillus displayed a cell wall of normal thickness in the contact zone, the two bacilli being separated by an area of lower electron density (Pl. 2, fig. 6). The thickness of these contact zones was approximately 700 Å. The cytoplasmic membrane was clearly visible immediately inside the cell wall. Most of the contact zones in the filamentous forms were characteristically thinner than those displayed by dividing normal bacilli being approximately 400 Å. When present, the constriction around this contact zone was very small. The distinct area of lower electron density in the contact zone of normal bacilli was absent (Pl. 3, figs 7, 8). No further differences with regard to thickness or appearance of the cell walls of the normal and filamentous forms was observed.

DISCUSSION

Most of the filamentous forms of *Bacillus cereus* described in the literature (Kominek & Halvorson, 1965; Hughes, 1956) have been the result of a phenotypic variation. The filamentous variant isolated from continuous cultures of *B. cereus* T described here showed a great genetic stability. Spores, isolated from the filamentous forms, gave rise only to filamentous forms upon germination. Lysozyme treatment resulted in a fragmentation of the filaments into rods of the same appearance as normal bacilli. These rods were viable and yielded filamentous forms when grown on nutrient agar. The two morphological forms did not differ significantly in their growth patterns or in the gross chemical composition of their cell walls. However, during the purification procedure accessory cell-wall components (Stolp & Starr, 1965) may have been lost; these are mainly polysaccharides and mucopolysaccharides in Gram-positive organisms. It is possible that there are certain differences in the accessory components even if the 'murein sacculus' (Weidel & Pelzer, 1964) appears to be of the same chemical composition in the two forms. Attempts to isolate polysaccharides from whole organisms by mild extraction procedures gave negative results, however, which makes it less probable that the difference between the two forms is a difference in the cell-wall polysaccharides.

In the electron microscope the links between cells in the filaments did not show a distinct zone of lower electron density, as was clearly observed in dividing normal forms, which thus indicated a separation into two distinct cell walls. The links between the cells in the filaments were of approximately the same thickness as the cell wall proper. The results of the ultrasonic treatment indicated a high mechanical resistance of these links. They were, however, sensitive to lysozyme. It is known that the lysozyme-sensitive β-1-4-glucosidic bond between glucosamine and muramic acid is of great

importance to the stability of the cell wall. The reason for the resistance of many bacterial cells to lysozyme is not known, but is usually ascribed to a masking effect of accessory components of the wall. A tentative interpretation of the present results would be that the filamentous forms lack the ability to split bonds in the septa which separate the cells and thereby to complete division. These bonds appear to be lysozyme-sensitive and contribute to a high mechanical strength of the septa.

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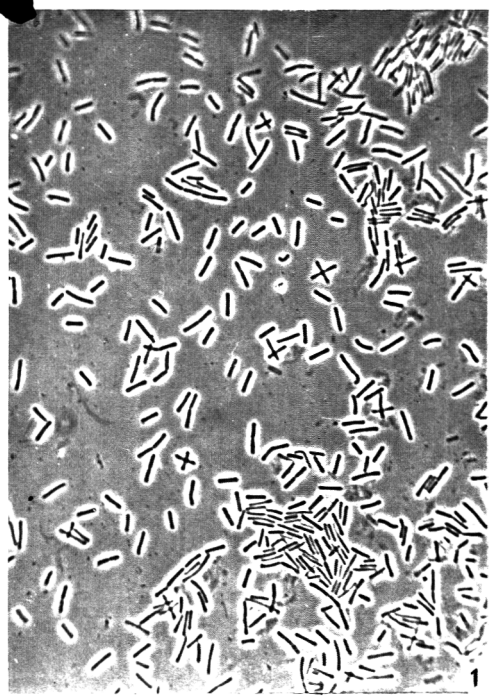
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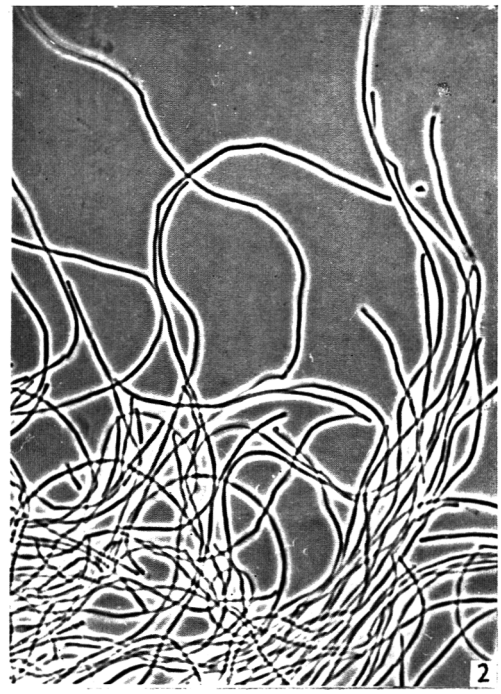
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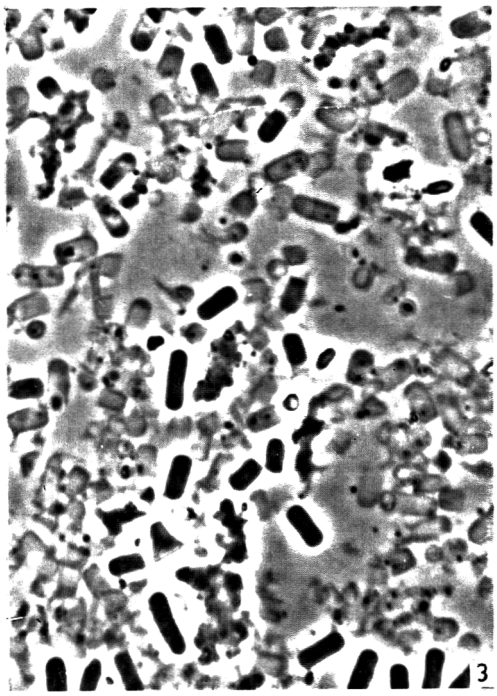
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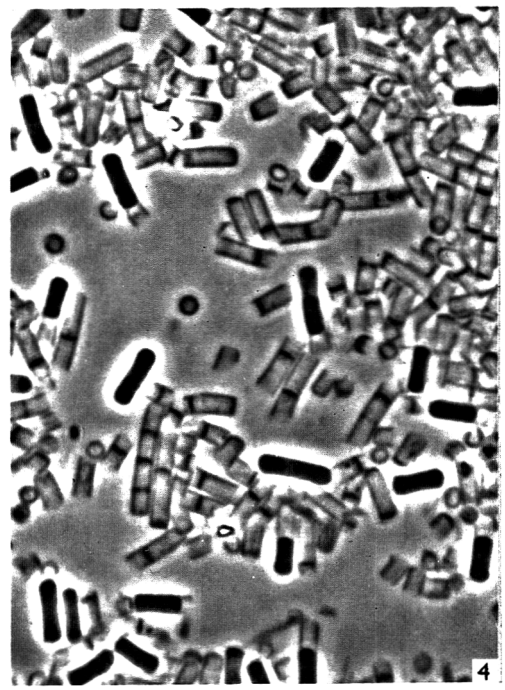
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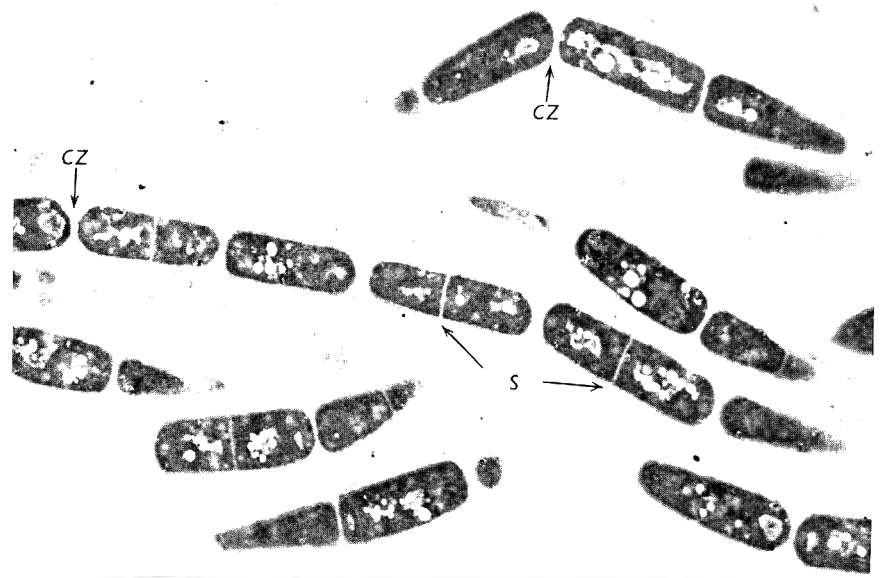

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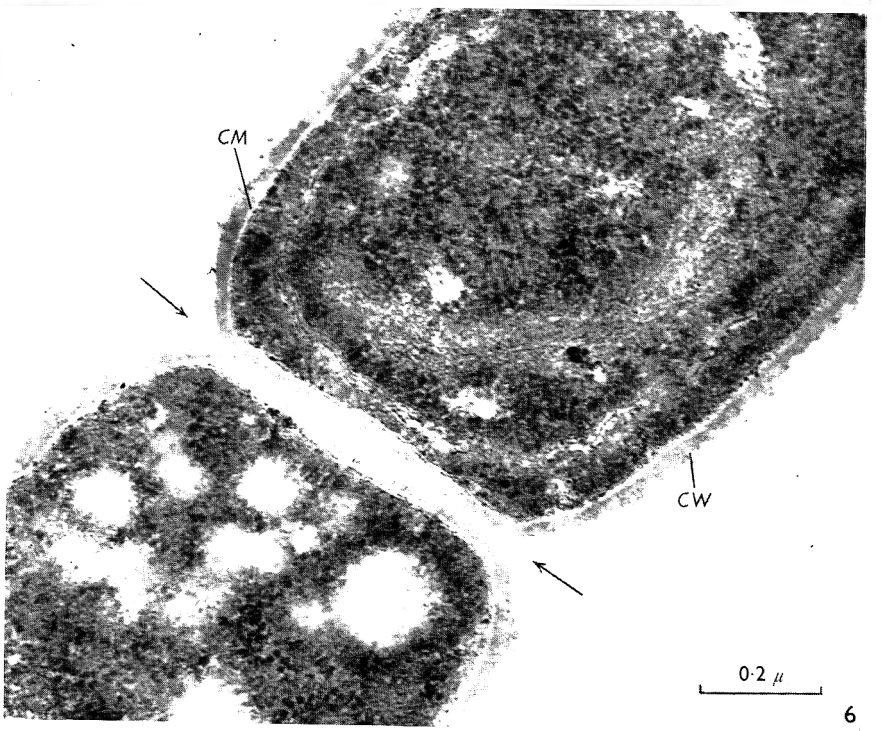

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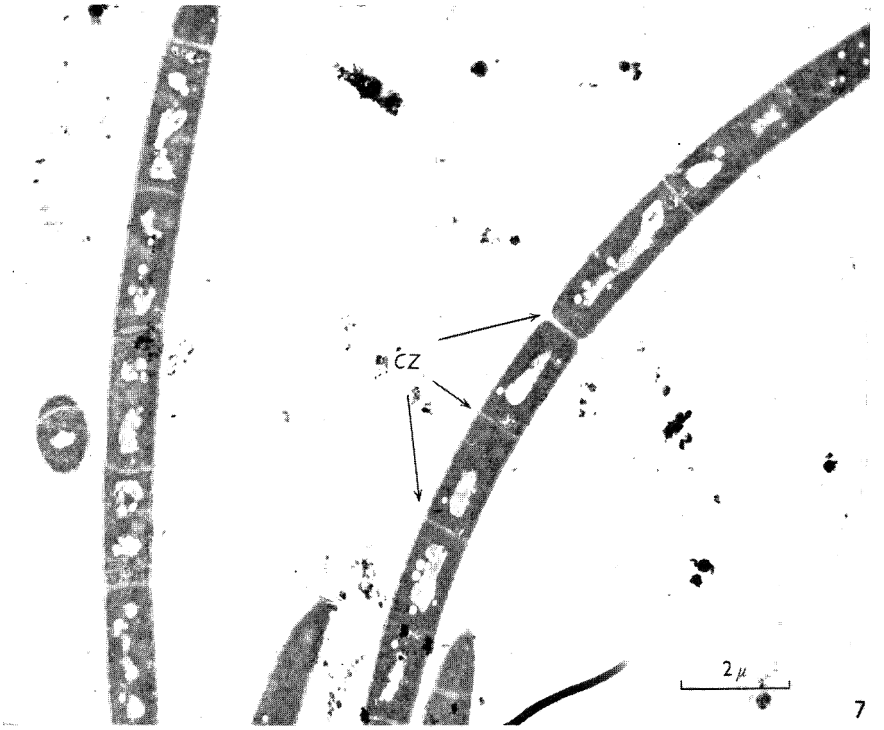

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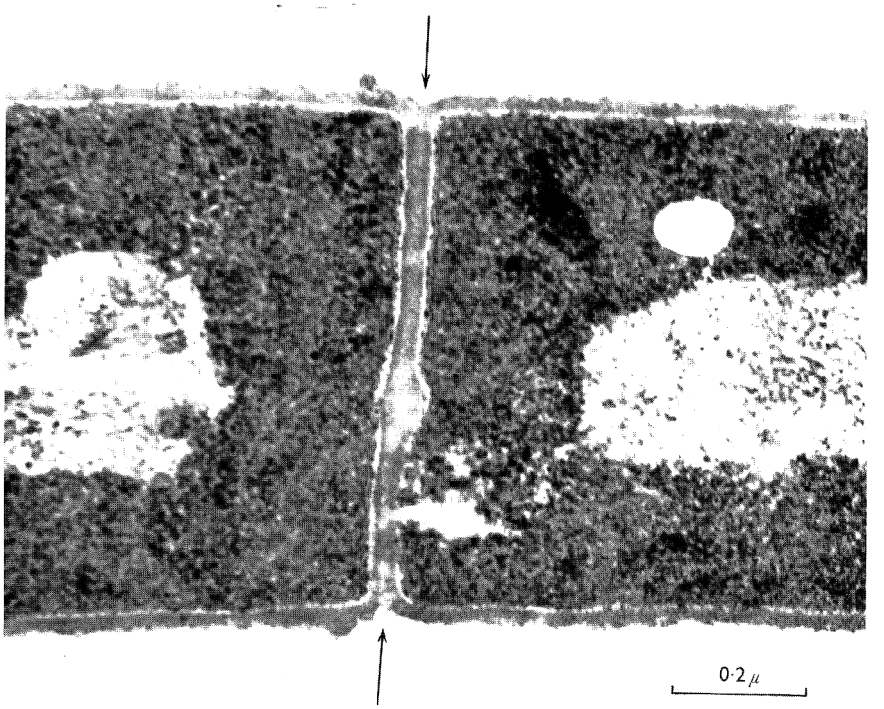

8

EXPLANATION OF PLATES

PLATE 1

(Phase-contrast microscopy; figs. 1, 2 ×530; figs. 3, 4 ×2100)

Fig. 1. Normal bacilli of *Bacillus cereus* T.

Fig. 2. Filamentous forms of *B. cereus* T.

Fig. 3. Normal bacilli of *B. cereus* T after ultrasonic treatment.

Fig. 4. Filamentous forms of *B. cereus* T after ultrasonic treatment.

PLATE 2

Fig. 5. Electron microscopic survey of normal bacilli of *Bacillus cereus* T. The bacilli are arranged in pairs with a straight septum (S) between the two bacilli and the rounded free ends connected with adjacent pairs by a short wide contact zone (CZ). Many lightly stained poly-β-hydroxybutyrate granules can be seen in the cytoplasm. (×9000.)

Fig. 6. Detail of normal bacilli of *Bacillus cereus* T demonstrating the wide contact zone (700 Å) between two pairs of bacilli (arrows). A zone of lower electron density than the normal cell wall is seen. Inside the cell wall (CW) a cytoplasmic membrane (CM) can be seen (×100,000).

PLATE 3

Fig. 7. Electron microscopic survey of filamentous forms of *Bacillus cereus* T demonstrating the straight contact zones (CZ) (×9000).

Fig. 8. Detail of contact zone (arrows) of filamentous forms of *Bacillus cereus* T demonstrating the straight and narrow (400 Å) contact zone. The distinct zone of lower electron density of the contact zone, as found in the normal bacilli, is absent. The cell wall and cytoplasmic membrane exhibit an appearance similar to those of normal bacilli. Sometimes a slightly thickened area could be seen in the contact zone with a width of up to 700 Å (×110,000).

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A Study of Some Motile Group D Streptococci

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SUMMARY

Thirteen strains of motile enterococci showed more similarity, in their physiological reactions, to *Streptococcus faecium* than to *S. faecalis*. A serological study of the type antigens divided the motile strains into four sets; (1) three strains previously described as *S. faecium* serotype 29, (2) one strain reacting as *S. faecium* serotype 38, (3) five strains showing a specific reaction with antiserum prepared to one of them ('serotype 4725'), (4) four untyped strains. Esterase and protein patterns from the soluble fractions of the motile strains were examined by electrophoresis in polyacrylamide gel and were different from those of non-motile strains of *S. faecalis*, *S. faecium* and *S. durans*. Extracts of the three motile strains of *S. faecium* type 29 showed a common esterase pattern, extracts from five strains of serotype 4725 showed three different esterase patterns. The motile strain of *S. faecium* serotype 38 gave extracts with a strong esterase band which differed from the very weak bands shown by extracts of non-motile strains. Differences in esterase pattern could be found between motile strains whether untyped, of different serotypes, or of the same serotype. Comparison of the 'protein patterns' of motile strains gave some indication that major protein bands occurred at similar positions after electrophoresis.

INTRODUCTION

A previous study by gel electrophoresis of soluble cell components (Lund, 1965) showed that 12 strains of *Streptococcus faecalis* and its varieties '*zymogenes*' and '*liquefaciens*' of eight serotypes had a similar pattern of separated protein bands, and showed strong bands with esterase activity. Strains of *S. faecium* of 19 serotypes gave 'protein patterns' with major bands having similar mobility (amongst these strains) but strong esterase bands were not detected. The pattern of protein bands of *S. faecium* and *S. durans* differed from that of *S. faecalis*. Of 21 strains of *S. faecium* and *S. durans* examined initially, one strain had a distinctive pattern of proteins and strong bands of esterase activity; this strain was the only motile one among those initially studied.

The purpose of the present work was to study, by gel electrophoresis, extracts of other motile strains of enterococci from diverse sources to compare their protein components and esterase enzymes, and to correlate this comparison with a study of some physiological and serological properties. Physiological properties and agglutination reactions of a large number of strains of motile enterococci have previously been studied by Graudal (1952, 1955, 1957*a*, *b*).

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METHODS

Organisms. The motile enterococci studied are listed in Table 1. They were compared with strains of *Streptococcus faecalis* and *S. faecium* which have previously been described (Lund, 1965). Cultures of *S. faecalis* and *S. faecium* were stored on slopes of Hartley's digest agar at 5°. Because cultures of motile organisms tended to die during storage they were maintained as stab cultures in Brain Heart Infusion agar Difco) at 5° and were subcultured at intervals of 1 month.

Table 1. *Strains of motile group D streptococci studied*

Strain (species name is that used in the reference cited)		Source	Reference
S. faecium (type 29, type strain)	P 14/6	Pig colon	Sharpe & Fewins (1960); Barnes (1964)
S. faecium	H/2/23	Human faeces	
S. faecium	119	Piglet	
S. faecalis	NCTC 4725	Probably human	Pownall (1935)
Streptococcus sp.	D 1000(b)	Representatives of Graudal's collection, isolated mainly from human faeces	Graudal (1957*a*, *b*)
Streptococcus sp.	D 1003(b) (described as 'pigmented' strain)		
Streptococcus sp.	D 1006(c)		
Streptococcus sp.	D 1010(c) (described as 'pigmented' strain)		
Streptococcus sp.	ATCC 12817	Oropharynx, patient undergoing treatment for pernicious anaemia	Hugh (1959)
Streptococcus sp.	ATCC 12818	Mouth, patient with untreated oral cancer	
Streptococcus sp.	ATCC 12819	As ATCC 12817	
Streptococcus sp.	ATCC 13638	Spinal fluid, patient with chronic meningitis	Liu *et al.* (1955)
S. faecalis	ATCC 12755	Milk	Sherman (1937); Hugh (1959)

Motility was demonstrated by stab inoculation of the organism into Brain Heart Infusion Broth (Difco) + 0·25% (w/v) Bacto-Agar (Difco) and incubation at 25°, 30° or 37° for 24 hr. The diffuse growth of motile strains could be distinguished from the more discrete growth of non-motile strains. Motility was confirmed by microscopical examination of living cells. Flagella were stained by Leifson's technique (Leifson, 1951).

Biochemical tests. The methods used for testing haemolysis, growth in presence of 40% bile, sensitivity to potassium tellurite, growth at 10°, growth at 45°, growth in presence of 6·5% NaCl, survival at 60° for 30 min. were those described by Barnes, Ingram & Ingram (1956). The appearance of colonies on thallous acetate-tetrazolium agar (TlTg) was studied using thallous acetate agar (Baltimore Biological Laboratory) and the method described by Barnes (1956). In all the above tests incubation was at 37° unless otherwise specified. Production of acid in carbohydrate media was studied by the method of Whittenbury (1963) utilizing a soft agar medium. The basal medium contained meat extract (Lab-Lemco), 5 g.; peptone (Evans), 5 g.; yeast extract (Difco), 5 g.; Tween 80, 0·5 ml.; agar (Davis), 1·5 g., distilled water to 1 l. As pH indicator

5·6 ml. of a 1·6% (w/v) solution of bromocresol purple in ethanol was added to 1 l. of medium and the pH adjusted to 6·8–7·0. Carbohydrate substrates (B.D.H. Laboratory reagent grade), as 5% (w/v) solutions in distilled water, were sterilized separately at 120° for 15 min. and added to the basal medium to give a final concentration of 0·5% (w/v). The melted medium was cooled to 45° before inoculating and then allowed to set. Results were recorded after incubation at 37° for 3 days. Tests for utilization of individual carbohydrates as sources of energy for growth were made by the technique of Deibel, Lake & Niven (1963). For aerobic tests 10 ml. quantities of media in T-tubes were inoculated and shaken at 104 strokes/min. with an amplitude of 2·25 in. on a reciprocal shaker; for anaerobic tests tubes containing 10 ml. of inoculated media were placed in a desiccator under an atmosphere of 95% nitrogen + 5% carbon dioxide. After incubation for 18 hr at 30° growth was estimated by turbidity measured in an 'EEL' nephelometer (Evans Electroselenium Ltd., Harlow, Essex). A positive result was taken as a turbidity, in the presence of added carbohydrate, equal to at least twice that in the basal medium.

Serological test for group D antigen. Extracts prepared as for polyacrylamide gel electrophoresis were tested for precipitin reaction with group D Streptococcus Grouping Serum (Burroughs Wellcome and Co.). Results were recorded 30 min. after testing.

Serological typing. The methods used were based on those described by Sharpe & Shattock (1952).

Type antisera. In preliminary experiments, extracts of motile strains were tested against *Streptococcus faecium* type antisera which had been prepared by Dr M. E. Sharpe, National Institute for Research in Dairying, Shinfield, Reading. In subsequent experiments antisera were prepared to motile strains P 14/6, ATCC 13638, NCTC 4725 and the non-motile strain P 17/8, using the following technique. Cultures were grown in 80 ml. of medium containing glucose, 1 g.; peptone (Evans), 1 g.; Lab-Lemco (Oxoid), 1 g.; NaCl, 0·5 g.; distilled water to 100 ml. pH 7·0–7·2 (medium A). After incubation at 37° for 18 hr cells were separated by centrifugation, washed twice in 50 ml. of 0·85% (w/v) NaCl in water and resuspended in 20 ml. solution containing 0·85% (w/v) NaCl and 0·1% (w/v) HCHO. The suspension was kept at room temperature for 24 hr, after which tests for viable organisms were negative. The suspension was adjusted to an opacity of 7–8 using standard opacity tubes (Burroughs Wellcome and Co.) and stored at −20°.

Two rabbits were inoculated by intravenous injections with each organism. An initial sensitizing dose of 0·4 ml. was given, and after 5 days the following series of injections was started: three injections of 0·4 ml. (week 1), three of 0·8 ml. (week 2), three of 1·0 ml. (week 3). Three days after the last injection a test bleeding showed satisfactory antibody production; 25 ml. of blood was withdrawn from each rabbit. After clot formation the separated serum was stored at −20° without preservative.

Absorbed antisera. For the initial experiments the absorbed type antisera to a range of serotypes of *Streptococcus faecium* had been prepared by Dr M. E. Sharpe. Further absorbed antisera were prepared by the following technique. The organism used for the absorption was grown in 200 ml. medium A at 30° for 18 hr, the cells harvested by centrifuging, washed and resuspended in 10 ml. 0·85% (w/v) NaCl + 0·1% (w/v) HCHO. The suspensions were then heated at 70° for 30 min. and washed on the centrifuge in saline-formaldehyde solution. The packed cells were resuspended in four

times their volume of antiserum and incubated at 37° for 2 hr, stirring frequently. After storage at +1° overnight the cells were sedimented by centrifugation, the antiserum removed and tested against an extract of the homologous organism.

Extraction of type antigens. Cultures in 20 ml. of medium A were incubated at 25° for 24 hr. Hydrochloric acid extracts were prepared by a technique based on Lancefield's (1933) method. The sediment from 20 ml. of growth medium was resuspended in 0·5 ml. of HCl (0·05N or 0·01N) in 0·85% (w/v) NaCl, heated in a boiling-water bath for 10 min., cooled, centrifuged and the clear supernantant neutralized by the addition of 1N- or 0·2N-NaOH to an end-point with phenol red; 1 drop of 1% (w/v) solution of Thiomersal (British Drug Houses Ltd.) was added as preservative.

Removal of flagella before extraction of type antigens. Cultures in 20 ml. of medium A were incubated at 25° for 24 hr, cells were separated by centrifuging at 1000 *g* for 20 min. and resuspended in 0·85% (w/v) saline. A sample stained by Leifson's (1951) technique showed the presence of flagella, many of which had become detached. In order to detach remaining flagella 10 ml. of the bacterial suspension was treated in a homogenizer (Measuring and Scientific Equipment Ltd.) using a blending speed of approximately 12,000 rev./min. for 2 min. After centrifuging at 10,000 *g* for 15 min. detached flagella tended to remain in the supernatant, the sedimented cells were resuspended in 10 ml. of fresh 0·85% (w/v) saline. This centrifuging and resuspension in fresh saline was repeated 4 times, giving a final sediment which consisted mainly of intact, deflagellated organisms. A hydrochloric acid extract was prepared as previously described, using for comparison a cell sediment prepared without removal of flagella.

Precipitin tests. Strong precipitin reactions were observed by the capillary tube technique of Swift, Wilson & Lancefield (1943). For weaker reactions it was necessary to use the precipitin ring technique (Sharpe & Shattock, 1952). In each case extracts prepared with 0·05N-HCl in 0·85% (w/v) NaCl (pH 1·6) gave a stronger precipitin reaction than those prepared with 0·01N-HCl in 0·85% (w/v) NaCl (pH 2·1); this differed from the results obtained by Maxted & Fraser (1966) with *Streptococcus faecalis* strains.

Gel diffusion tests. These were made on microscope slides 3 in. × 1 in. covered with 2 ml. of the following medium: barbitone-acetate buffer (Oxoid), 8·25 g., thiomersal (B.D.H.), 0·1 g., Ionagar no. 2 (Oxoid), 10 g., distilled water to 1 l. Wells were cut in the agar, filled with antiserum or antigen extract and the slides placed at 0–5° for 24 hr in a moist atmosphere for precipitin lines to develop.

Immuno-electrophoresis. Lantern cover glasses 3¼ in. × 3¼ in. were covered with 12 ml. of the medium described for gel diffusion tests. Wells were cut and filled with antigen extract. For electrophoresis the tank buffer contained barbitone-acetate buffer (Oxoid) 8·25 g.; distilled water to 2 l.; a potential difference of 8–10 V. per cm. was applied for 1·5 hr. Trenches were made, filled with antiserum, and the preparation was placed at 0–5° in a moist atmosphere for 24 hr to allow development of precipitin lines.

Effect of periodate on type antigens. Attempts were made to detect sensitivity of the antigens to periodate, using a method similar to that of Sharpe (1964). Neutralized hydrochloric acid extract 0·5 ml., or the acetone-precipitated fraction of this extract, was treated with an equal volume of 0·4M-acetate buffer pH 4 containing 0·08M-sodium periodate. After incubation at 37° for 16 hr 0·05 ml. of 5% (v/v) ethylene glycol was added to destroy the periodate, the pH was adjusted to 7 with N-NaOH

and the extract was tested against the homologous antiserum. Controls were included in which antigen extract was replaced by water.

Effect of trypsin on type antigens. Crystalline trypsin was added to the neutralized hydrochloric acid extract to give a concentration of 2 mg./ml. After incubation at 37° for 3 hr the extract was tested against the homologous antiserum.

Preparation of cell extracts for electrophoresis. Cultures were grown in 800 ml. volumes of Brain Heart Infusion Broth (Difco Laboratories) in static culture for 16 hr at 30°. The bacteria were harvested by centrifugation, washed, and the wet pellet of cells resuspended in half its weight of tris-citrate buffer pH 8·7 (see below). The suspension was frozen at −20°, disrupted in an X-press (AB Biox, Box 235, Nacka 2, Sweden), melted and clarified by centrifugation as previously described (Lund, 1965). The final extracts, with a protein content of 30–50 mg./ml., as estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) were stored at −20°.

Electrophoresis in polyacrylamide gel. Conditions for electrophoresis, staining for protein and for esterase enzymes (hydrolysing α-naphthyl acetate) and for photography were as previously described (Lund, 1965). The gel was prepared in buffer containing tris, 38 mM, citric acid 2·5, mM (pH 8·7 at 25°); the buffer for electrode vessels contained boric acid, 7·22 g./l., borax, 15·75 g./l. (0·28 M in terms of borate) pH 8·8 at 25°.

RESULTS

Biochemical properties of motile strains

Table 2 summarizes the properties of motile strains of enterococci and includes a comparison of the motile and non-motile strains in serotype 38.

The term 'enterococci' [introduced by Sherman (1937) to include streptococci giving positive results in the 'Sherman tests' (Deibel, 1964)], excludes *Streptococcus bovis* and *S. equinus*. In general the motile strains gave positive results in the Sherman tests except for 1 strain, ATCC 12755 which failed to grow at 45°. The nature of haemolysis, growth in presence of 0·04% (w/v) potassium tellurite, production of acid from sorbitol and L(+)-arabinose, and appearance of colonies on thallous acetate-tetrazolium agar are well-established tests used to differentiate *S. faecalis* from *S. faecium* and *S. durans* (Deibel, 1964). Sensitivity of the motile strains to potassium tellurite was intermediate between that of *S. faecalis* and that of *S. faecium* and *S. durans*, in agreement with the observation of Graudal (1957*b*). Like *S. faecium*, motile strains grew well in the presence of L(+)-arabinose, and marked acid production occurred in 24 hr. In contrast with *S. faecium*, many of the motile strains showed some production of acid in the presence of sorbitol, but generally the change in colour of the indicator was slower and less well-marked than in the case of *S. faecalis*. Strain NCTC 4725 was received as a strain of *S. faecalis*. It differed from typical *S. faecalis* in (i) appearance on thallous acetate-tetrazolium agar, (ii) fermentation of L(+)-arabinose and not of sorbitol, (iii) failure to utilize citrate or glycerol + fumarate as energy sources. The results in Table 2 show that this strain differed in several respects from typical *S. faecalis* and in common with other motile strains showed more similarity to *S. faecium* than to *S. faecalis* in these tests.

Table 2. *Biochemical reaction of motile enterococci compared with type strains of S. faecalis and S. faecium*

(Reactions of non-motile *S. faecium* type 38 are included for comparison with the motile type 38 strain.)

Motile strains	Serotype	'Sherman' tests*	Haemolysis on horse blood agar	Growth on blood agar + 0·04 % K. tellurite†	Appearance of colonies on thallous acetate-tetrazolium agar	Production of acid in medium containing: Sorbitol	L (+) Arabinose	Mannitol	Utilization of substrates as source of energy: Citrate Aerobic	Citrate Anaerobic	Gluconate Aerobic	Gluconate Anaerobic	Glycerol + fumarate Aerobic	Glycerol + fumarate Anaerobic
P 14/6	29	+	o or α	±	White to pale pink colonies‡	±	+	+	−	−	+	+	−	−
H/2/23	29	+	α	±	White to pale pink colonies	+	+	+	−	−	−	+	−	−
119	29	+	α	±		+	+	+	−	−	−	−	−	−
NCTC 4725	'4725'	+	α	+		±	+	+	−	−	+	+	−	−
ATCC 12819	'4725'	+	α	±		+	+	+	−	−	+	−	−	−
D 1000(b)	'4725'	+	α	−		+	+	+	−	−	−	+	−	−
D 1006(c)	'4725'	+	α	±		+	+	+	−	−	−	−	+	−
D 1010(c)	'4725'	+	α	±		+	+	+	−	−	−	+	−	−
ATCC 12817	Untyped	+	α	±		+	+	+	−	−	−	+	−	−
ATCC 12818	Untyped	+	α	±		+	+	+	−	−	−	+	−	−
ATCC 12755	Untyped	§	α	±		−	+	−	−	−	−	−	−	−
D 1003(b)	Untyped	+	α	±		+	+	+	−	−	−	+	−	−
ATCC 13638	38	+	α	±		±	+	+	−	−	−	+	−	−
Non-motile strains														
P 17/8	38	+	α	−		−	−	−	nt	nt	nt	nt	nt	nt
30/66	38	nt	α	−		−	−	−	nt	nt	nt	nt	nt	nt
Reactions of typical strains of:		(Results recorded as number of strains giving a positive reaction, in proportion to number of strains tested.)												
S. faecalis		+	o or β	+	Red colonies	$\frac{12}{13}$+	$\frac{0}{12}$+	$\frac{12}{12}$+	$\frac{0}{9}$+	$\frac{7}{9}$+	$\frac{5}{6}$+	$\frac{6}{6}$+	$\frac{0}{9}$+	$\frac{9}{9}$+
S. faecium		+	o or α	−	White to pale pink colonies	$\frac{2}{18}$+	$\frac{15}{18}$+	$\frac{15}{18}$+	$\frac{0}{8}$+	$\frac{0}{8}$+	$\frac{1}{9}$+	$\frac{1}{9}$+	$\frac{0}{8}$+	$\frac{0}{8}$+
S. durans		+	β	−		$\frac{0}{2}$+	$\frac{0}{2}$+	$\frac{0}{2}$+	nt	nt	nt	nt	nt	nt

* The term 'Sherman tests' is used to include the following: growth in presence of 40 % bile; growth at 10°; growth at 45°; growth in presence of 6·5 % NaCl; survival at 60° for 30 min.

† ± denotes production of small, greyish colonies, in contrast with good growth of black colonies of *S. faecalis*.

‡ Strain P 14/6 produced variant, red colonies.

§ Failed to grow at 45°.

nt Not tested.

Serological typing

Of the 13 motile strains, three belonged to *Streptococcus faecium* serotype 29 of which one, P 14/6, is the type strain. Extracts of the remaining 10 strains were tested against antisera which were available to 17 serological types of *S. faecium*; only one strain gave a reaction, ATCC 13638, which belonged to serotype 38 (type strain non-motile P 17/8). To confirm these results antisera were prepared to type strain P 14/6 (type 29, motile), type strain P 17/8 (type 38, non-motile), ATCC 13638 and also to the untyped strain, NCTC 4725. Precipitin reactions with these antisera are shown in Table 3.

Table 3. *Precipitin reactions of extracts of motile enterococci and of a non-motile type 38 strain of S. faecium*

	Antiserum to			
0·05 N-HCl saline extract of—	P 14/6 *S. faecium* type 29, type strain	P 17/8 (non-motile) *S. faecium* type 38, type strain	ATCC 13638	NCTC 4725
P 14/6	+	−	−	+
H/2/23	+	−	−	+
119	+	−	−	+
P 17/8 (non-motile)	−	+	+	−
ATCC 13638	−	+	+	−
NCTC 4725	−	−	−	+
ATCC 12819	−	−	−	+
D 1000(b)	−	−	−	+
D 1006(c)	−	−	−	+
D 1010(c)	−	−	−	+
ATCC 12817	−	−	−	−
ATCC 12818	−	−	−	−
ATCC 12755	−	−	−	−
D 1003(b)	−	−	−	−

In each case absorption of the antiserum with a heterologous reacting organism abolished the precipitin reaction with the homologous organism. Gel diffusion tests showed reactions of identity between precipitin lines of homologous and heterologous extracts reacting with antiserum to NCTC 4725. The term 'type 4725' will be used to describe organisms reacting with antiserum to NCTC 4725, but not with antiserum to P 14/6.

The extracts reacting with antiserum to NCTC 4725 included those of the *Streptococcus faecium* type 29 strains P 14/6, H/2/23 and 119. Antiserum to P 14/6, which produced a strong precipitin reaction with the homologous extract, gave no reaction with an extract of NCTC 4725 (Table 3). The precipitin line produced by extract of P 14/6 and antiserum to NCTC 4725 did not give a reaction of identity with the line produced by P 14/6 and the homologous antiserum (Fig. 1). This indicates that two antigenic groups were present in acid extracts of strain P 14/6, one of which reacted with antiserum to NCTC 4725 but failed to induce detectable antibody formation. The two antigens could be distinguished by immuno-electrophoresis of an acid extract of strain P 14/6. The antigen revealed by the homologous antiserum showed a lower

mobility than that revealed by antiserum to NCTC 4725 (Fig. 2). Immuno-electrophoresis of extracts of 'type 4725' strains revealed a single precipitin with a similar mobility in each extract under these conditions.

Gel diffusion tests showed that the precipitin line produced by *Streptococcus faecium* type 38 strain P 17/8, and by ATCC 13638 against antiserum to either of these strains gave a reaction of identity; by immuno-electrophoresis a single antigen with the same mobility was detected in extracts of each strain.

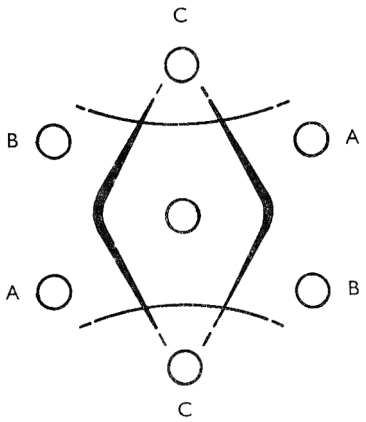

Fig. 1

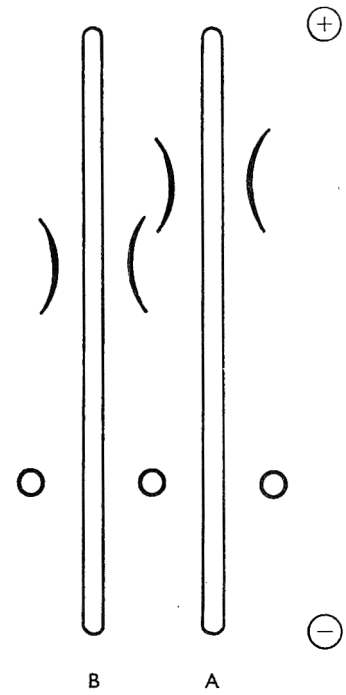

Fig. 2

Fig. 1. Diagram of gel diffusion test of extract of strain P 14/6 (*S. faecium* type 29, type strain, motile) against homologous antisera and against antiserum to strain NCTC 4725. Centre well contained 0·05 N-HCl extract of strain P 14/6. Outer wells A and B contained antisera, from two different rabbits, to strain P 14/6; C contained antiserum to strain NCTC 4725.

Fig. 2. Diagram of immuno-electrophoresis of extract of strain P 14/6. An 0·05 N-HCl extract of strain P 14/6 was placed in the three wells. After electrophoresis trench A was filled with antiserum to strain NCTC 4725, trench B was filled with antiserum to strain P 14/6.

The nature of the type antigens studied. The experiments relating to the chemical nature of the antigens were inconclusive. Treatment with 0·08 M-sodium periodate for 16 hr at 30° failed to destroy the antigens, although precipitin reactions of periodate-treated extracts tended to be weaker than those of control extracts. Treatment with 2 mg./ml. of trypsin for 3 hr at 37° failed to inhibit precipitin reactions.

The immunization procedure for serological typing of enterococci involves the use of formalin-killed suspensions. In the case of motile organisms this procedure may involve production of antibodies to flagella. During the production of acid extracts by the Lancefield method it is not known whether the serological reaction of the flagellar protein would be destroyed. The failure of trypsin to abolish the precipitin

reaction was inconclusive, since the flagellar protein of *Salmonella typhimurium* has been reported to be insensitive to trypsin and to other proteolytic enzymes (Stocker & Campbell, 1959). Cultures of strain P 14/6 (type 29) and of strain NCTC 4725 were harvested and the flagella removed before preparation of acid extracts for typing. The resulting extract of P 14/6 and a control extract of untreated organisms gave equally strong precipitin reactions with homologous antisera and with antiserum to NCTC 4725; in the case of strain NCTC 4725, an extract prepared after removal of flagella and a control extract also gave equally strong precipitin reactions. It is concluded that the antigens studied in acid extracts of these motile streptococci were not derived from the flagella.

Electrophoresis of soluble components of disrupted organisms

(i) *Esterases*

Preliminary experiments (Lund, 1965) showed the presence of esterase bands in extracts of motile strain P 14/6. The relative intensity of these bands was affected by the presence of buffer during cell disruption. In experiments described below organisms were suspended in tris-citrate buffer before disruption.

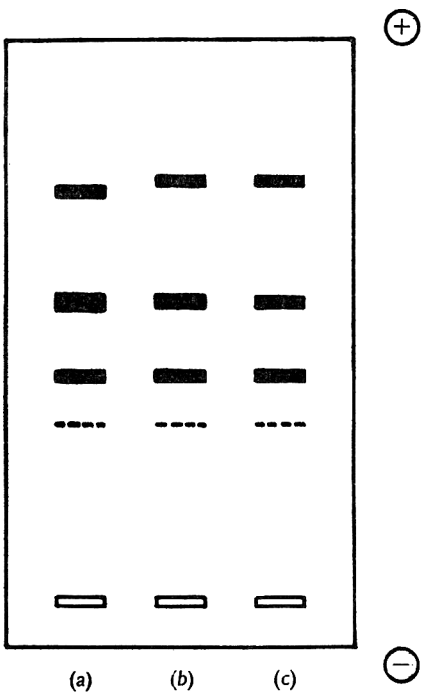

Fig. 3

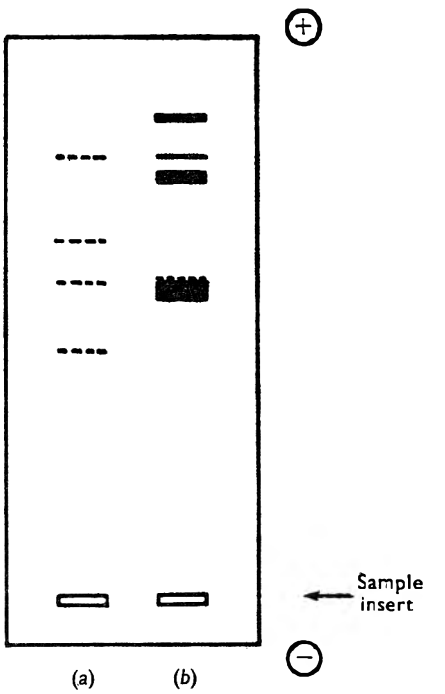

Fig. 4

Fig. 3. Diagram of esterases of motile, type 29 strains of *S. faecium* separated by electrophoresis on polyacrylamide gel. (*a*) = strain P 14/6 (*S. faecium*, type 29, type strain); (*b*) = strain H/2/23; (*c*) = strain 119.

Fig. 4. Diagram of esterases of a non-motile (type 38) strain of *S. faecium* and a motile (type 38) strain of streptococcus, studied by electrophoresis on polyacrylamide gel. (*a*) = strain P 17/8 (non-motile); (*b*) = strain ATCC 13638 (motile).

Type 29 strains. P14/6, H/2/23, 119, all motile, showed similar esterase patterns (Fig. 3).

Type 38 strains. Esterase bands of the motile strain ATCC 13638, and non-motile type 38 strain P17/8 (type strain), are illustrated in Fig. 4. The motile strain showed strong esterase bands, the non-motile strain showed weak bands with esterase activity and with different mobilities from those of the motile strain.

'*Type 4725*' *strains.* Esterase patterns are shown in Figs 5 and 6, inserts (*e*) to (*j*). Strains reacting with antiserum to NCTC 4725 gave a range of different esterase patterns, only strain D 1010 (*c*) gave a pattern identical to that of NCTC 4725.

Untyped strains gave esterase patterns shown in Fig. 5 and Pl. 1, fig. 1, inserts (*a*) to (*d*). Strains D 1003 (*b*) and ATCC 12755 gave esterase bands at similar positions.

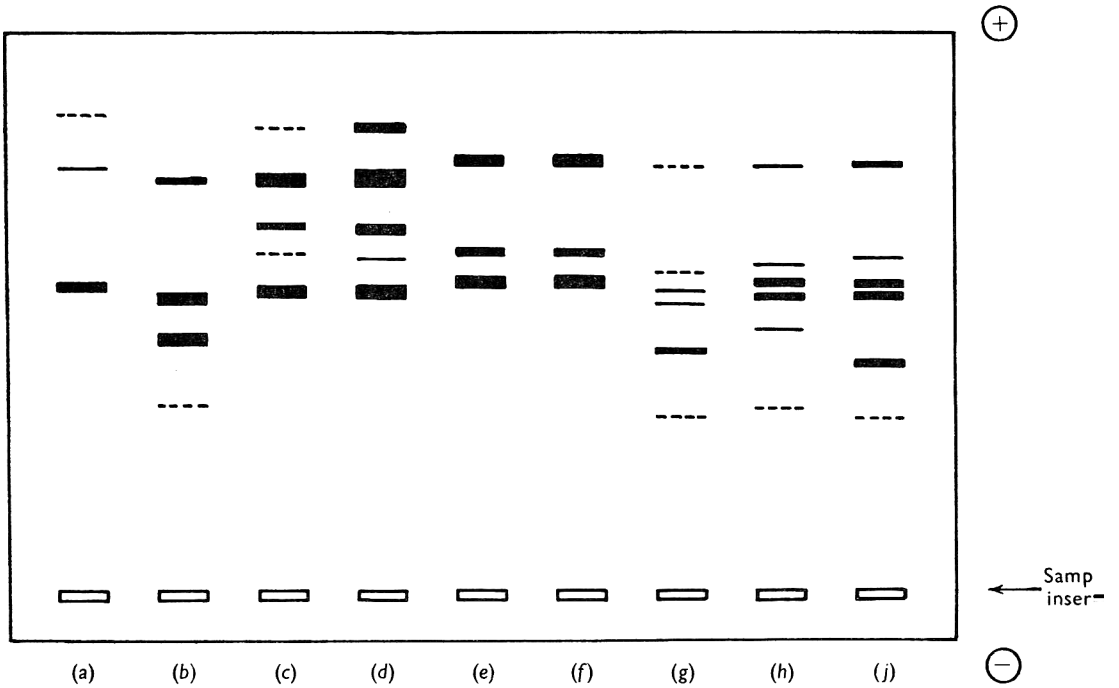

Fig. 5. Diagram of esterases of a range of motile group D streptococci, studied by electrophoresis on polyacrylamide gel. Inserts (*a*)–(*d*) contained extracts of untyped strains; inserts (*e*)–(*j*) contained extracts of strains reacting with antiserum to NCTC 4725.

(*a*) = strain ATCC 12817;
(*b*) = strain ATCC 12818;
(*c*) = strain D 1003 (b);
(*d*) = strain ATCC 12755;
(*e*) = strain NCTC 4725;
(*f*) = strain D 1010 (c);
(*g*) = strain ATCC 12819;
(*h*) = strain D 1000 (b);
(*j*) = strain D 1006 (c).

(ii) *Protein bands*

Initial experiments showed that the pattern of protein bands from a motile strain (P14/5) differed from that of other strains of *Streptococcus faecalis* and *S. faecium* (Lund, 1965). The major differences were most clearly seen in the case of bands with $\bar{E}_f$ values (Fowler, Coble, Cranmer & Brown, 1963) between 25 and 60 (approx.), the region indicated by the bracket in Pl. 1, fig. 2. No difference was found between the protein patterns of extracts prepared in water and those prepared in tris-citrate buffer.

Protein patterns of other motile strains are shown in Pl. 1, fig. 2. In general the motile strains resembled the original strain (P 14/6) in showing five clear bands with $\bar{E}_f$ values between 25 and 60. In one case (strain ATCC 12755) the forward band was relatively weak, in other cases there were slight differences in the position of these bands and some strains showed a sixth band in this region.

Type 38 strains. There was a clear difference between the protein patterns of motile and non-motile strains within this serotype; strain ATCC 13638 gave a pattern similar to that of the remaining motile strains, P 17/8 gave a pattern resembling that of other non-motile strains of *Streptococcus faecium* (Lund, 1965).

The effect of presence of flagella. It did not seem likely that subunits of flagellar protein contributed to the protein pattern since flagella tended to be broken off and separated from the cells during harvesting and washing. Deliberate removal of flagella, by a method similar to that described in the section on serological methods, before preparation of extracts of strain P 14/6 in the X-press, had no observable effect on the protein pattern obtained.

DISCUSSION

Although streptococci are generally considered to be non-motile, many workers have studied motile strains belonging to group D, isolated from a wide variety of sources (e.g. Pownall, 1935; Auerbach & Felsenfeld, 1948; Ødegaard & Gardborg, 1953; Liu, Lindberg & Mason, 1955; Hugh, 1959; Langston, Guttierez & Bouma, 1960; Courtieu, Le Tellier, Guillermet, Imbert & Longeray, 1964; de Saint Aubert, Dubouclard, Roumiantzeff & Vaugon, 1964). Cowan & Steel (1965) stated 'we confirmed Pownall's (1935) observation that many group D streptococci are motile'. The most extensive studies of motile enterococci appear to have been made by Graudal [1952, 1955, 1957*a*, *b*, Graudal & Birch-Anderson, 1958] who examined 129 strains, mainly isolated from human faeces, and concluded (1957*b*) that motile organisms constituted a special subgroup distinct from *Streptococcus glycerinaceus* (*S. faecalis*), *S. faecium* and *S. durans*. He further subdivided the motile organisms into a yellow pigmented group and a non-pigmented group. (In the case of strains of the pigmented group studied in this present work, slight pigment formation was observed in colonies grown on horse blood agar at 30° for several days.) Hugh (1959) studied six motile strains, including four of the ATCC strains used in this present work, and concluded that they were a variety of *S. faecalis*, without referring to the distinction between *S. faecalis* and *S. faecium*. He reported that the organisms fermented arabinose and not sorbitol, reactions generally characteristic of *S. faecium*.

In the present work several motile strains were found to produce acid when growing in the presence of sorbitol. The change in colour of the indicator was usually less marked than in the case of typical *Streptococcus faecalis* and possibly it would not be observed in slightly different reaction conditions. The results in Table 2 show that the motile strains differed in several respects from typical *S. faecalis* and showed a greater similarity to *S. faecium* in these tests.

Serology. Three of the motile strains had previously been described as *Streptococcus faecium* type 29 (Sharpe & Fewins, 1960; Barnes 1964). The remaining motile strains were tested against available antisera to 17 *S. faecium* types. Only one further strain gave a reaction (ATCC 13638, type 38); antisera were prepared to confirm these results, and antiserum to strain NCTC 4725 was prepared in order to elucidate

further the relationships between serotype and esterase pattern in this group of organisms.

In relating the serological studies of motile organisms to those of non-motile enterococci the nature of the antigens detected in motile bacteria by this typing technique should be considered. Elliott (1960) showed that type-specific antigens of three strains of *Streptococcus faecalis* and one of *S. durans* were located in the cell wall and were probably carbohydrate. The chemical nature of the carbohydrate type antigen of these and other strains of *S. faecalis* has been the subject of later publications (Bleiweis & Krause, 1965; Willers & Michel, 1966). Sharpe (1964) compared the serological type strains of *S. faecalis* from several workers and confirmed that the type antigen was derived from the cell wall. The inactivation of these antigens by periodate was taken as evidence of their carbohydrate nature. This also appeared to be true for the type antigens of many strains of *S. faecium* (Barnes, 1964), in most cases these antigens were destroyed by treatment with 0·01 M-potassium periodate. The antigens of motile streptococci studied by these techniques showed no evidence of inactivation by periodate, treatment of extracts with sodium periodate at a higher concentration and for a longer time than used by Sharpe (1964) or Barnes (1964) had very little effect on the subsequent precipitin reaction. Among the range of *S. faecium* strains studied by Barnes, several were reported to give type antigens resistant to periodate, these included strains of types 29 and 38, two of the three 'serotypes' studied in the present work.

The location of the antigen extracted from motile enterococci in these experiments has not been determined. Preliminary results indicate that it occurs at the cell surface since whole cells were used for immunization and for absorption of antisera. It is not flagellar since removal of flagella before acid extraction of strains P 14/6 and NCTC 4725 gave no detectable decrease in precipitin reaction. An aim of the present work was to relate the study of esterase patterns to the most widely used method of serotyping the enterococci; no study has been made of the H antigens. The work of Graudal (1957*a*) seems to indicate a surprising lack of variation in flagellar antigens of motile, enterococci. Using agglutination reactions to study 129 strains he reported a great number of serological O-types, but 128 strains constituted one H-type and 1 strain another H-type.

Esterases. The finding that motile strains show diverse patterns of esterase enzymes indicates that this technique may be a useful aid to characterization of these strains. The results with strains of type 38 illustrate the difference which may occur between motile and non-motile strains within the same serotype, the former (ATCC 13638) having strong esterase bands, the latter (strain P 17/8) having a different, much fainter esterase pattern. Motile strains within the same serotype may show different esterase patterns ('serotype 4725'); this contrasts with the results found for *Streptococcus faecalis* (Lund, 1965), where a similar esterase pattern was found in strains of *S. faecalis* and its varieties '*zymogenes*' and '*liquefaciens*', and in strains from five serotypes.

Protein patterns. The protein patterns, in contrast to those of the esterases, indicated some features which seem to be common to these motile strains. Despite the diverse esterase patterns observed it is possible that much of the enzyme protein of these organisms is very similar. The motile strain of serotype 38 showed a different protein pattern from the non-motile strain of this serotype.

Relationship of motile strains to Streptococcus faecalis and S. faecium. The protein patterns indicate that these motile strains form a group distinct from *S. faecalis* and *S. faecium*. The esterase patterns show a distinction from *S. faecalis*, strains of which give esterase patterns different from those of any of the motile strains, and from *S. faecium*, strains of which show much fainter esterase bands.

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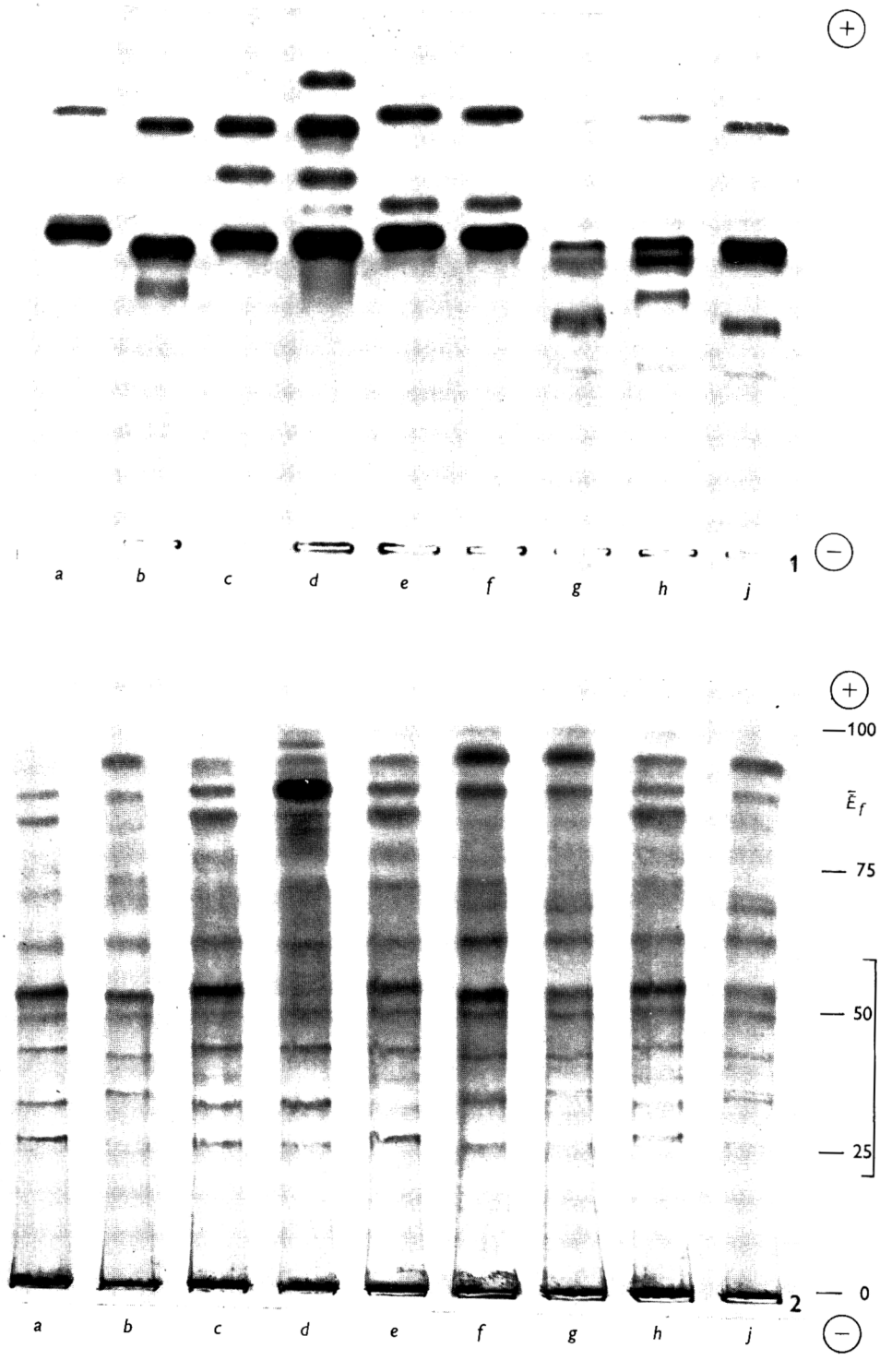
EXPLANATION OF PLATE

(Studies of soluble components of disintegrates of motile group D streptococci by electrophoresis on polyacrylamide gel.)

Fig. 1. Esterases of untyped motile streptococci (inserts *a–d*) and of strains reacting with antiserum to NCTC 4725 (inserts *e–j*). After electrophoresis the gel was incubated with α-naphthyl acetate and Fast Blue BB to detect esterase enzymes. Photograph of gel shown diagrammatically in Fig. 5 (approximately actual size).

Fig. 2. Protein bands of untyped motile streptococci (inserts *a–d*) and of strains reacting with antiserum to NCTC 4725 (inserts *e–j*). After electrophoresis the gel was stained with naphthalene black to detect protein bands. Inserts contained the same series of extracts as in Fig. 5 and Pl. 1, fig. 1 (approximately actual size).



(*Facing p.* 80)

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A Mutant of *Salmonella* Possessing Straight Flagella

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SUMMARY

A mutant of *Salmonella typhimurium* produced straight flagella in phase 2 (antigen-*1,2*) and normal flagella in phase 1 (antigen-*i*). The straight flagella were observed by light microscopy and electron microscopy either with or without formalin fixation. Flagellar bundles of the mutant bacteria prepared in 0·25 % methylcellulose (w/v) and examined by dark-field microscopy were also found to be straight. It was shown by electron microscopy that the component flagella of the straight flagellar bundle were in most instances irregularly twisted about each other. Heteromorphous bacteria which had straight flagella and either normal or mini-small-amplitude flagella were seen at a frequency of 10–13 % among the bacterial clones in phase 2. The bacteria with straight flagella were non-motile but they were sensitive to bacteriophage χ, which is known to infect motile bacteria of Salmonella species. In transduction, using phage P22 grown on a normal flagellar strain and the phase 2 straight strain as recipient, transductional clones with normal flagella in both phase 1 and phase 2 were obtained. The transductional clones showed the antigen of the recipient in phase 1 and that of the donor in phase 2. This indicated that the straight mutant originated by a mutation of the structural gene of phase 2 flagellin. In absorption-agglutination experiments with antisera prepared against flagella of either normal-*1,2* or straight-*1,2* no antigenic difference between normal and straight flagella could be detected.

INTRODUCTION

Bacterial flagella are spiral filaments functioning as locomotive organelles. Their wavelength and amplitude are characteristic of each bacterial strain, and various morphological types of flagella on bacteria isolated from nature (Leifson, 1960) have been described. Several types have been obtained as mutational variants of other flagellar shapes (Iino & Mitani, 1966). Among these morphological types, the extreme one which is uncoiled has been called 'straight'. Although it had been reported that certain strains of *Listeria monocytogenes* (Leifson & Palen, 1955) and *Arthrobacter citreus* (Leifson, 1960) possessed straight flagella, Leifson (1961) showed that the straight flagellar shape of these strains was due to the formalin fixation procedure used; when this was eliminated the flagella were spirally shaped. However, one exception was a strain of *Serratia indica* on which small-amplitude and straight flagella were seen in unfixed preparations, while normal flagella were observed in the formalin-fixed ones (Leifson, 1961). The present report deals with a new type of mutant of *Salmonella typhimurium* which possesses straight flagella, whether or not fixed by formalin.

METHODS

The Salmonella strain used in the present work was a mutant of *Salmonella typhimurium* TM2 (Lederberg & Iino, 1956). The strain is diphasic, showing *i*-antigen in phase 1 and *1,2*-antigen in phase 2. Mutant SJ770 was isolated from TM2 by screening on semi-solid medium (Enomoto & Iino, 1963) after *N*-methyl-*N*-nitro-*N*-nitrosoguanidine treatment following method no. 3 of Eisenstark, Eisenstark & van Sickle (1965).

Bacteriophage χ, which is able to infect motile salmonellas but not paralysed or non-flagellate ones (Meynell, 1961), was received from Dr B. A. D. Stocker (Department of Medical Microbiology, Stanford University, California, U.S.A.).

Bacterial motility was observed by phase-contrast microscopy using the hanging-drop technique. Clonal motility of bacteria was examined on plates of semi-solid medium containing 0·2 % (w/v) agar and 8 % (w/v) gelatin in broth (pH 7·2). For antiserum selection, semi-solid medium was supplemented with anti-H-serum, with an agglutination titre of 1/5000, to a final concentration of 0·2 % (v/v).

The flagella were stained by the method of Leifson (1951). Dark-field microscopy and the general procedures of electron microscopy used were as described previously (Mitani & Iino, 1965; Iino & Mitani, 1966).

To decrease drying artifacts which may distort and break flagella, a method for the electron microscopy of mutant flagella was applied (suggested by D. Lang), incorporating the sedimentation of bacteria by gravity on an interface between the bacterial suspension containing cytochrome *c* and a dense inert fluorochemical, and subsequent adsorption. Bacteria were grown in Bacto-antibiotic medium 3 (BA-3; Difco Lab., Detroit, Michigan, U.S.A.) overnight at 37° without shaking, and 0·1 ml. of the bacterial culture inoculated into 5 ml. new BA-3 medium. After incubation for 3 hr at 37°, the bacteria were harvested by centrifugation at 1200 *g* for 10 min. and resuspended in 0·01 % cytochrome-*c* solution (pH 6·1) containing 0·25 % methylcellulose. This bacterial suspension was incubated up to 20 hr at 2–3°. One ml. of the bacteria + cytochrome-*c* mixture was added to a vessel (15 mm. in diameter × 50 mm. long) containing 0·7 ml. of Fluorochemical FC-42 (sp.gr. 1·87 g./cm^3; surface tension (air) 16 dynes/cm.; Minnesota Mining and Manufacturing Co., Saint Paul, Minnesota, U.S.A.). After 5 min. the bacteria at the interphase were picked up by platinum grids (Siemens type) and fixed by the vapour of 2 % OsO_4 for 30 min., unless otherwise stated. Shadowing was made with platinum at an angle of 15°. The grids were examined in a Siemens Elmiskop *1A* at 80 kV.

Transduction was done with phage P22 grown on the indicated host cultures. The general procedures of cultivation and transduction were according to the methods of Stocker, Zinder & Lederberg (1953) and Lederberg & Iino (1956).

Specific flagellar antisera were prepared according to Edwards & Ewing (1955). Antigen type was identified qualitatively by slide agglutination tests with antisera having a titre of 1/100. Quantitative measurements were made by tube agglutination tests (Kauffmann, 1954).

RESULTS

Motility of the mutant

When organisms from a broth culture of *Salmonella typhimurium* TM2, mutant SJ770, were spread on semi-solid media, both compact colonies and swarms appeared (Pl. 1, fig. 1). By the light microscope in a hanging-drop preparation, the bacteria from compact colonies were seen to be non-motile, while those from swarms moved translationally. Upon subcultivation both compact colonies and swarms segregated out motile bacteria and non-motile bacteria repeatedly. The frequency of the interchanges between the two types was of the order of 10^{-4}/bacterium/division. Examination of the flagellar antigen of these subclones showed that the change in motility corresponded exactly with the antigenic phase i.e. motile subclones were always in phase 1 (antigen-*i*) and non-motile subclones were always in phase 2 (antigen-*1,2*).

Light microscopy of the flagella of mutant SJ770

Phase 1 and phase 2 broth cultures of mutant SJ770 were treated separately with methylcellulose (0·25 %, w/v) for 4 hr at 23° and observed by dark-field microscopy. In phase 1 cultures, the flagellar bundles appeared to be fuzzy straight tails on the actively moving bacteria, and to be spirally shaped with normal wavelength and amplitude in the slowly moving or stationary bacteria (Pl. 1, fig. 2). Flagellar bundles in phase 2 culture were straight and not regularly coiled at all (Pl. 1, fig. 3). However, these differed from the fuzzy straight tails observed in rapidly moving normal bacteria in resembling stiff rods and in being found on the stationary bacteria. The fraction of the bacteria with visible flagellar bundles increased as the time of incubation was prolonged. Comparable results were obtained in a study of stained preparations. Phase 1 bacteria stained by Leifson's method showed only normal flagella whether the organisms were untreated or fixed with formalin (5 %, v/v in broth; Pl. 1, figs 4, 5). On the other hand, stained preparations of phase 2 bacteria showed straight flagella, whether or not the bacteria were fixed before staining by formalin (Pl. 1, figs 6, 7).

Electron microscopy of straight flagella

Phase 2 organisms of mutant SJ770 were prepared, both with and without methylcellulose, and examined in the electron microscope. Dispersed straight flagella were seen in the preparation without methylcellulose (Pl. 1, fig. 8). The average flagellar number per bacterium was $7·2 \pm 0·7$, determined by examination of about 170 individual bacteria. By extensive observation of the flagellated bacteria, it was found that 10–13 % were heteromorphous with regard to their flagellar morphology. These heteromorphous bacteria, besides straight flagella, possessed either normal or 'mini-small-amplitude' flagella. The term mini-small-amplitude is proposed to describe a new flagellar shape in which the wavelength and amplitude are 0·78–1·5 μ and 0·08–0·1 μ, respectively. The overall shape of mini-small-amplitude flagella is analogous to that of the small-amplitude type (Iino & Mitani, 1966), except that the wavelength is approximately one-third of that of the small-amplitude type. Within a clone, heteromorphous bacteria which carried both straight and normal or straight and mini-small-amplitude flagella were usually observed at the same frequency. The number of normal or mini-small-amplitude flagella in these clones was less than three per bacterium

(Pl 2, fig. 9). Less frequently (in about 2 % of the organisms), heteromorphous bacteria which carried straight, normal and mini-small-amplitude flagella were seen. Furthermore, at a frequency of about 1 in 500, single flagella were observed which were heteromorphous, themselves possessing either straight and mini-small-amplitude or straight and normal regions.

Flagellar shape was observed after 3 and 20 hr of treatment of the bacteria with 0·25 % methylcellulose. In the former preparation, flagellar bundles were hardly seen, while in the latter 94·7 % of the bacteria formed flagellar bundles. The overall shape of the bundle was not helical but straight, comparable to the observations by dark-field microscopy. The component flagella of the bundle were irregularly twisted at different intervals in bundle to bundle (Pl. 2, figs 10, 11).

Stability of the mutant SJ770

Because of the immobility of bacteria with straight flagella, revertants which recovered motility in phase 2 could be selected from the bacterial population of mutant SJ770 as swarms on semisolid agar plates containing phase 1 antiserum. When $1{\cdot}5 \times 10^7$ bacteria from a single colony of mutant SJ770 in phase 2 were spread on semisolid agar and incubated at 37° for 48 hr, no swarms were detected in 20 independent experiments. When the innoculum was increased to 5×10^8 bacteria, one to five swarms were detected in each experiment. Fifteen such swarms, selected in independent experiments, were transferred to broth and the motility and flagellar shape of the component bacteria were examined. It was found that these reverted clones consisted of actively motile bacteria with flagella of normal wavelength and amplitude.

Genetic analysis of the straight flagellar mutant

Transduction was carried out using a phase 1 culture of mutant SJ770 (normal-*i*: straight-*1,2*) as recipient and phage prepared on a phase 2 culture of Salmonella

Table 1. *Transduction of a phase 2 culture of Salmonella strain SL688 (b:e,n,x) with a phase 1 culture of S. typhimurium SJ770 (i: straight-1,2)*

Recombinants were selected on plates of semisolid agar medium containing anti-*i* serum. Donor: 3×10^9 phage particles; recipient: 1×10^9 bacteria.

	Flagellar antigens		Flagellar shape in		No. of clones
	Phase 1	Phase 2	Phase 1	Phase 2	
Donor	*b*	*e,n,x*	Normal	Normal	—
Recipient	*i*	*1,2*	Normal	Straight	—
Recombinants	*i*	*e,n,x*	Normal	Normal	31
	b	*1,2*	Normal	Straight	36

strain SL683, which produces normal flagella in both phase 1 (antigen-*b*) and phase 2 (antigen-*e,n,x*) (Iino & Enomoto, 1966). The use of phase 1 bacteria as recipient has two advantages: first, both phase 1 and phase 2 antigen type recombinants should be detectable, and secondly, even if genotypically normal-*1,2* type clones appear by reversion, the *1,2* antigens will not be expressed unless phase variation from phase 1 to phase 2 occurs simultaneously (Lederberg & Iino, 1956).

A mixture of 3×10^9 phage particles propagated from the donor and 1×10^9 recipient bacteria were spread on semisolid agar plates containing anti-*i* serum. Transductional

clones identified as swarms on the plates were isolated, and their antigen type and the shape of their flagella examined. The results are listed in Table 1. Among 67 clones examined, 36 possessed the phase 1 antigen of the donor *b*, but in phase 2 they showed the recipient type antigen, *1,2*. Their flagellar shapes were normal in phase 1 and straight in phase 2. The remaining 31 clones possessed the phase 2 antigen of the donor, *e,n,x*, and the phase 1 antigen of the recipient, *i*. Their flagellar shapes were normal in both phase 1 and phase 2. These results indicate that the straight flagellar character in phase 2 of mutant SJ770 is replaced by the normal when the phase 2 antigen type determinant of the normal strain is transduced into the straight flagellar mutant.

To examine the possibility of the rare occurrence of normal-*1,2* type recombinants, the transduction was repeated on a larger scale on semisolid agar plates containing *b*, *i*, and *e,n,x* antisera. A transduction mixture containing 3×10^{10} phage particles propagated in the donor and 1×10^{10} recipient bacteria was spread on the plates. In this experiment, in which approximately 300 normal-*e,n,x* recombinants per plate were anticipated, the appearance of swarms, which would indicate the occurrence of normal-*1,2* type recombinants, was not detected. Thus, as far as has been tested, the mutant site of the straight-flagella character was inseparable from the determinant of phase 2 flagellar antigen by recombination.

Flagellar antigens of the mutant

The H-antigen of the straight flagellar mutant was identified as *1,2* by slide-agglutination tests. For detailed comparison of the H-antigen of normal and straight flagella, anti-*1,2* sera were obtained against *Salmonella typhimurium* TM2 and its straight flagellar mutant SJ770. The agglutination titres of the anti-*1,2* sera determined with both *1,2* type bacteria were examined before and after reciprocal absorption. The titres of the antisera before absorption were 2^{14} in both antisera. After cross-absorption, no agglutination was observable over the range of dilutions of $1/2^3$ to $1/2^{15}$. Therefore, the *1,2* antigens of TM2 and its straight flagellar mutant SJ770 show identity in the absorption-agglutination test.

Sensitivity of the straight flagellar mutant to bacteriophage χ

A χ-phage suspension propagated on a broth culture of *Salmonella typhimurium* TM2 and having a titre of 10^8 particles/ml. was diluted sequentially tenfold down to

Table 2. *Efficiency of plating of bacteriophage χ on normal, straight and paralysed flagellar strains of Salmonella typhimurium*

S. typhimurium strain	H-antigen type	Flagellar character	EOP*	SD†
TM2	*1,2*	Normal	1·00	—
SJ770	*i*	Normal	0·93±	0·09
SJ770	*1,2*	Straight	0·95±	0·10
SJ60	*1,2*	Paralysed	0·00	—

* EOP = efficiency of plating: values on the normal-*1,2* were taken as 1·00.
† SD = standard deviation.

10^2 particles/ml. Each of the dilutions was mixed with an equal volume of a broth culture (10^7 bacteria/ml.) of TM2-normal-*1,2*, SJ770-normal-*i*, SJ770-straight-*1,2* or SJ60-*1,2*, a paralysed mutant (*mot*$^-$) of TM2 (Enomoto, 1966) and 0·1 ml. of each mixture

was spread on a 0·6% (w/v) nutrient agar plate. After incubation for 10 hr at 37°, the number and morphology of the developed plaques were examined. In three independent experiments, plaques were not detected at all on the plates of SJ60. However, when the phages were plated on TM2-normal-*1,2*, SJ770-normal-*i* and SJ770-straight-*1,2* the plaques appeared with 100% efficiency in all three strains (Table 2). The size and morphology of the plaques were also indistinguishable among the three: they were clear and circular, having a diameter of 0·5–1·5 mm.

DISCUSSION

The straight mutant flagella described here differ from those reported previously (Leifson & Palen, 1955; Leifson, 1960, 1961) in that they are straight whether or not they have undergone formalin fixation. Dark-field microscopy and electron microscopy provided additional support for the idea that the straight flagella character observed by light microscopy of the stained materials is not an artifact caused by stretching of the spiral but is an intrinsic property of the mutant.

One characteristic of the mutant is that the straight flagella appear only in phase 2. Transductional analysis further demonstrated that the mutant site of the straight flagella is inseparable by recombination from the phase 2 antigen determinant. From the accumulated data (Iino, 1962; McDonough, 1965) it appears that the specificity of the flagellar antigen in each phase is determined by the structural gene of flagellin, the component protein of flagella, i.e. *H1* in phase *1* and *H2* in phase 2. Other flagellar shape mutants which have been studied in Salmonella were all found to express their mutant characters only in phase 1 or phase 2, and their mutant sites were located in the structural gene of flagellin of the corresponding phase (Iino, 1962; Iino & Mitani, 1966).

Differences in the tryptic peptide maps of flagellin between the flagellin of a curly flagellar mutant and that of the parental wild-type strain have been found (Enomoto & Iino, 1966). Although chemical analysis has not been done with the flagellin on the straight flagella, it is reasonable to infer that the straight flagellar characteristic is due to mutation of the structural gene of phase 2 flagellin, resulting in a change in the primary structure of the flagellin of the mutant. This difference in primary structure is manifested in the conformation of the flagellin molecule itself and in the mode of polymerization of the flagellins to flagellar fibres. The immunological identity of the H-antigens of the normal strain and the flagellar shape mutants, and the absence of immunological cross-reactions between flagella with identical shapes indicate that the determinants of the H-antigen and of the characteristic flagellar shape are present at different sites in the flagellin molecule.

A characteristic feature of the straight flagellar mutant is the heteromorphism of flagella on a single bacterium, i.e. one organism with normal or mini-small-amplitude flagella and straight ones in a phase 2 clone. The frequency of this heteromorphism varied from clone to clone in the range of 10–13%. Moreover, bacteria possessing only mini-small-amplitude or normal flagella were not detected. Mini-small-amplitude flagella cannot be defined as a particular shape because some intermediate flagella from straight to more or less mini-small-amplitude were seen. It is very difficult to determine whether the straight mutant produces mini-small-amplitude flagella together with straight or irregular ones, because of possible artifacts appearing during

preparation for electron microscopy. Bacteria with only normal flagella were seen only one-tenth as often as straight-normal heteromorphous bacteria; the former are presumed to occur by phase variation from phase 2 to phase 1 (Lederberg & Iino, 1956). The frequent appearance of heteromorphous bacteria observed in phase 2 clones of mutant SJ770 is hardly attributable to flagellar phase variation or mutation. Together with the flagellar heteromorphism observed in the heteromorphous mutant in *Salmonella abortus-equi* (Iino & Mitani, 1966), the phenomenon offers an interesting problem concerning the morphological differentiation of cellular organelles. A straight flagellar strain of *Serratia indica* reported by Leifson (1961) is also heteromorphous: it produces both straight and small-amplitude flagella.

Changes in the spiral shape of flagella by mutation are very often associated with changes in the mode of cellular movement (Iino & Mitani, 1966). This is most dramatic in the straight flagellar mutant; in liquid medium the mutant bacteria are completely non-motile. The formation of a flagellar bundle during methylcellulose treatment does not effectively restore mobility to the mutant bacterium. This indicates that the spiral structure of flagella is essential for the effective movement of the flagellated bacteria.

Bacteriophage χ has been known to be able to infect salmonellas when the bacteria are flagellated and motile. It cannot infect paralysed strains which harbour mutations at the *mot* loci even though they do have flagella structurally indistinguishable from those of the parental motile strain (Meynell, 1961). Although the straight flagellar bacteria are non-motile in liquid media, their response to χ-phage is quite different from the paralysed mutant bacteria in that they have retained the same degree of sensitivity to χ-phage as normal bacteria. This may be explained by assuming that cellular locomotion *per se* is not a prerequisite for infection by χ-phage but rather that the presence of motile flagella is. Thus, in the straight flagellar mutants, the flagella *per se* may have retained motility but, because of loss of the spiral structure, this movement may not be capable of effecting locomotion of the bacterial bodies.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. A broth culture of *Salmonella typhimurium* mutant SJ770 was diluted and about 400 organisms/0·1 ml. were spread on a semisolid agar and incubated for 10 hr at 37°. Swarms and compact colonies appeared on the plate. Swarms correspond to phase 2 clones and compact colonies to phase 1. × 0·6.

Figs 2, 3. Dark-field micrographs of the organisms of mutant SJ770. In fig. 2 a normal flagellar bundle of a phase 1 organism and in fig. 3 straight flagellar bundle of a phase 2 organism are seen. × 4000.

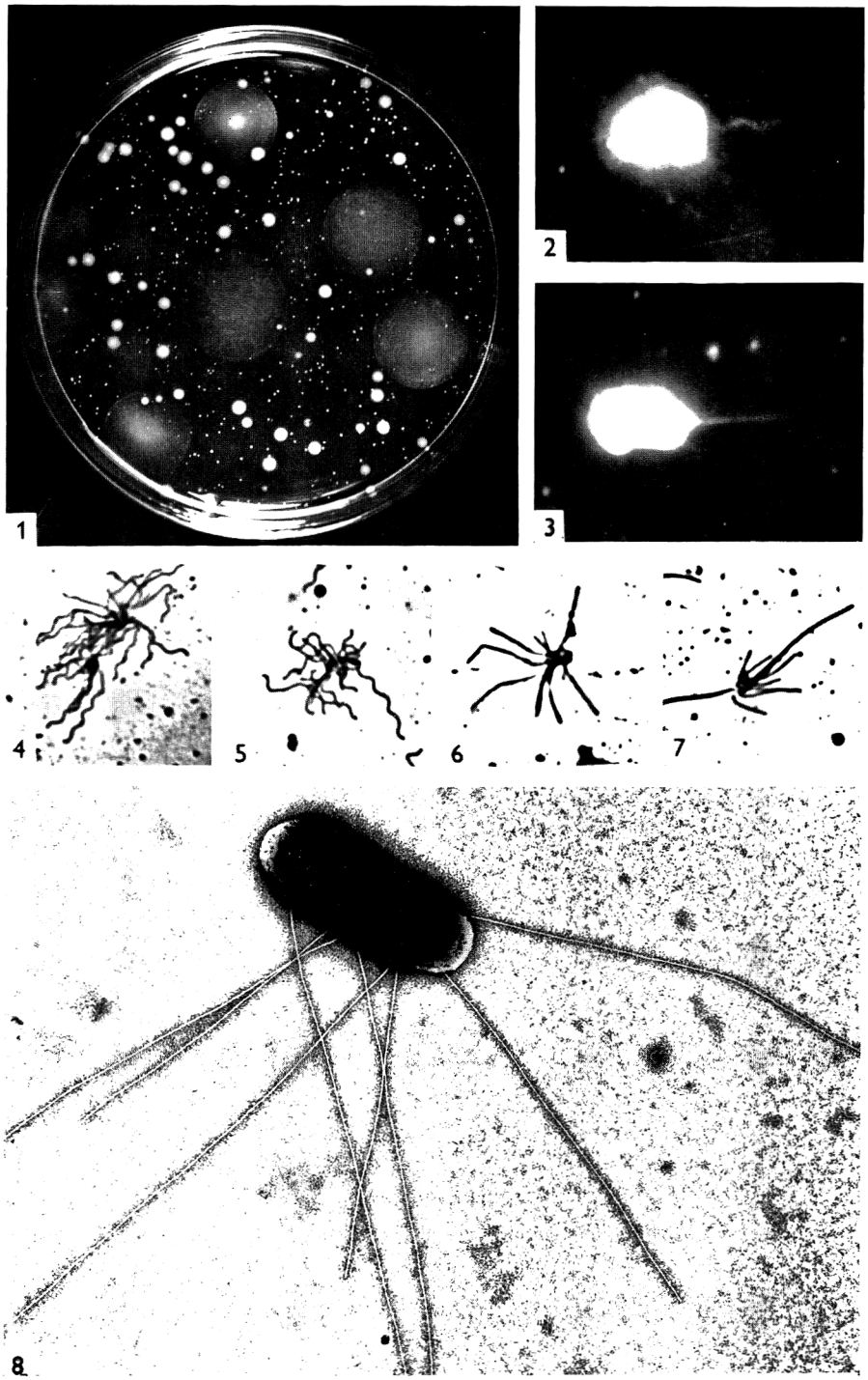
Figs 4–7. Organisms of mutant SJ770 whose flagella were stained by Leifson's method. In figs 4, 5 are shown phase 1 organisms with normal flagella, and figs 6, 7 phase 2 organisms with straight flagella. Figs 4, 6 are unfixed preparations, and figs 5, 7 are formalin-fixed × 1000.

Fig. 8. Electron micrographs of a phase 2 organism of mutant SJ770. Organisms were negatively stained with 1% bovine serum albumin (pH 7·3). × 10,400.

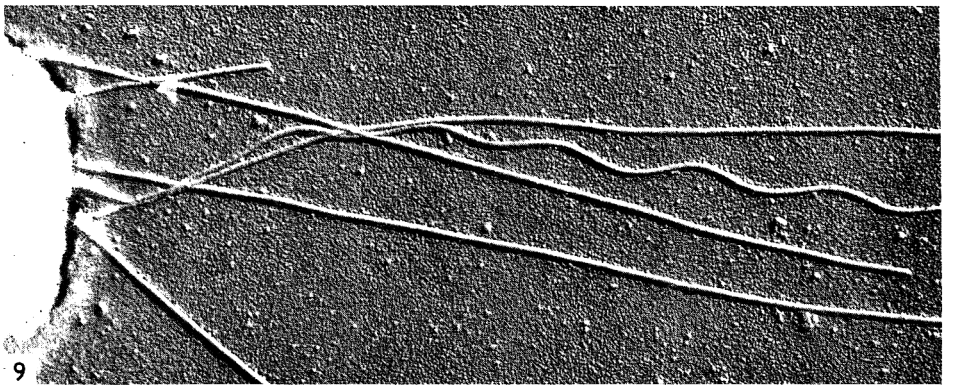
PLATE 2

Fig. 9. Electron micrograph of a heteromorphous organism present in phase 2 of mutant SJ770 prepared by shadowing. × 13,000. Straight flagella and a typical mini-small-amplitude flagellum are seen attached to single organism.

Figs 10, 11. Electron micrographs of phase 2 organisms of mutant SJ770 incubated with 0·25% methylcellulose for 20 hr at 2°–3° and prepared by shadowing. The figures show bundles of straight flagella which are irregularly twisted together at different intervals.



(*Facing p.* 88)



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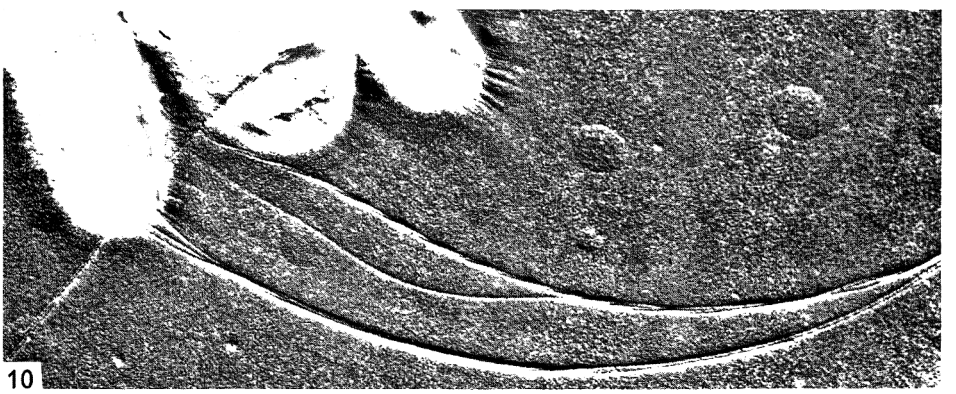

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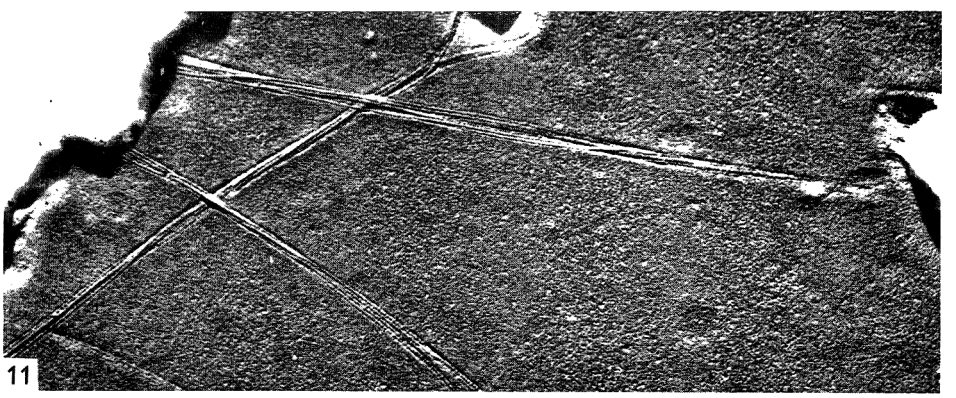

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Specific Piliation Directed by a Fertility Factor and a Resistance Factor of *Escherichia coli*

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SUMMARY

Production of pili directed either by the sex factor F or by the drug-resistance factor R 100 in *Escherichia coli* K 12 is regulated by a gene which acts by producing a repressor, and the mutant R 100–1 no longer produces this repressor. The specific pilus determined by R 100–1 resembled the F pilus morphologically, but differed in its affinity for F-specific RNA phages. Mutants of F and R 100–1 which had lost the ability to synthesize pili could each restore to the other the ability to produce pili on mixed infection of the same host.

INTRODUCTION

Filamentous appendages called pili (Brinton, 1965) or fimbriae (Duguid, Anderson & Campbell, 1966) are produced by most of the enterobacteriaceae under the control of chromosomal genes (Maccacaro, Colombo & Nardo, 1959; Brinton, Gemski, Falkow & Baron, 1961). However, a special kind of pilus is determined by the sex factor, F, which can be recognized because it specifically adsorbs the spherical particles of certain RNA phages, e.g. R 17 (Crawford & Gesteland, 1964), M 12 (Brinton, Gemski & Carnahan, 1964) and f 2 (Valentine & Strand, 1965). Brinton (1965) has suggested that the F pilus acts as the conjugation tube through which the chromosome of the male cell is transferred into female (F^-) bacteria and whether or not this is the case, the presence of F pili is certainly essential to conjugation mediated by F.

Other extrachromosomal genetic elements such as Resistance Factors (R factors) responsible for contagious resistance to antibiotics in enteric bacteria (Ochiai, Yamanaka, Kimura & Sawada, 1959; Akiba, Koyama, Isshiki, Kimura & Fukushima, 1960) confer on F^- bacteria the ability to conjugate, as well as to transfer chromosomal genes (Sugino & Hirota, 1962). To make it possible to observe the formation of specific pili, and thus to clarify the genetic relationship between pili and ability to conjugate, a strain of *Escherichia coli* K 12 was used which had none of the ordinary surface appendages like flagella or chromosomally determined pili. Certain R factors, termed i^+, co-ordinately inhibit fertility, susceptibility to F specific phages and

formation of the specific f+ antigen (Hirota, Nishimura, Ørskov & Ørskov, 1964) in F+ bacteria, but mutants, designated *i*⁻, have been isolated which lack this suppressive effect on F (Egawa & Hirota, 1962). As shown by Hirota, Fujii & Nishimura (1966), the *i*⁻ mutant R100-1, of one R factor, R100, can also restore full F function to bacteria carrying defective mutants of the F factor such as have been described by Cuzin (1962) and Nishimura (1964). No specific pili were present on F+ or F⁻ bacteria when they carried R100, but pili which adsorbed F specific phage could be seen on F+ bacteria, normal or defective, carrying R100-1. The *i*⁻ mutant, R100-1, in F⁻ bacteria brought about conjugation and gave chromosomal recombinants almost as frequently as F itself, suggesting that conjugation is regulated with R100 and that mutation in the *i* gene of the R factor derepresses its own conjugation function as well as that of F. Moreover, specific pili could now be seen on F⁻(R100-1) bacteria. Hirota *et al.* (1964) were able to show a serological relationship between an antigen determined by R100-1 and the F antigen, for an antiserum prepared against R100-1 in an F⁻ culture specifically agglutinated F+ bacteria. Meynell & Datta (1966) have reported that in a series of R factors of independent origin, those that inhibited the functions of F themselves determined the production of a pilus sufficiently like the F pilus to adsorb F specific RNA phage. Experiments in Osaka demonstrated the presence of a pilus on F⁻(R100-1) bacteria, but no adsorption of phage could be detected. In view of the discrepancy between this result and the conclusion to be drawn from Hirota *et al.* (1964) and Meynell & Datta (1966), it was decided to re-examine the pili determined by R100-1 for adsorption of F specific phage.

METHODS

In Osaka

Bacteria. Escherichia coli K12 strain JE2217, used as host for F and R in most of the experiments, had lost type I pilli and flagella by two independent mutations, being a recombinant resulting from a cross between W2802, a non-flagellate (*fla*⁻) mutant of Hfr Cavalli (Cavalli-Sforza, 1950), kindly provided by Dr J. Lederberg, Stanford University, California, and JE346 which is a *pil*⁻ mutant of JE344 unable to produce chromosomally determined pili. Strain JE344 was an F⁻ derivative obtained by acridine treatment of JE343 (Hirota, 1960). Inability to swarm on semi-solid agar (Stocker, Zinder & Lederberg, 1953) was taken to indicate absence of flagella, and failure to haemagglutinate chick red cells absence of type I pili (Duguid, Smith, Dempster & Edmunds, 1955). The absence of both flagella and type I pili in strain JE2217 was also confirmed by electron microscopy. Strain JE2442, a non-lactose fermenting (*lac*⁻) recombinant resulting from a cross between JE2217 and JE1428, Hfr Cavalli *lac-52*, was used as host for temperature-sensitive F-*lacT62*.

Salmonella paratyphi B carrying F13 was kindly provided by Dr S. Iseki, Gunma University, Maebashi.

F factors. F8 and F13 have been described by Hirota & Sneath (1961). The temperature-sensitive mutant, F-*lacT62* (Jacob, Brenner & Cuzin, 1963), was kindly provided by Dr F. Cuzin, Institut Pasteur, Paris. Infertile mutants of F8 were obtained by growing JE2217 (F8) in broth containing 10^{13} particles/ml. of phage M12 and selecting phage-resistant bacteria. One such mutant, F8-D15, *def-15*, was examined, together with mutants similarly obtained from F-*lacT62*D.

R factors. R 100 is an R factor conferring resistance to streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc) and sulphonamide (Su), which appeared in a strain of *Shigella flexneri* 2 b 222 isolated by Dr R. Nakaya and which has been observed to confer a low degree of fertility on F^- bacteria (Sugino & Hirota, 1962). Its i^- mutant R 100–1 i^- was obtained by selecting, from cultures of Hfr Cavalli carrying R 100, clones which retained the high fertility of the Hfr (Egawa & Hirota, 1962; Sugino & Hirota, 1962). R 100–31, i^-, *def*–31 and R 100–70, i^-, *def*–70 are mutants of R 100–1 i^- which have lost their infectivity, and thus their ability to bring about conjugation.

A hybrid factor resulting from recombination between F 13 and an incomplete R factor, R 23 (Harada, Kameda, Suzuki & Mitsuhashi, 1964), was obtained from Dr K. Harada, Gunma University.

F specific phage. Phage M 12 (Hofschneider, 1963) was kindly provided by Dr P. H. Hofschneider, Max-Planck Institut, München, Germany. Phage sensitivity was tested by the cross-brushing method (Lederberg, 1947), where a loopful of bacterial culture is streaked across a strip of nutrient agar plate previously inoculated with a loopful of phage.

Media. L-broth (Lennox, 1955) and Difco Brain Heart Infusion (BHI) broth were used, and the solid medium was BHI agar.

Electron microscopy. Bacterial cultures in L-broth were diluted 10-fold in fresh medium and grown for 3 hr at 37° without shaking. To test for adsorption of phage M 12, the phage was added at multiplicities ranging from 10 to 100, together with $5{\cdot}3 \times 10^{-3}$ M-$CaCl_2$ (Loeb & Zinder, 1961) and the mixture was incubated for 10 min. before transferring a sample to the grid. To examine detached pili, confluent growth on BHI agar was harvested in Davis minimal medium (Davis & Mingioli, 1950), minus glucose, sedimented by low-speed centrifugation, and the supernatant examined. For shadowcast preparations, duplicate specimens for the electron microscope were prepared by placing drops of suspension on formvar-coated grids, and drawing off the excess. The grids were then rinsed twice with distilled water and allowed to dry in air, and then shadowcast with platinum-palladium alloy. Negative staining (Brenner & Horne, 1959) was carried out by mixing samples of suspension with phosphotungstate before transferring them to the grids.

In London

Bacteria. Escherichia coli K 12 strains JE 254 and JE 255, which carried R 100 and R 100–1 respectively, were provided by Dr Y. Hirota. These bacteria were R^+ derivatives of strain W 4354 *met*$^-$, which is 58$^-$161 cured of F by acridine treatment (Hirota, 1960). Other bacterial strains, all lines of *E. coli* K 12, were J 5–3 *pro*$^-$*met*$^-$, an acridine-cured derivative of an F^+ strain, and RC 12 *thr*$^-$*leu*$^-$*thi*, which came from the F^- strain W 1177. These strains are fully described in Meynell & Datta (1966). Strains 58–161 F^+ and HfrH were used as indicators for the phages.

F specific phages. Three RNA phages, MS 2 (Davis, Strauss & Sinsheimer, 1961), μ 2 (Dettori, Maccacaro & Piccinin, 1961) and f 2 (Loeb & Zinder, 1961), were tested.

Sensitivity of a bacterial strain to lysis was tested both by spotting a drop of high-titre phage preparation on the surface of a nutrient agar plate spread with a loopful of broth culture, and by assaying dilutions of the phage preparation for plaques in a soft agar overlay. The presence of any phage-sensitive bacteria in cultures was detected by incubating mixtures of bacteria with excess phage, and plating for infective centres

(phage-infected bacteria) on the sensitive indicator strain, HfrH, after passing the mixture through antiphage serum to inactivate residual free phage. The media and experimental techniques are given fully in Datta, Lawn & Meynell (1966).

RESULTS

In Osaka

Strains JE2217 and JE2443, which produced neither flagella nor type I pili, were examined in the electron microscope after infection with the different F and R factors.

The results are shown in Table 1 and Plates 1 and 2, and can be summarized as follows.

Specific piliation directed by the F factor. F^-R^- bacteria showed no pili (Table 1, lines 1 and 2; Pl. 1, fig. 1). Infection with F8 led to the formation of a few pili per bacterium, to which the F specific phage M 12 could attach (Table 1, lines 3 and 4; Pl. 1, figs 2, 3 and Pl. 2, fig. 4). These pili were generally longer than type I pili, and some measured 20μ or more. Addition of anti-f^+ serum to the cultures causes agglutination of the pili; this is a specific effect of the antibody and does not occur with normal serum, showing that the F pilus constitutes at least part of the specific f^+ antigen (Ishibashi, 1967).

Salmonella paratyphi B carrying F 13 could be seen to produce similar pili.

Detached pili in the supernatant of centrifuged broth cultures showed an outer diameter of about 100 Å and were often aggregated. They were still capable of adsorbing particles of phage M 12, and the protein coats of the attached phage particles were empty, in contrast to what has been reported for phage f2 (Valentine & Strand, 1965).

Inhibition of F piliation by R factors. The wild type i^+ R factor, R 100, co-ordinately inhibits the functions of F, so that an F^+(R 100) culture loses its sensitivity to lysis by F specific phage and agglutinability by F specific antiserum, and conjugates at much reduced frequency (Hirota *et al.* 1964). Such F^+(R 100) bacteria could not be seen to produce any F pili (Table 1, line 8). When, however, instead of R 100, its i^- mutant, R 100–1, was used, which does not suppress the function of F, F pili were formed to which phage M 12 could readily be seen to adsorb (Table 1, line 9). Thus, all the several effects of the i^+ character of R 100 appear to result from inhibition of formation of the F pilus.

Piliation directed by R factors. No pili could be detected on bacteria infected with the wild-type R factor, R 100 (Table 1, line 5), and no detached pili could be found in the supernatant of centrifuged broth cultures. The i^- mutant, R 100–1, brings about conjugation and transfer of drug resistance at much higher frequency than R 100 (Table 1, line 6) and with R 100–1, bacteria bearing pili could readily be seen (Pl. 2, fig. 5). The pili on F^-(R 100–1) bacteria were morphologically distinguishable from both flagella and type I pili, and disappeared when the R factor was eliminated by acridine treatment. They evidently differed from F pili, however, for there was no adsorption of phage M 12. The frequency of piliation in F^-(R 100–1) cultures was about one tenth of that in F^+ cultures, and a larger proportion of the pili produced were very short (0·1–1·0μ).

Piliation directed by a hybrid of F and an R factor. When R factors are transduced by salmonella phages, they are generally transferred only in part, and the transductants

Table 1. *Piliation directed by F and R factors*

Line	Host bacteria	Infected with: F	Infected with: R	Phage M 12	Conjugal fertility	No. of bacteria observed	Adsorption of M 12 on pili	% Bacteria with pili numbering: 0	1	2	3	4	5	6	7	No. of unattached pili	Detached pili in the supernatant of mass culture
1	JE2217	—	—	r	−	344	−	100	0	0	0	0	0	0	0	0	−
2	JE2443	—	—	r	−	157	−	100	0	0	0	0	0	0	0	0	−
3	JE2217	F 8	—	s	++	224	+	34·4	28·6	23·2	8·0	2·7	2·7	0·4	0	177	++
4	JE2443	F 8	—	s	++	132	+	32·6	34·1	15·9	9·1	3·0	3·0	0·8	1·5	15	++
5	JE2217	—	R 100	r	+	262	−	100	0	0	0	0	0	0	0	0	−
6	JE2217	—	R 100–1	r	+	401	+	93·3	6·2	0·2	0·2	0	0	0	0	6	+
7	JE2443	F 13 × R 23 hybrid		s	+	104	+	41·3	29·8	17·3	6·7	1·9	1·0	1·0	1·0	160	+
8	JE2217	F 8	R 100	r	+	311	−	100	0	0	0	0	0	0	0	0	−
9	JE2217	F 8	R 100–1	s	++	130	+	46·9	23·8	18·5	7·7	1·5	0	1·5	0	72	++
10	JE2217	F 8–D 15	—	r	−	219	+	96·8	2·3	0·9	0	0	0	0	0	0	+
11	JE2443	F–T 62D	—	r	+	191	−	100	0	0	0	0	0	0	0	0	−
12	JE2217	—	R 100–31	r	−	225	−	100	0	0	0	0	0	0	0	0	−
13	JE2217	—	R 100–70	r	−	232	−	100	0	0	0	0	0	0	0	0	−
14	JE2217	F 8–D 15	R 100–1	s	++	71	+	63·4	23·9	5·6	4·2	2·8	0	0	0	96	+
15	JE2443	F–T 62D	R 100–1	s	++	105	+	81·0	13·3	3·8	1·9	0	0	0	0	1	+
16	JE2217	F 8–D 15	R 100–31	s	++	79	+	83·5	11·4	5·1	0	0	0	0	0	18	+
17	JE2217	F 8–D 15	R 100–70	s	++	141	+	29·1	34·0	18·4	8·5	5·6	1·4	2·8	0	429	+

fail to receive some of the drug resistances as well as the ability to pass on the R factor by conjugation (Watanabe & Fukasawa, 1961). Such an incomplete R factor, R23, is part of a complete R factor, R10 (Tc Cm Sm Su), conferring only its tetracycline resistance. A hybrid factor resulting from recombination between F13 and R23 was obtained by Harada *et al.* (1964), and shown to give sensitivity to F specific phage. Strain JE2443 carrying this hybrid factor produced pili to which phage M12 could be seen to adsorb with the same efficiency as to F pili (Table 1, line 7).

Defective mutants of F and R factors. The reduced fertility mutant F8–D15 was found to give only an occasional piliated bacterium, to which phage M12 adsorbed (Table 1, line 10). Defective mutants similarly derived from the temperature-sensitive F-*lac T62* factor gave, at 30°, under optimal conditions for reproduction of the F-*lac T62* factor very low frequencies of F-*lac* transfer (*c.* 10^{-5} per donor) and no chromosomal recombinants nor piliated bacteria could be found (Table 1, line 11). Nor could piliated bacteria be seen with R100–31 or R100–70, mutants of R100–1 which showed reduced frequency of drug-resistance transfer, and were taken to be less effective in promoting conjugation (Table 1, lines 12 and 13). Thus, mutants of F or R factors defective in conjugal activity are also defective in piliation.

Restoration of defective F piliation by R100–1. When bacteria carrying the defective F factor, F8–D15, were also infected with R100–1, this restored their ability to transfer the *gal* gene of F8 and to give chromosomal recombinants; in addition, the numbers of piliated bacteria increased to nearly the level found with F8 itself (Table 1, line 14). The pili present on F8–D15 (R100–1) bacteria resembled typical F pili in readily adsorbing phage M12. Similar results were obtained with R100–1 and the defective mutants of F-*lac T62* (Table 1, line 15).

Complementation evidently occurred between defective F factors and defective R factors for bacteria carrying both F8–D15 and one or other of R100–31 and R100–70, produced pili like typical F pili (Table 1, lines 16 and 17). This is in accord with the previous observation that Hfr strains that had lost fertility and phage sensitivity through a mutation in the F factor (Lederberg & Lederberg, 1956) regained both properties when they were infected with R100–1 itself or with one of its defective mutants (Nishimura, 1964; Hirota *et al.* 1966).

In London

Presence of F phage sensitive bacteria in cultures of bacteria carrying R100 and R100–1. When phage MS2 was added at 5×10^9 particles/ml. to cultures of JE254 (R100) which had been freshly grown to a bacterial concentration of 2×10^8/ml., and 8 min. were allowed for adsorption before free phage was neutralized by addition of antiserum, the numbers of plaques produced on the indicator strain, HfrH, suggested that about 1 in 1000 of the bacteria had been infected. The conclusion that phage-sensitive bacteria were present in this strain was supported by finding that further incubation of the cultures for about 2 hr led to a considerable increase of phage.

When JE255 (R100–1) was tested in the same way for phage-sensitive bacteria, a much larger number of infective centres was obtained, corresponding to 2–10% of the bacteria in different experiments, and the increase of phage after incubation of the cultures was proportionately greater. It was not possible to estimate the precise proportions of bacteria which could be infected, for cultures of JE255 in liquid medium undergo spontaneous aggregation into clumps (Hirota *et al.* 1964); thus an individual

plaque could just as well arise from a clump of several infected bacteria as from a single one. Broth cultures inoculated from morphologically smooth colonies agglutinated as markedly as cultures of bacteria producing rough colonies. The aggregation in broth was evidently directly due to the pilus, for pili are poorly formed on solid medium, and broth cultures of JE254 (R 100), which contained very few piliated bacteria, were evenly dispersed.

From these results, it appeared that the pilus determined by the R factor, R100, did have some affinity for phage MS2. Electron-microscopic examination of mixtures of JE255 bacteria and phage MS2, kindly performed by Dr A. M. Lawn, Lister Institute of Preventive Medicine, showed in addition to type I pili, spherical particles of the phage attached to the specific pili. Seventy per cent of the bacteria bore pili with particles of phage MS2 attached, but fewer phage particles were attached to each of these pili than in the usual preparations of F^+ bacteria (A. M. Lawn, personal communication).

Table 2. *Relative efficiencies of plating of male-specific RNA phages MS2, μ2 and f2 on JE 255(R100–1)*

Phage stocks were prepared by picking single plaques on either 58–161 F^- or JE255 and growing the phage on the same strain in broth. The efficiencies of plating are with reference to the numbers of plaques produced on 58–161 F^+ and are average values from several experiments.

Phage	Single plaque isolate last grown on	Efficiency of plating on JE255
MS2	58–161 F^+	2×10^{-3}
	JE255	2×10^{-3}
μ2	58–161 F^+	1×10^{-3}
	JE255	2×10^{-2}
	58–161 F^+*	2×10^{-2}
f2	58–161 F^+	8×10^{-2}
	JE255	1×10^{-1}

* Plaque produced by stock previously grown on JE255.

The numbers of phage MS2 infective centres obtained with strain JE255 (R 100–1) suggested that the proportion of infectable bacteria might be high enough to allow the phage to lyse cultures macroscopically on solid medium, and, on testing, drops of undiluted phage stock produced patches of clearing, and dilutions gave plaques. Strain JE255 was less sensitive, however, than F^+ indicator strains like 58–161 F^+ or HfrH, for the clearing with undiluted phage was less complete and individual plaques were more turbid and heterogeneous in size. The efficiency of plating was considerably lower than on F^+ bacteria and most of the plaques were extremely small. Table 2 shows the results of testing the three F specific RNA phages, MS2, μ2 and f2, in parallel on 58–161 F^+ and JE255. It can be seen that the efficiency of plating on JE255 differed for each phage; the values remained approximately the same in repeated titrations and reproducible titres were obtained with the phage stocks, thus excluding the possibility that the differences between the phages were due to chance fluctuations resulting from occasional failure to observe and count the smallest plaques.

Heterogeneity in plaque size, such as was observed here, is often indicative of inefficient adsorption. The rates of adsorption to JE255 and 58–161 F^+ were directly

compared using preparations of the three phages made on JE255. Phage was mixed for 10 min. at 37° with bacteria freshly grown to a concentration of 5×10^8/ml. in broth, and the supernatant was assayed after centrifugation. With strain 58–161 F^+, 77% of phage MS2 (added at $1{\cdot}5 \times 10^7$/ml.), 76% of phage $\mu 2$ (added at 4×10^7/ml.) and 90% of phage f2 (added at $2{\cdot}6 \times 10^7$/ml) were absorbed; with JE255, adsorption was too poor for a decrease in free phage to be detected with any of the three phages.

Strain JE255 is a derivative of 58–161 cured of its F factor by acridine treatment. In case the particular kind of pilus, able to adsorb phages MS2, $\mu 2$ and f2, determined in this strain was due to some peculiarity of the strain itself, R100–1 was tested in two other lines of *E. coli* K12, strain RC12 and strain J5–3, and these were both lysed macroscopically by phage MS2.

DISCUSSION

The i^+ R factor, R100, which suppresses conjugation in F^+ bacteria by producing a repressor of F function (Egawa & Hirota, 1962) prevents the formation of F pili on F^+(R100) bacteria. However, with the i^- mutant, R100–1, F^+ bacteria continue to conjugate normally and can be seen to produce pili apparently identical with those of F^+R^- bacteria. Using bacterial strains without other surface appendages such as flagella or type I pili, it has been possible to observe that F^-(R100–1) bacteria produce pili which are morphologically like F pili but which can be distinguished from them by a lesser affinity for F specific RNA phages. With strains JE2217 and JE2443 used in Osaka, no adsorption of phage M12 to these pili could be detected. With strains JE255, RC12 and J5–3, examined in London, some attachment of phages MS2, $\mu 2$ and f2 could be demonstrated, but it was markedly less than with an F pilus.

The i^+ to i^- mutation leading from R100 to R100–1 is associated both with a loss of inhibition of F function and with the appearance of piliated bacteria in F^-(R) cultures. Since pili are produced by F^- bacteria when they carry R100–1 but not when they carry R100, it may be concluded that the cytoplasmic repressor produced by the *i* gene of R100 (Egawa & Hirota, 1962; Hirota *et al.* 1964) suppresses its own piliation as well as that of F. These regulatory changes may be compared with the operon model for enzyme synthesis in *E. coli* (Jacob & Monod, 1961), R100–1 being a constitutive mutant R factor unable to synthesize the cytoplasmic repressor of R and F mediated piliation. Meynell & Datta (1966) reported that those R factors which determine the production of a pilus resembling the F pilus are the same as those whose own repressor of function also repressed the function of F. Minor differences between the pili determined by these R factors and by F would not have been detected in their experiments, but in the present case it has been possible to discern a difference in structure between R100–1 pili and F pili as reflected in their different affinities for F specific phages. At the same time, using both F^+ and $(R100\text{–}1)^+$ bacteria as indicator strains, it was possible to detect differences between each of the three F specific phages, MS2, $\mu 2$ and f2.

The interactions between F and R100 in pilus production are set out in Table 3. Wild-type F produces pili constitutively, while R100–1 is a constitutive mutant of R100 failing to synthesize repressor. F^+(R100–1) bacteria produce F pili, while F^-(R100–1) bacteria produce R100 pili, which, although serologically related (Hirota *et al.* 1964), differ somewhat in structure from F pili. The structural genes determining pilus production in F and in R100 may be thought of as allelic, for they are subject to

the same control of function and their products, although not identical, are alike in kind. Defective mutants of F and of R 100–1 unable to determine the production of pili have evidently mutated in genes controlling an organelle producing pili specific for F or R 100; pilus production is restored to the cell when a normal F or R 100–1 factor is introduced, showing that the absence of pili is due to a mutation which is recessive to the wild type, as opposed to production of repressor.

Table 3. *Control of pili formation by F and R factors*

Genotypes of F and R	Pili in established cultures	Pili on newly infected bacteria	Type of pilus
F(i^-)	Present	.	F
Ri^+/F(i^-)	Absent	.	.
Ri^-F(i^-)	Present	.	F
Ri^+	Absent	Present*	R
Ri^-	Present	.	R
Ri^+/Ri^-	Absent	.	.
Ri^-/F(i^-)*def*	Present	.	F
Ri^-*def*/F(i^-)*def*	Present	.	F

* Datta, Lawn & Meynell (1966).

It is also clear that genetic alterations resulting in loss or acquisition of F or R pili are strictly correlated with loss or gain of ability to transfer the chromosome by conjugation. Thus, the presence of pili, determined by F or by R factors, is essential for conjugation, whether or not the pilus itself acts as the tube through which the donor chromosome is transferred to the recipient bacterium as postulated by Brinton (1965).

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EXPLANATION OF PLATES

PLATE 1

Electron micrographs by platinum-palladium shadow-casting technique (× 20,000).

Fig. 1. JE2217 (F⁻ *pil*⁻ *fla*⁻).

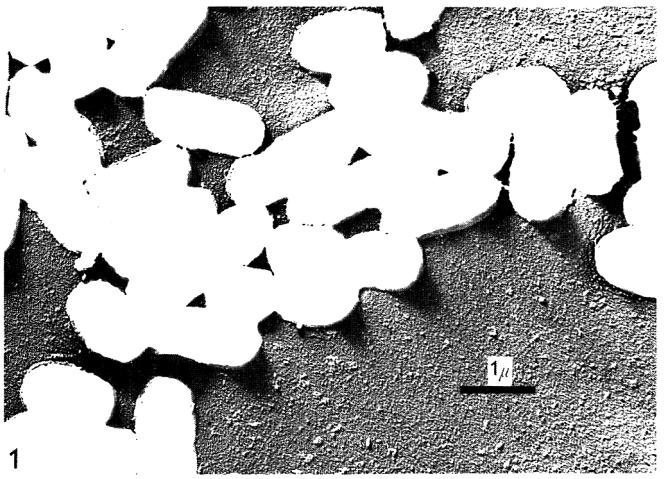
Fig. 2. JE2217 carrying F8.

Fig. 3. Phage M12 adsorbed to JE 2217 (F8).

PLATE 2

Fig. 4. Electron micrograph of detached F pili with absorbed phage M12: phosphotungstate negative staining technique (× 100,000).

Fig. 5. Electron micrograph of JE2217 carrying R100-1: platinum-palladium shadow-casting technique (× 20,000).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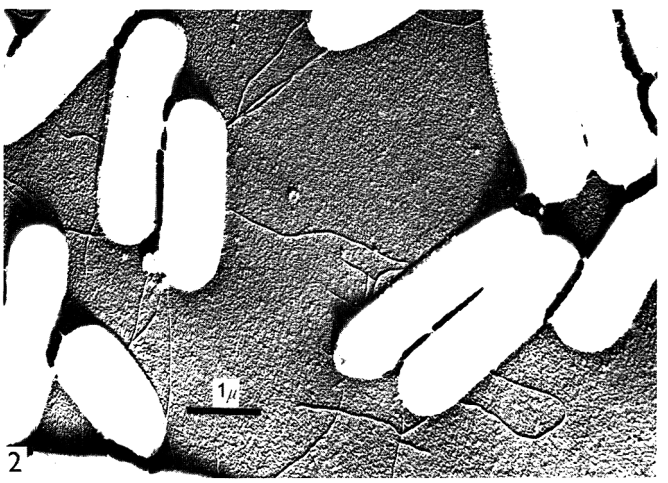
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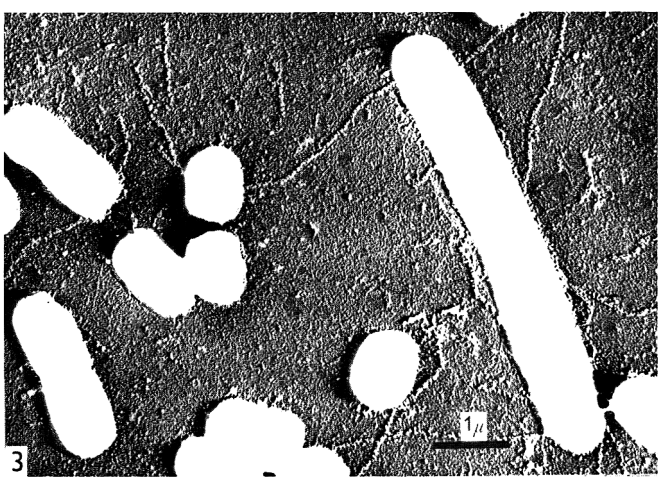
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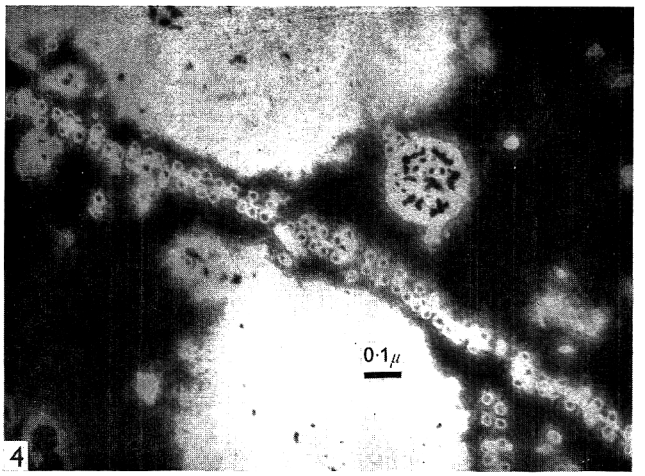
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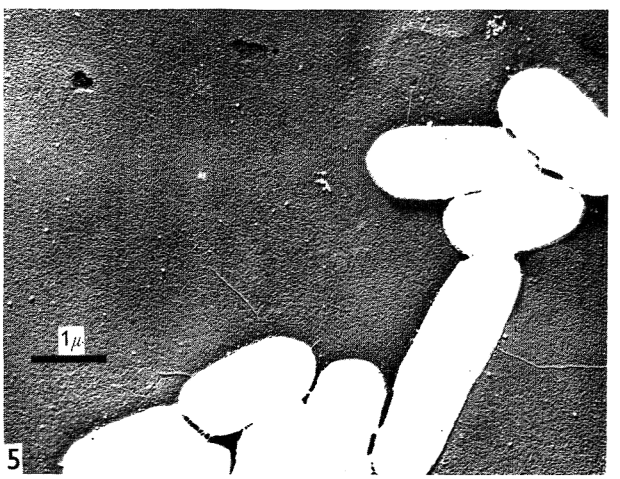
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J. gen. Microbiol. (1967), **49**, 99–108
With 1 *plate*
Printed in Great Britain

The Expression of R-gene Resistance to *Phytophthora infestans* in Tissue Cultures of *Solanum tuberosum*

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SUMMARY

The expression of R-gene resistance in tissue cultures of Solanum species was investigated. Tissue aggregates (4 mm. diam.) of *Solanum tuberosum* var. Majestic (rr) and Orion (R 1) and other R-gene Solanum lines behaved in a similar way to the intact plants from which they were derived, in response to *Phytophthora infestans* race 4. Tissue aggregates of *S. tuberosum* var. Orion supported good growth of *P. infestans* race 1, to which the intact Orion plant is susceptible, but did not support more than rudimentary growth of race 4, to which the intact Orion plant is resistant. Sectioning revealed that *P. infestans* race 4 made general growth in Majestic tissues, but was almost totally excluded from Orion tissues. A slide technique showed that Orion tissue suspensions, unlike those of Majestic, inhibited the growth of *P. infestans* race 4 germ tubes. This led to a demonstration that toxicity to *P. infestans* zoospores was developed in Orion but not in Majestic tissue cultures in response to infection with *P. infestans* race 4. It was concluded that R-genes may be expressed in tissue culture, that the failure of the Orion tissue to support growth of *P. infestans* may be due to expression of the R 1 gene, and that this may operate through the development of post infectional toxicity.

INTRODUCTION

Hypersensitive or R-gene resistance in Solanum species leads, after initial penetration of a small group of cells, to the rapid exclusion of incompatible races of *Phytophthora infestans* (Ferris, 1955; Müller 1950). This is the case with *S. tuberosum* var. Orion, where *P. infestans* race 4 is unable to make any growth on either leaves or tubers as a result of the function of the R 1 gene (Lapwood & McKee, 1961). In contrast, race 4 makes general and unrestricted growth in the susceptible *S. tuberosum* var. Majestic plant. Müller & Börger (1940) postulated that in the potato, hypersensitive resistance was due to a rapid development of antibiotics in the tissues in response to infection. The substances responsible were termed 'phytoalexins', and the phytoalexin concept has since been developed further for *S. tuberosum* and for other plant/fungus combinations (Cruickshank, 1963). Ingram & Robertson (1965) showed that tissue cultures of *S. tuberosum* var. Majestic supported good growth of *P. infestans* race 4, whereas var. Orion tissues supported only rudimentary growth of this fungus. The situation corresponded exactly with observations made with intact plants, and it was suggested that the failure of the Orion tissues to support fungal growth was due to the expression of the R 1 gene in tissue culture. The expression of R-genes in tissue

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cultures of Solanum species, and in particular in tissue cultures of *S. tuberosum* var. Orion, has now been further investigated.

METHODS

Organisms *Phytophthora infestans* race 1 was isolated from a leaf of *Solanum tuberosum* var. Orion, and race 4 from a leaf of var. Duke of York during July 1964. Race 1 was found to be pathogenic to leaflets and to half tubers of both *S. tuberosum* var. Majestic and var. Orion, while race 4 was pathogenic only to Majestic, and non-pathogenic to Orion. These and other pathogenicity tests were made by inoculating leaflets and half tubers contained in humidity chambers with 1×10^3 sporangia suspended in 0·01 ml. of distilled water.

Tubers of *Solanum tuberosum* var. Majestic (rr) and var. Orion (R 1) were maintained as tissue cultures. For specific experiments tissue cultures were initiated from stems and tubers of *S. tuberosum* var. Ulster Torch (R 1), tubers of var. Pentland Beauty (R 3) and stems of *Solanum demissum* (R 4) and *Solanum stoloniferum* (R 2). Normal (i.e. not cultured) material was obtained from field-grown plants in the case of the *S. tuberosum* varieties and from pot-grown plants in the case of *S. demissum* and *S. stoloniferum*.

Media used for the establishment and maintenance of tissue cultures, and for the maintenance of *Phytophthora infestans* stock cultures, were as described by Ingram & Robertson (1965). The tissue culture medium, either in liquid form or solidified with 1 % (w/v) Davis agar, was used in all experiments. This medium did not support colonial growth of *P. infestans* from spores.

Establishment and maintenance of tissue cultures. *Solanum tuberosum* tuber callus cultures were established according to the procedure of Ingram & Robertson (1965). This procedure was modified slightly for the initiation of stem-tissue cultures as follows: 3 cm. internode segments from mature plants were surface sterilized in 95 % (v/v) ethanol in water for 1 min., then in a saturated calcium hypochlorite solution for 7 min., and washed several times in glass-distilled water. A 1 cm. length of tissue was then cut from the centre of each segment and placed on 15 ml. of solidified medium contained in a 6 in. × 1 in. Pyrex glass culture tube, and incubated at 25°. Callus growth from the cut ends of the inocula was normally visible after 1–4 weeks, and after 6–8 weeks it was removed from the original explant and transferred to fresh medium. For a supply of experimental material calluses were transferred to shake culture where the growing tissue mass broke up to give suspensions of single cells and tissue aggregates of up to 5·0 mm. diameter (Ingram & Robertson, 1965).

Preparation of spore suspensions. Seven-day-old bean-medium slopes of *Phytophthora infestans* grown at 22° were used as source of spores. Sporangial suspensions were prepared by washing 7-day cultures with 10 ml. liquid tissue culture medium. In this medium the sporangia germinated directly and did not produce zoospores. When zoospores were required, 7-day 22° cultures were left overnight at 10°, after which sporangia were removed in 10 ml. sterile distilled water and the suspension incubated at 10° for 2 hr. Zoospores were produced when the suspensions were brought to room temperature, and could be pipetted from the top of the liquid, empty and ungerminated sporangia having settled out.

Inoculation of tissue aggregates. Small aggregates of tissue, about 4·0 mm. in diam.,

were removed from liquid culture with a wire loop and placed on the surface of the solidified tissue culture medium in Petri plates. Sporangia of *P. infestans*, 1×10^3 suspended in 0·02 ml. liquid medium, were applied to the tissues with a sterile pipette. Incubation was at 22°. Wherever possible all treatments in an experiment were placed in the same Petri plate and replicated at least 10 times. In this way environmental variation between treatments was virtually eliminated.

Histological investigations. Tissue aggregates (4 mm. diam.) were transferred from liquid culture to agar plates and each aggregate inoculated with 1×10^3 sporangia of *Phytophthora infestans* race 4. After incubation for 7 days at 22° the infected tissues were removed from the agar, fixed in formalin + acetic acid + ethanol, dehydrated by the tert.-butanol procedure and embedded in paraffin wax (Johansen, 1940). Sections (8 μ thick) were cut and after being mounted were stained, either with 0·01 % cotton blue in lactophenol and mounted in lactophenol, or with Delafield's haematoxylin (Gray, 1954) and mounted in Canada balsam. Attempts to stain the material with carbol thionin blue (Stoughton, 1930) produced indifferent results.

Slide germination experiments. These experiments were done under completely sterile conditions at 22°. Thoroughly clean 3·0 in. × 1·0 in. glass microscope slides were supported on glass rods over a thin film of distilled water in Petri dishes. The slides were inoculated near the centre with 0·1 ml. of a cell suspension and with 0·05 ml. distilled water containing 1×10^4 Phytophthora zoospores. The film of distilled water maintained a high humidity in the Petri dishes, so that there was little evaporation from the drops of liquid on the slides, even after 7 days. Sample slides were examined microscopically as required, and after examination were usually discarded because of the loss of sterility. It was found that a small drop of cotton blue in lactophenol often facilitated microscopic examination, and when germ tubes were to be measured a drop of this stain was added at the same time to all slides to be examined, to halt growth of the fungus.

Tissue cultures used in these experiments were from tubers of *Solanum tuberosum* var. Majestic and var. Orion. Cultures were grown from 5·0 ml. tissue suspension inoculum in 20 ml. liquid medium contained in 100 ml. Erlenmeyer flasks. The cultures were incubated on the shaker for 8 weeks, and 10 ml. fresh medium was added after 4 weeks of growth. Dense cultures consisting predominantly of single cells or small groups of cells (up to 20) were selected for use, and a small quantity of the cell suspension removed with a sterile Pasteur pipette. A single drop of cell suspension delivered from such a pipette normally had a volume of about 0·05 ml. In individual experiments cells for all replicates and treatments of a tissue variety were taken from the same culture.

Tissue culture filtrate experiments. Erlenmeyer flasks (100 ml.) each containing 25 ml. of a dense 8-week *Solanum tuberosum* tissue suspension were used. Each flask was inoculated with 2×10^5 sporangia of *Phytophthora infestans* and incubated on a shaker at 22°. About 0·5 ml. liquid ('filtrate') was removed from each flask just before inoculation and at 24, 48, 72, 96 and 168 hr after inoculation. This liquid was assayed against *Phytophthora infestans* zoospores on glass slides. Each slide was inoculated with 0·1 ml. of the liquid and with 1×10^4 zoospores in 0·05 ml. distilled water, and incubated at 22° for 4 hr. Percentage germination of zoospores was calculated from the total spore counts in three low power (750 μ diameter) microscope fields for each of two slides (about 100 spores/field) for each treatment.

RESULTS

Growth of Phytophthora infestans race 4 on Solanum tissue aggregates of various R-genotypes

The growth of *Phytophthora infestans* race 4 from sporangia was tested on tissue aggregates derived from *in vitro* cultures of several R-gene Solanum lines. These lines were: *Solanum tuberosum* var. Majestic (rr, tuber), var. Orion (R 1, tuber), var. Ulster Torch (R 1, tuber and stem), var. Pentland Beauty (R 3, tuber), *S. demissum* (R 4, stem), *S. stoloniferum* (R 2 [McKee, 1962], stem). The results of the tests, together with the reaction to sporangial infection of leaflets and half tubers of the varieties concerned are given in Table 1. Tissue cultures derived from resistant Solanum plants did not support growth of *P. infestans* race 4, while tissue cultures derived from susceptible plants supported good growth of this fungus. (The R 2, R 3 and R 4 genes, unlike the R 1 gene, are only partially expressed in tubers [Lapwood & McKee, 1961].)

Table 1. *The growth of Phytophthora infestans race 4 from sporangia on tissue cultures and on leaflets and half tubers of the Solanum lines from which the tissue cultures were derived*

Reactions: S = susceptible reaction, profuse growth of the fungus; R = resistant reaction, little or no growth of the fungus; H = resistant reaction of leaflets, production of hypersensitive flecks; N.T. = not tested

		Reaction to infection			
		Tissue cultures		Plant parts	
Solanum variety	R-genotype	Tuber	Stem	Half tubers	Leaflets
S. tuberosum					
var. Majestic	rr	S	N.T.	S	S
var. Orion	R 1	R	N.T.	R	H
var. Ulster Torch	R 1	R	R	R	H
var. Pentland Beauty	R 3	S	N.T.	S	H
S. demissum	R 4	N.T.	S	N.T.	S
S. stoloniferum	R 2	N.T.	R	N.T.	H

The growth of Phythophthora infestans race 1 on tissue aggregates of Solanum tuberosum var. Orion

Solanum tuberosum var. Orion tissue aggregates inoculated with 1×10^3 sporangia of *Phytophthora infestans* race 1 (to which the intact Orion plant is susceptible) were compared, on the same plate, with aggregates similarly inoculated with sporangia of race 4. After incubation for 3 days considerable mycelial development had taken place on those tissue aggregates inoculated with sporangia of race 1, and at 7 days the colonies were between 1·5 and 2·5 cm. diam. Only rudimentary mycelial growth took place on tissues inoculated with sporangia of race 4, even after 10 days (Pl. 1, fig. 1). Both isolates were able to make good growth on similarly inoculated Majestic tissue aggregates.

Histology of tissue aggregates infected with Phytophthora infestans race 4

Serial sections of three Majestic and four Orion tissue aggregates were examined with the Delafield's haematoxylin stain 7 days after infection with sporangia of

Phytophthora infestans race 4. Both types of tissue were found to consist basically of large thin-walled cells. These were roughly spherical in shape and 30–100μ diameter. Within the matrix of large cells meristematic pockets up to 800μ diameter were occasionally noted. These consisted of a group of small cells, densely filled with cytoplasm, surrounded by a crushed zone, 4 or 5 cells thick. Tracheid-like, reticulately thickened cells were also observed; these were few in number, were not orientated, and usually occurred in groups of two or three. The bands of thickening did not stain red with phloroglucinol and hydrochloric acid, but gave a cellulose reaction with Schultze's solution (50 g. $ZnCl_2$ and 16 g. KI in 17 ml. water + excess iodine). Starch was not a normal cell constituent in the tissue aggregates, but small grains between 7·5μ and 15·0μ diam. were often present in isolated groups of cells. Tissue aggregates generally had a number of deep, debris-filled fissures. The *P. infestans* exhibited quite different growth patterns in the tissue aggregates of the two varieties; each will be described separately.

In Solanum tuberosum var. Majestic. After incubation for 7 days the *Phytophthora infestans* had made good growth on these tissues, which had become soft and fragile. Within the tissues growth of the fungus was general and diffuse, with a loose unrestricted network of inter- and intracellular hyphae permeating the whole of the aggregate. In some instances the fungal hyphae proliferated within a single cell or small group of cells so that these became packed with mycelium. Two of the Majestic tissue aggregates examined had extensive areas of broken and separated cells, which appeared to have resulted from the direct activity of the *P. infestans.*

In Solanum tuberosum var. Orion. After 7 days the *Phytophthora infestans* made virtually no visible growth on the Orion aggregates, which remained quite firm. Sections showed that hyphae were almost totally absent from the tissues, though a few isolated cells near the surface of the aggregates had become infected and were packed with mycelium (Pl. 1, fig. 2). No fungus was detected in cells adjacent to these or in cells away from the surface of the aggregates. Occasionally the *P. infestans* proliferated in debris-filled fissures, and the infection of tissues lining such structures was of a more general type, with inter- and intracellular hyphae extending to a depth of three or four cells. No tissue maceration was noted in any of the Orion material examined.

Two meristematic zones in one of the Orion tissue aggregates exhibited an unusual infection pattern. Both zones were exposed in part at the surface of the aggregate and, in contrast with surrounding tissues, were densely packed with *Phytophthora infestans* mycelium. Massive development of the fungus had taken place in the meristematic cells and in the surrounding zones of crushed cells. Similar meristematic pockets in Majestic tissue aggregates did not show this massive development of the fungus, but were diffusely infected in a similar way to the rest of the tissue aggregate in which they were situated.

Haustoria. The structure of the host/parasite interface was not clear in Delafield's haematoxylin preparations, but some detail of the region of contact between cells and individual hyphae was obtained when sections were stained with cotton blue and mounted in lactophenol. Preparations of tissue aggregate material were examined for haustoria by using this stain; no haustoria of the type described by Blackwell (1953) was detected. (Dr A. E. Godwin [personal communication] has seen similar haustoria.) However, small haustorium-like projections from hyphae were occasionally

noted; these were 1·5–2·0μ long and penetrated cell walls. They lacked both the sheath and the basal constriction reported by other workers as being typical of *Phytophthora infestans* haustoria.

Slide germination experiments

In these experiments glass slides were simultaneously inoculated with a cell suspension of the resistant *Solanum tuberosum* var. Orion or the susceptible var. Majestic, and with zoospores of *Phytophthora infestans* race 4.

Growth of zoospores germ tubes. The lengths of 25 separate germ tubes, in a sample slide from each treatment, where 1×10^4 zoospores had been incubated in the presence of 0·1 ml. cell suspension of Majestic or Orion, were measured after incubation for 24 hr at 22°. There was significantly less growth of the germ tubes in the presence of Orion cells than in the presence of Majestic cells (Table 2). Substitution of 0·1 ml. of liquid medium in which no tissues had been grown, for the cell suspensions, resulted in a similar growth of germ tubes to that which had taken place in the presence of Majestic cells.

Table 2. *Lengths of zoospore germ tubes for each treatment, where Phytophthora infestans race 4 zoospores were incubated for 24 hr in the presence of cells of Solanum tuberosum var. Majestic and var. Orion and in unused liquid tissue culture medium*

	Mean length of 25 germ tubes (μ)	pH* value
Var. Majestic	$554{\cdot}4 \pm 26{\cdot}27$	5·0
Var. Orion	$452{\cdot}8 \pm 31{\cdot}50$ $t_{48} = 8{\cdot}22$	4·9
Liquid medium	$549{\cdot}6 \pm 30{\cdot}13$	5·4

* pH value of tissue cultures or uninoculated medium.

Penetration of cells. Cell slides of Majestic and Orion tissues were examined at 24, 48, 72 and 96 hr after infection with *Phytophthora infestans* zoospores for penetration of cells by germ tubes. The number of penetrations was very low so that no reliable comparison could be made between the two varieties. However, the number of penetrated cells became greater with time, and certain clearly defined stages in cell penetration by germ tubes were noted.

At 24 hr some of the germ tubes had become attached to cell walls by a small swelling resembling an appressorium, and could not be detached with the aid of a fine needle. From most of these appressoria an infection hypha with a constricted base had penetrated the cell wall (Fig. 1 A). At 48 hr infection hyphae had usually branched within the infected cell, and in many cases had grown out through the wall at the other side (Fig. 1 B). The second point of wall penetration was also accompanied by an appressorium-like swelling at the point of contact between the hypha and the cell wall. At 96 hr hyphae had usually made considerable growth within the infected cells (Fig. 1 C). All stages of penetration were represented in the 96 hr slides.

Germination of zoospores of Phytophthora infestans in tissue-culture filtrates

Suspension cultures of *Solanum tuberosum* var. Majestic and var. Orion were infected with 2×10^5 sporangia of *Phytophthora infestans* race 4 and incubated on a shaker at 22°. Samples of the culture liquid (filtrate) were assayed against *P. infestans* race 4 zoospores on glass slides immediately before infection, and at 24, 48, 72, 96 and 168 hr after infection.

Germination of zoospores in the Majestic filtrate was 100% at all times of testing. In Orion filtrate, germination of the zoospores was 100% immediately before infection and after 24 hr, but decreased to only 7% at 96 hr and to 0·0% at 168 hr (Table 3; Fig. 2). At 48 hr a few of the ungerminated zoospores burst. The proportion of burst zoospores became greater with time, and was 100% at 168 hr. Similar tests showed that the infected Orion culture liquid was equally toxic to zoospores of *P. infestans* race 1 (Table 4), but had no effect on the germination of spores of either *Glomerella cingulata* or *Botrytis allii*.

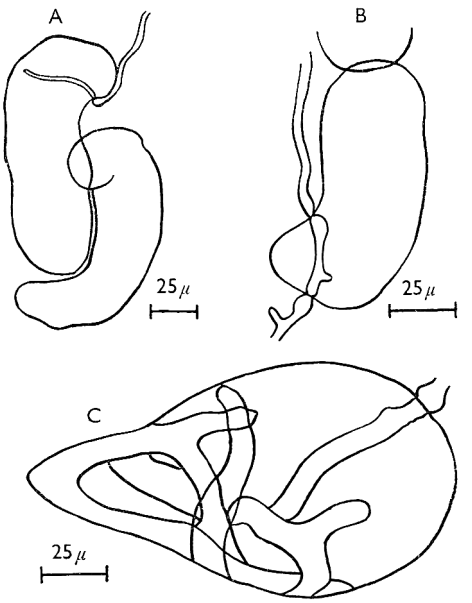

Fig. 1. Penetration of cells of *Solanum tuberosum* by germ tubes of *Phytophthora infestans* race 4. A, 24 hr; B, 48 hr; C, 96 hr.

Table 3. *Germination of Phytophthora infestans race 4 zoospores in liquid taken from infected tissue cultures of Solanum tuberosum var. Majestic and var. Orion after various times*

Liquid from the variety	Time (hr)					
	0	24	48	72	96	168
	Germination (%)					
Var. Majestic	100	100	100	100	100	100
Var. Orion	100	100	50	26	7	0

The toxicity of the 168 hr culture liquid to *P. infestans* race 4 zoospores was modified by heat treatment: a sample which had been held at 100° for 1 min. brought about no bursting of zoospores, while a sample which had been held at 100° for 5 min. allowed a small number of zoospores (less than 1%) to germinate.

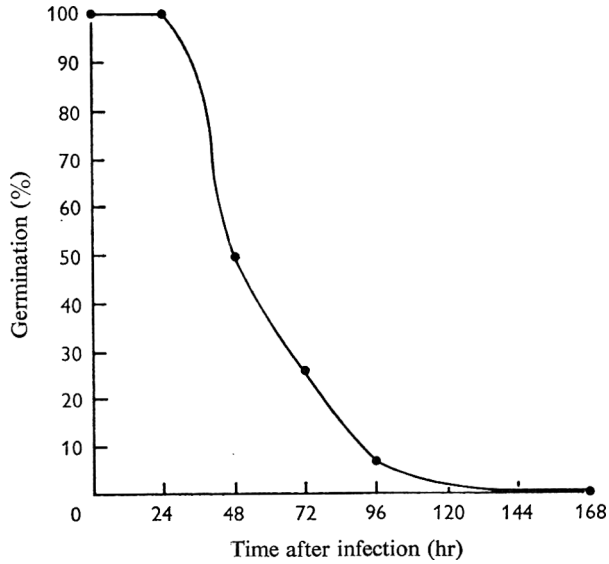

Fig. 2. Germination of *Phytophthora infestans* race 4 zoospores in liquid taken from an infected tissue culture of *Solanum tuberosum* var. Orion after various times.

Table 4. *Germination of Phytophthora infestans race 1 zoospores in liquid taken from infected tissue cultures of Solanum tuberosum var. Majestic and var. Orion after various times*

Liquid from the variety	Time (hr) 0	24	48	72	96	158
	Germination (%)					
Var. Majestic	100	100	100	100	100	100
Var. Orion	100	100	50	31	7	0

DISCUSSION

The suggestion that R-genes may be expressed in tissue culture (Ingram & Robertson, 1965) is supported by the demonstration that *Phytophthora infestans* race 4 grew well on tissue culture aggregates derived from susceptible Solanum lines but made little growth on tissue cultures from resistant Solanum lines. Evidence for the R-gene nature of the *Solanum tuberosum* var. Orion tissue cultures is provided by the demonstration that tissue aggregates, which did not support growth of *P. infestans* race 4, did support good growth of *P. infestans* race 1, an isolate which has been shown to be pathogenic to the intact Orion plant.

Sectioning revealed that *Phytophthora infestans* race 4 made unrestricted growth in the *Solanum tuberosum* var. Majestic tissue aggregates. This fungus was largely excluded from the *S. tuberosum* var. Orion aggregates, although a small number of cells were heavily infected. The infected cells situated near the surface of the tissue mass may previously have suffered damage, owing to their exposed position, or the *P. infestans* may have in some way overcome their resistance. Infection of cells lining

fissures in the Orion aggregates might have resulted from the massive build up of fungal inoculum in the debris which invariably filled such structures.

The proliferation of *Phytophthora infestans* in meristematic pockets in Orion tissue aggregates is difficult to comprehend, unless it be assumed that young and actively dividing cells were less resistant than those which had reached maturity. Ingram (1966) showed the formation by some Orion tissue cultures of sectors which were only partially resistant to *P. infestans* race 4. It is possible that those meristematic pockets in which the *P. infestans* proliferated may have been centres of initiation of such sectors.

The mechanism whereby Orion tissue aggregates were able to exclude *Phytophthora infestans* race 4, and cell suspensions to inhibit the growth of germ tubes, is suggested by the results of the tissue culture filtrate experiments. Development of post-infection toxicity in Orion tissue cultures and not in Majestic tissue cultures was found. To achieve an almost total exclusion of the infecting fungus, development of post-infectional toxicity would need to take place very rapidly. However, the development of toxicity in infected Orion tissue suspensions was very slow. This may have been due to an inability to detect the toxic principle until it had been produced in considerable quantities, because the ratio between the volume of the tissue culture liquid and the infected tissues was very high.

In some ways the reaction of the Orion tissue suspensions resembled the phytoalexin reactions described by Müller (1958) and Cruickshank (1963). Certain inconsistencies exist, however. For example, although the filtrate was toxic to zoospores from two strains of *Phytophthora infestans*, there was no toxicity to spores of *Glomerella cingulata* or *Botrytis allii*. The development of toxic substances in response to infection of *Solanum tuberosum* tissue cultures with *P. infestans* race 4 must be investigated further before any definite conclusions can be drawn. Tissue cultures differ in many ways from the intact potato plant (Steward & Pollard, 1956) and no direct comparison can be drawn between the two. However, if the results of the tissue culture experiments represent a model system of the processes normally involved in R-gene resistance, it is possible that the tissue culture method may provide results which will be of value in interpreting results already obtained, or in planning further experimentation to define more clearly the precise nature of the R-gene resistance of the potato.

My thanks are due to the Agricultural Research Council for financial support, and to Professor N. F. Robertson for advice and discussions.

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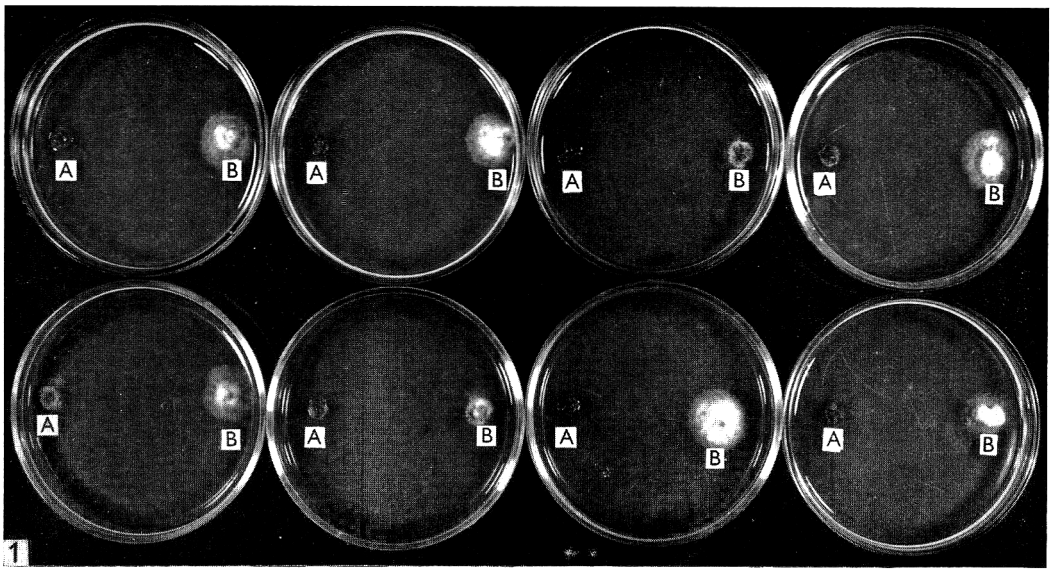
EXPLANATION OF PLATE

Fig. 1. Eight replicate examples of Petri plates inoculated with tissue aggregates of *Solanum tuberosum* var. Orion and *Phytophthora infestans* (5 days). A, Tissues infected with *P. infestans* race 4; B, tissues infected with *P. infestans* race 1.

Fig. 2. Cell near the surface of a var. Orion tissue aggregate, packed with mycelium of *Phytophthora infestans* race 4.



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The Aerosol Survival of *Escherichia coli* JEPP Sprayed from Protecting Agents into Nitrogen Atmospheres under Changing Relative Humidity Conditions

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(*Accepted for publication* 19 *April* 1967)

SUMMARY

The aerosol survival of *Escherichia coli* JEPP, a particularly sensitive strain, has been studied in a nitrogen atmosphere (> 99·9%) when sprayed from suspension in solutions of sodium glutamate or glycerol, and collected into phosphate buffer with or without M-sucrose. The response caused by changes in relative humidity (RH) to 30% and 100% before collection was also examined. These two protecting agents were selected because glycerol readily permeated the cytoplasmic membrane, whereas sodium glutamate did not. *E. coli* JEPP sprayed as a suspension in sodium glutamate and collected in phosphate buffer showed two minima in the survival *versus* RH curves: (i) a comparatively narrow zone between 90 and 82% RH; (ii) a broad instability zone between 82 and 50% RH. The presence of sucrose in the collecting fluid increased the survival for storage above 50% RH, but decreased it below 50% RH. For storage above 50% RH a change to 100% RH before collection increased the survival for collection in phosphate buffer but not in phosphate buffer+sucrose; below 50% RH this change decreased the survival. A change to 30% RH before collection had little effect. In similar experiments with glycerol a gradual decline in survival occurred as the RH value was decreased and no critical minima were apparent. Addition of sucrose to the phosphate buffer collecting fluid increased survival. A similar improvement was obtained by changing the RH to 100% before collection into phosphate buffer. The combined effect of having sucrose in the collecting fluid and an RH change were not additive, and were somewhat antagonistic for aerosols stored at low RH values. A change to 30% RH before collection decreased the survival. These results are compared with those for *E. coli* JEPP sprayed from suspension in distilled water and in solutions of raffinose, and show that at high RH values glycerol provided the best protection. At low RH values glycerol was inferior to sodium glutamate and raffinose, which were similar. The importance of the manner in which water re-enters the bacterium on collection is discussed.

INTRODUCTION

The aerosol survival of *Escherichia coli* has been shown to be dependent upon a number of factors (Cox, 1966*a*, *b*); these include an air stress at low relative humidity (RH) caused by the toxic action of oxygen. Hess (1965) found a similar result for *Serratia marcescens*. At high RH values, regions were found where *E. coli* survival

was particularly sensitive to RH, in a manner that was dependent upon the spray and collecting fluids. Relative humidity changes to 100 or 30% immediately before collection also influenced the degree of survival, either beneficially or detrimentally, depending upon the strain of *E. coli* used and the spray and collecting fluids. The present paper reports the survival of *E. coli* JEPP, a particularly sensitive strain, when sprayed from suspension in solutions of sodium glutamate and of glycerol. Previous studies have shown that raffinose, which is a good protective agent for *E. coli* B and *E. coli commune*, was not particularly good for the JEPP strain stored at high RH (Cox, 1966*a*, *b*). Sodium glutamate and glycerol were chosen for comparison with raffinose since their permeabilities are such that it is possible to obtain different distributions within the bacterial system. Raffinose could be confined to outside the cell wall, sodium glutamate permeated the cell wall but perhaps not the cytoplasmic membrane, and glycerol permeated both the cell wall and the cytoplasmic membrane. The importance of the distribution of the protective agent was discussed previously (Cox, 1965).

METHODS

These techniques were as previously reported (Cox, 1966*a*, *b*), except that instead of spraying the suspension of bacteria immediately upon the addition of the protective agent to the suspension of bacteria in distilled water, the sodium glutamate was allowed to equilibrate with the bacteria at 26·5° for 1 hr and the glycerol for 30 min. before spraying.

RESULTS

Tests with sodium glutamate

Figure 1 shows the survival of *Escherichia coli* JEPP sprayed from 0·13 M-sodium glutamate in distilled water into nitrogen at an aerosol age of 25 min. for collection by raised impinger (May & Harper, 1957) into phosphate buffer with and without M-sucrose. For collection in phosphate buffer two minima were apparent between 90% and 50% RH. Beyond these regions, survival similar to that for raffinose-protected bacteria (Cox, 1966*a*) was achieved. Addition of sucrose to the phosphate buffer collecting fluid tended to smooth out the marked instability between 90% and 50% RH, but below 50% RH the sucrose collecting fluid was inferior to phosphate buffer alone. This effect of the collecting fluid was similar to that found for *E. coli* JEPP sprayed from distilled water and from raffinose solution (Cox, 1966*a*). Figure 2 gives the result when the aerosol was changed from its storage RH to 100% RH immediately before collection. Above 50% RH this treatment increased the survival for collection in phosphate buffer, but generally decreased it for collection in phosphate buffer + sucrose, with the result that the collection difference shown in Fig. 1 was decreased. Below 50% this change in RH decreased the survival for collection in both fluids. A change to 30% RH immediately before collection gave results that were generally similar to those of Fig. 1, except that for storage above 82% RH this change in RH caused a decrease of survival for collection in both fluids. Table 1 presents the results obtained for different equilibration times before spraying and shows the dramatic nature of the effect. Plasmolysis data indicated that the bacteria became plasmolysed within 5 min. following the addition of sodium glutamate (final concentration 0·5M) to a suspension of bacteria in distilled water. Hence the influence of equilibration

time was not simply that due to sodium glutamate penetrating the cell wall. The bacteria were not observed to undergo plasmoptysis ('deplasmolysis').

Tests with glycerol

Figure 3 gives the survival of *Escherichia coli* JEPP sprayed from 0·3 M-glycerol in distilled water into nitrogen at an aerosol age of 25 min. collected by raised impinger (May & Harper, 1957). The data show greatest survival at high RH with a gradual decrease in survival as the RH was lowered. Unlike raffinose (Cox, 1966*a*, *b*) and sodium glutamate, no critical minima were apparent. Collection in phosphate buffer was inferior to that when sucrose was added over the range RH 100–30%, unlike

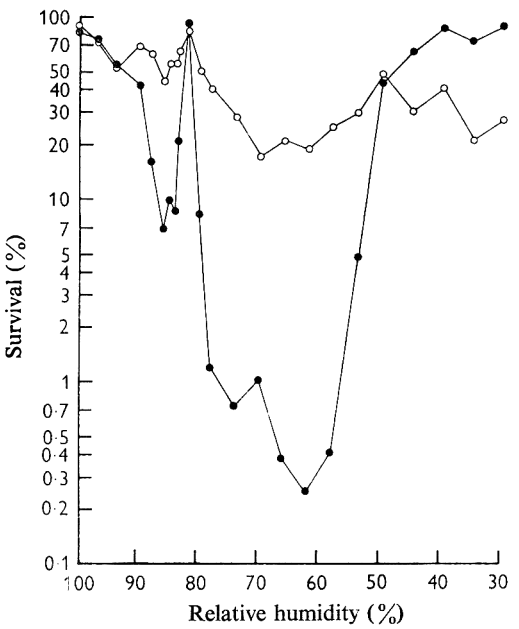

Fig. 1

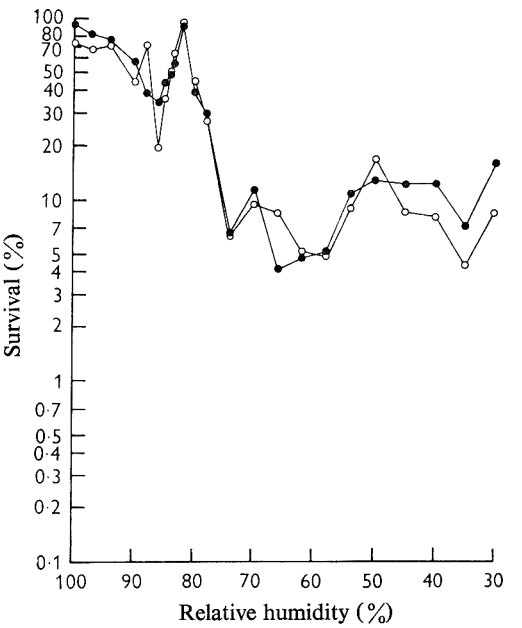

Fig. 2

Fig. 1. Aerosol survival of *Escherichia coli* JEPP in nitrogen, sprayed from 0·13 M-sodium glutamate, at an aerosol age of 25 min. at the storage relative humidity and 26·5°. ●, Collection in phosphate buffer; ○, collection in phosphate buffer+sucrose (M).

Fig. 2. Aerosol survival of *Escherichia coli* JEPP in nitrogen, sprayed from 0·13 M-sodium glutamate, at an aerosol age of 25 min. at the storage relative humidity and 26·5° followed by a change to 100% relative humidity prior to collection. ●, Collection in phosphate buffer; ○, collection in phosphate buffer+sucrose (M).

raffinose and distilled water (Cox, 1966*a*) and sodium glutamate. At high and intermediate RH values glycerol and sodium glutamate showed similar protecting abilities for collection in phosphate buffer+sucrose. At low RH glycerol-protected bacteria gave decreased survival as compared to those sprayed from suspension in distilled water and raffinose solution (Cox, 1966*a*) and in sodium glutamate. In Fig. 4 results are given for a change from the storage RH to 100% RH immediately before collection. Over the range 100–30% RH, this change brought about increased survival for collection in phosphate buffer; there was little effect for collection in phosphate buffer+sucrose for storage above 65% RH, but below this RH value, the RH

change decreased survival. As a consequence of this treatment together with collection in phosphate buffer, glycerol was superior to sodium glutamate as a protective agent above 60% RH, and was superior to raffinose above 70% RH. Below this RH region, even with the RH changed to 100% before collection, glycerol was inferior to distilled water, raffinose (Cox, 1966*a*) and sodium glutamate. A change to 30% RH before collection decreased the survival as compared with that of control bacteria over the range RH 100–30%; addition of sucrose to the phosphate collecting fluid again increased survival.

Table 1. *The influence of equilibration time with 0·13 M-sodium glutamate on the aerosol survival of Escherichia coli JEPP stored in nitrogen at 82% RH and 26·5°: aerosol age 25 min.*

Equilibration time (min.)	Collecting fluid	Survival (%)		
		Storage RH	100% RH*	30% RH*
0	PB†	0·82	8·0	1·7
	PBS†	25	6·8	0·15
30	PB	7·3	34	34
	PBS	57	45	28
60	PB	91	90	60
	PBS	83	95	100

* Aerosol stored at 82% RH and shifted to 100 or 30% RH prior to collection.
† PB = phosphate buffer; PBS = phosphate buffer + sucrose (M).

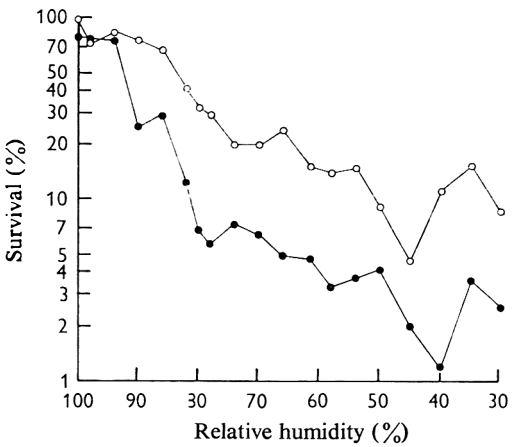

Fig. 3

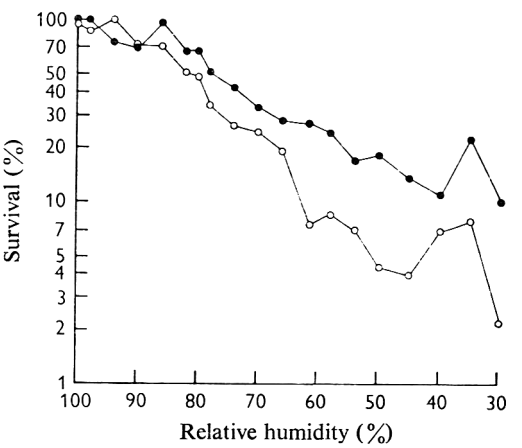

Fig. 4

Fig. 3. Aerosol survival of *Escherichia coli* JEPP in nitrogen, sprayed from 0·3 M-glycerol at an aerosol age of 25 min. at the storage relative humidity and 26·5°. ●, Collection in phosphate buffer; ○, collection in phosphate buffer + sucrose (M)

Fig. 4. Aerosol survival of *Escherichia coli* JEPP in nitrogen, sprayed from 0·3 M-glycerol, at an aerosol age of 25 min. at the storage relative humidity and 26·5° followed by a change to 100% relative humidity prior to collection. ●, Collection in phosphate buffer; ○, collection in phosphate buffer + sucrose (M).

The results of Table 2 indicate that increasing the concentration of glycerol in the spray fluid from 0·3 to 1 M did not improve its protective action. Replacing phosphate buffer + sucrose by 2 or 4 M-glycerol in phosphate buffer did not result in higher survival of the aerosol sample.

Table 2. *The effect of spray fluid and collecting fluid on the 25 min. aerosol survival of Escherichia coli* JEPP *in nitrogen at 26·5°.*

		Collecting fluids			
		A	B	C	D
RH (%)	Spray fluid	Survival (%)			
90	Distilled water	0·82	2·3	—	—
	0·3 M-glycerol	25	75	—	—
	1 M-glycerol	29	76	72	24
80	Distilled water	0·5	1·5	—	—
	0·3 M-glycerol	24	58	—	—
	1 M-glycerol	11	33	25	37
70	Distilled water	17	2·6	—	—
	0·3 M-glycerol	6·4	20	—	—
	1 M-glycerol	7·5	28	15	28
40	Distilled water	50	7·4	—	—
	0·3 M-glycerol	1·2	11	—	—
	1 M-glycerol	1·0	6·3	3·1	5·1

Collecting fluids: A, phosphate buffer, B, phosphate buffer + M-sucrose; C, phosphate buffer + 2 M-glycerol; D, phosphate buffer + 4 M-glycerol.

DISCUSSION

Cox (1966*a*) showed that the survival of *Escherichia coli* B, *E. coli* JEPP and *E. coli commune*, when sprayed from suspension in distilled water or raffinose solution into nitrogen, was less at high RH than at low RH, *E. coli* JEPP being particularly unstable at high RH. The poorer survival at high RH was expressed as RH zones in which *E. coli* was especially unstable. The presence of raffinose in the spray fluid increased the survival values without completely eliminating the critical RH zones. This instability was shown, by the use of different collecting fluids or RH changes from the storage RH to 100% or 30% RH immediately before collection, to be caused by loss of viability during or following collection (Cox, 1966*a*, *b*). However, unlike *E. coli* B and *E. coli commune*, strain JEPP remained unstable in the region of 82% RH (Cox, 1966*b*). The present results show that a protective agent having a permeability different from that of raffinose, modified the response of survival to RH. Glycerol, being able to penetrate the cytoplasmic membrane, was the best protective agent at high RH, but at low RH was inferior to distilled water, and in this sense could be considered to be toxic. However, when compared with the survival of *E. coli* JEPP sprayed from distilled water into air (Cox, 1966*a*), glycerol would be said to have a slight protective action. This apparent anomaly is because two different phenomena are occurring: in air, oxygen is toxic at low RH for *E. coli* (to be published) and glycerol protects to some degree against this stress.

Good survival at high RH can be obtained by addition of glycerol to the spray fluid, together with a shift in RH value to 100% before collection, while at low RH either raffinose (Cox, 1966*a*, *b*) or sodium glutamate are better protective agents than glycerol. Since protection of *E. coli* JEPP at high RH appears to require an agent able to permeate the cytoplasmic membrane, components of the cytoplasm seem to be implicated in decreased survival at high RH value. At low RH an agent such as

raffinose, which does not penetrate the cell wall, protects (Cox, 1966*a*, *b*). Hence different lethal mechanisms seem to be operating at high and low RH for *E. coli* JEPP stored in nitrogen; however, these mechanisms must be influenced by the manner in which water re-enters the bacteria following collection as shown by the effects of the collection procedures. *E. coli* B can also die through different lethal mechanisms at high and low RH, as shown by Cox & Baldwin (1966). Like *E. coli* JEPP, *E. coli* B and *E. coli commune* survival can also depend upon the way in which water re-enters the bacteria during collection (Cox, 1966*a*, *b*). This feature of collection is not confined to aerosol droplets (about 1 μ diam.) but also applies to comparatively large droplets (about 100 μ diam.) supported upon fine glass fibres (Cox, 1965; Silver, 1965).

The author thanks Mr I. H. Silver for his interest and advice and thanks Mr C. M. Saunders for technical assistance.

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The Toxic Effect of Oxygen Upon the Aerosol Survival of *Escherichia coli* B

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(*Accepted for publication* 19 *April* 1967)

SUMMARY

At high relative humidity (RH) similar survivals were obtained for storage in oxygen, air or nitrogen. At low RH the survival in nitrogen was much greater than that in air or in a 20% (v/v) oxygen + 80% (v/v) nitrogen mixture, in which the survivals were similar. In oxygen alone, survival was even lower than in air. Hence oxygen, or a trace contaminant in it, was responsible for the poorer survival in air than in nitrogen. The mechanism of death caused by oxygen is discussed.

INTRODUCTION

The aerosol survival of *Escherichia coli* has been shown by Cox (1966*a*, *b*; 1967) to depend upon several variables. Amongst these was the nature of the atmosphere, in that at low relative humidity (RH), but not at high RH, survival was much greater in nitrogen than in air (Cox, 1966*a*; Cox & Baldwin, 1966). The previous suggestion that this survival difference was owing to the toxic action of oxygen (Cox, 1966*a*) is now examined. Hess (1965) found that at low RH, but not at high, oxygen caused enhanced death of aerosolized *Serratia marcescens* and *Escherichia coli* B.

METHODS

The methods used were as previously reported (Cox, 1966*a*) except that in all experiments a rotating drum container (Goldberg, Watkins, Boerk & Chatigny, 1958) was used for storing the aerosol cloud, and only *Escherichia coli* B sprayed from distilled water was studied. The drum apparatus used for the present work was similar to that used by Cox (1966*a*).

RESULTS

Results are given in Table 1 and show that at high RH similar survivals were obtained for storage in oxygen (> 99·5%), air or nitrogen (> 99·9%). At low RH the survival in nitrogen was much greater than in air or in a 20% (v/v) oxygen + 80% (v/v) nitrogen mixture, in which the survivals were similar. In oxygen alone survival was even lower than in air. Hence oxygen, or possibly a trace contaminant in it, was responsible for the poorer survival in air than in nitrogen. Analytical data of the oxygen did not suggest any obvious contaminants that were likely to have a toxic action at the parts per million concentrations at which they were present. However, such possibilities are not excluded completely since oxidizing agents such as ozone and the oxides of nitrogen at concentrations of a few parts per million might be toxic for *Escherichia coli* B in aerosols at low RH values. Also, oxygen + other

oxidizing agents might have a marked synergistic effect. It is possible therefore that survival in air might be greatly affected by its composition, which might vary appreciably from place to place.

Table 1. *The aerosol survival of Escherichia coli* B *sprayed from distilled water into different gas phases*

RH* value	Gas phase	Aerosol storage time (min.) 0	15	30	60
		Survival (%)			
90	Oxygen	100	27	11	8
90	Air	100	22	12	8
90	Nitrogen	100	20	14	11
40	Oxygen	100	7	2	1
40	Air	100	40	24	14
40	80% (v/v) Nitrogen +20% (v/v) oxygen	100	32	16	11
40	Nitrogen	100	90	85	80

* Storage relative humidity.

DISCUSSION

The results presented here and in the papers of Cox (1966*a*), Cox & Baldwin (1966) and Anderson (1966) for the aerosol survival of *Escherichia coli* B in air at low RH show discrepancies in the degree of survival. These occurred even though similar methods of growth, aerosol generation, storage, collection and assay were used. In nitrogen, similar survival values were obtained; the 30 min. survival of 85% (Table 1) compared favourably with that of 84% reported by Cox (1966*a*). These comparisons suggest that other factors operate to modify the toxic action of oxygen. One of these factors might be the exact composition of the air used.

Oxygen toxicity is not confined to *Escherichia coli* and *Serratia marcescens* in aerosols, since freeze-dried *E. coli* is also susceptible to oxygen poisoning (Lion & Bergmann, 1961*a*, *b*; Lion, 1963) as is *S. marcescens* (Bateman, McCaffrey, O'Connor & Monk, 1961; Benedict *et al.* 1961; Dewald, 1966*a*, *b*). To consider the mechanism of death caused by oxygen the following observations are relevant. Experiments in conjunction with Dr B. B. Singh (Royal Military College of Science, Shrivenham) using electron spin resonance showed that a free-radical reaction mechanism was involved for *E. coli* B in the freeze-dried state in air. Under similar conditions free radicals were detected by Dimmick, Heckley & Hollis (1961) and by Lion, Kirby-Smith & Randolph (1961). Such a mechanism may also occur for bacteria in the aerosol. Benbough (1967) suggested that the action of oxygen on *E. coli* B in the aerosol and in the freeze-dried state caused damage to flavin-linked enzymes. Free radicals may be involved, since metabolic inhibitors and free-radical scavengers protected *E. coli* B sprayed from distilled water into air at low RH (Benbough, 1967). It is perhaps surprising that *E. coli* B which suffered this damage and also suffered a temporary and severe loss of ability to synthesize β-galactosidase (Anderson, 1966) as well as a loss of control of potassium ions (Anderson & Dark, 1967) was able to reproduce phage T7 to a marked extent (Cox & Baldwin, 1964, 1966; Webb, Dumasia & Singh Bhorjee, 1965). This production of phage was very much greater than the ability of the bacteria to

form colonies. Since DNA, RNA and protein synthesis are required for phage production these processes must occur in *E. coli* B killed by oxygen. Therefore the damage reported by Anderson (1966), Benbough (1967) and Anderson & Dark (1967) may not have been directly implicated in the inability of *E. coli* B to replicate when aerosolized from distilled water and recovered from air at low RH. As mentioned previously in this paper, more than one death mechanism operates for *E. coli* in the aerosol. That caused by oxygen operated differently to the other death mechanisms which occurred at high RH, as shown by Cox (1966*a*, *b*; 1967) and by Cox & Baldwin (1966); Benbough's (1967) results supported these findings. These other death mechanisms, independent of the presence of oxygen, are not discussed here; they are reviewed by Anderson & Cox (1967).

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The Presence of Type 12 M-Protein Antigen in Group G Streptococci

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SUMMARY

Three strains of group G streptococci isolated from a community in which glomerulonephritis is common were found to have an M-protein antigen indistinguishable from the type 12 M-protein of group A, type 12, streptococci.

INTRODUCTION

The M-antigen of *Streptococcus pyogenes* (the group A streptococcus) is an essential factor in the virulence of the organism as indicated by its ability to kill mice and to multiply in human blood; and antibodies to M-protein form the basis of type-specific immunity (Lancefield, 1962). Other protein antigens, such as the T and R antigens, which are commonly present in *S. pyogenes*, may also be found in streptococci of other Lancefield groups (Lancefield & Dole, 1946; Crowley, 1944; Maxted, 1949, 1953). The M-proteins, however, are generally believed to occur only in members of Lancefield group A and in variants of that group which have lost their A-carbohydrate (Wilson, 1945; McCarty & Lancefield, 1955).

During the bacteriological investigation of a large epidemic of acute glomerulonephritis in Trinidad in 1965 (Simon *et al.* 1965), three strains of group G streptococci were isolated which appeared to possess the group A, type 12, M-protein. These three cultures formed part of a collection of streptococci of groups A, C and G isolated by one of us (E.P.) from patients with glomerulonephritis and from non-nephritic school children. The laboratory in Chicago found that one of the group G streptococci gave a strong precipitin reaction with M 12 antiserum. The M 12 antigen in this strain and in the other two cultures described in this report was detected independently at Colindale during an investigation into the specificity of a highly absorbed fluorescent conjugate of group A, type 12, antiserum (Heimer, 1966). Subsequently, two additional strains have been isolated and identified in Chicago. Because of the association with acute glomerulonephritis of group A streptococci which possess the M 12 antigen (Rammelkamp & Weaver, 1952; Wilmers, Cunliffe & Williams, 1954), it was of some importance to establish precisely the nature of the antigen present in these group G streptococci.

METHODS

Streptococci. Group G strains. Culture no. 2832 was isolated from the throat of a child with acute glomerulonephritis in San Fernando General Hospital, Trinidad. (A group A streptococcus, with no detectable M-antigen, but with the T-agglutination pattern 3/13/B3264, was isolated from a skin sore of the same patient.) Cultures 2439 and 2440 were isolated from skin sores of children without nephritis during a school survey also in the San Fernando area. These three organisms had the type 12 antigen and will be referred to as G12.

Group A strains. Culture no. 1077 was a type 12 laboratory strain originally isolated from a case of glomerulonephritis. Culture no. 41448 was a mouse-virulent laboratory strain, also of type 12, originally isolated from a case of scarlet fever. Strain NCTC 8305 (type 24) was used as a control in bactericidal tests.

Media. A modified Todd–Hewitt broth was used; it was a meat infusion with buffer and glucose added, and the whole sterilized by heat.

Blood agar was Hartley digest agar + 7 % (v/v) horse blood.

Bactericidal tests. Streptococci capable of multiplying in heparinized normal human blood are killed when small amounts of type-specific antiserum containing M-antibody are added. The test used here was essentially that described by Maxted (1956), except that the mixtures were rotated end-over-end at 37° and 0·02 ml. samples inoculated into blood agar pour plates after 3–4 hr.

Antisera. These were made by injecting rabbits intravenously with washed whole-cell vaccines made by concentrating 18 hr broth cultures tenfold and heating them to 60° for 1 hr. A 1 ml. dose of this vaccine was given intravenously on two successive days each week for 8 weeks.

Antigen extracts. The overnight growth from 50 ml. of broth was extracted with 0·6 ml. 0·2 N-HCl in boiling water for 10 min., cooled, neutralized with 0·2 N-NaOH and centrifuged until clear.

Precipitin tests were done in capillary tubes by the method first described by Swift, Wilson & Lancefield (1943).

Absorption of sera. For absorption with whole organisms, equal volumes of packed bacteria and serum were mixed, left for 4 hr at 37°, and then centrifuged until clear.

Absorption with extracted antigen was done as follows. Four volumes of ethanol (95 %, v/v in water) were added to 1 volume of antigen extract. After centrifugation, the supernatant fluid was removed and the precipitate taken up in 1 volume of serum. The mixture was left at 37° for 2 hr and then at 4° overnight before centrifuging until clear.

Double gel-diffusion. Five ml. of 1 % (w/v) 'Oxoid' Ionagar No. 2 in water at pH 7.4 was poured into Petri dishes of 2 in. diameter. Wells 4 mm. in diameter were arranged radially 5 mm. from a centre well.

Mouse-protection tests. White mice (18–20 g.) were given 0·25 ml. of serum intraperitoneally and challenged 24 hr later with 0·5 ml. of a 16 hr broth culture suitably diluted in saline with 10 % (v/v) of broth added.

Fluorescent staining. Smears were made directly from 16 hr blood agar plate cultures, stained with a highly specific fluorescent conjugate of a group A, type 12, antiserum, and examined microscopically with illumination from a Mazda ME/D 250 W. mercury vapour lamp and a chance Pilkington O.X. 1 excitation filter with an Ilford 805 Q barrier filter.

RESULTS

Capillary precipitin tests were done with acid extracts of the three group G strains and all the M-antisera for *Streptococcus pyogenes* types available at Colindale (types 1–3, 5, 6, 9, 11, 12, 14, 15, 17–19, 22–26, 29–31, 33, 36, 37, 39, 41, 43, 46–51). All three precipitated with type 12 antiserum and with no other.

The extracted antigen from the group G strain 2832 was precipitated with ethanol and used to absorb a sample of group A, type 12, M-antiserum made with strain 1077. The serum before and after absorption was tested against an acid extract of strain 1077. The antigen from the group G strain completely removed the type 12 precipitating antibody from the serum (Table 1).

Table 1. *Removal of precipitating antibody, and of bactericidal power from a streptococcus group A, type 12, by absorption with streptococcus group G, type 12, protein*

Bactericidal test: 0·3 ml. blood + 0·02 ml. serum + 0·02 ml. culture inoculum.

	Precipitation with antiserum		Bactericidal test			
			Inoculum total colony count	Colony count in 0·02 ml. sample after 3 hr incubation with serum		
Streptococcal strain	Group A, type 12	Group A, type 12, absorbed with G 12 protein		Normal rabbit	Group A, type 12	Group A, type 12, absorbed with G12 protein
1077, group A, type 12	++	–	40	80	0	55
			400	T.N.C.*	6	T.N.C.
8305, group A, type 24	–	–	55	180	80	350
			550	T.N.C.	T.N.C.	T.N.C.

* T.N.C. = Colonies too numerous to count.

If this were a true M-antigen-antibody system, the bactericidal power of the serum, when added to normal human blood, should also be removed by the group G extract. Normal rabbit serum and group A, type 12, antiserum, before and after absorption with G12 antigen, were each mixed with samples of heparinized normal human blood, and the killing efficiency of these mixtures tested against group A, type 12, streptococci. The bactericidal power of the group A, type 12, serum was removed by absorption with the G12 antigen from strain 2832 (Table 1). The group A, type 24, strain used as control grew unrestrictedly in all blood + serum mixtures.

The three group G12 strains were each used as inocula in bactericidal tests with similar blood + serum mixtures and proved equally susceptible to the killing efficiency of group A, type 12, antiserum.

Antisera were made against each of the three group G, type 12, strains and tested for precipitating antibody against stock M-extracts of all known types of group A streptococci. The group A, type 12, extract reacted well with each of these sera, but no extract of any other type gave a similar precipitate. The sera were also tested for their bactericidal power against a group A, type 12, strain, a group A, type 24, strain, and also against each of the group G vaccine strains. The bactericidal action of two of

the antisera against the A 12 and each of the G 12 strains was good. The third antiserum, made against strain 2832, was not so bactericidal, but showed some bacteriostatic effect. The results were reproducible with blood from several donors; one such test is shown in Table 2. In this test a single inoculum of each test strain was used throughout.

It was possible that the group G strains might have two M-protein antigens, the A 12 and another found only among group G strains. If this were so, and the group G cocci surviving in the bactericidal system did so because they possessed this second antigen, a mixture of the group A, type 12, antigen and the G 12 antiserum in a single bactericidal system might be expected to give greater killing. Bactericidal tests done with such a mixture of antisera showed no greater killing than with either antiserum alone.

Table 2. *A comparison of the bactericidal power of streptococcus group A, type 12, antiserum and antisera prepared against streptococcus group G, type 12, strains*

All samples received a single inoculum of the strain tested. This was 0·02 ml. of an 18 hr culture diluted 10^{-4} and averaged 200–300 organisms for each strain.

Streptococcus			Antisera made with strains			
Strain	Group and type	Normal rabbit serum	2439 G 12	2440 G 12	2832 G 12	1077 A 12
			Colony numbers			
41448	A 12	T.N.C.*	31	1	1500	2
J17D	A 24	T.N.C.	T.N.C.	T.N.C.	T.N.C.	T.N.C.
2439	G 12	T.N.C.	64	25	1000	23
2440	G 12	T.N.C.	40	8	350	0
2832	G 12	T.N.C.	46	45	400	38

* T.N.C.: colonies too numerous to count.

Gel-diffusion tests were done to show the identity of the type antigens of the group G and group A organisms. With group G, type 12, antiserum in the centre well and extracts of the A 12 and G 12 strains in the peripheral wells a strong and continuous line of identity was seen. When each of the G 12 antisera was tested in the centre well against similar extracts, a continuous line of identity was again seen, as well as several other lines of precipitation with the G 12 extracts (probably due to the group antibody, since these antisera were unabsorbed).

It has been shown repeatedly that anti-M sera, when injected into mice, give excellent passive protection against a subsequent challenge with a virulent group A streptococcus of homologous type. Antisera against the G 12 strains should therefore protect mice challenged with group A, type 12, streptococci. The group A and the group G antisera were tested against strain 41448 (group A, type 12). Mice in groups of 20 were each given 0·25 ml. of antiserum intraperitoneally 18 hr before the inoculation of the virulent streptococci. The test was done with two dilutions of the challenge strain. The G 12 antisera protected mice as effectively as the group A, type 12, antiserum against fatal infection with the virulent group A, type 12, streptococci. The results of a representative test with one G 12 serum are shown in Table 3.

Because of the widely held belief that M-antigens are to be found only in group A streptococci, they have probably not been looked for extensively in members of other groups. A survey was therefore made of a larger number of group G strains, both from

stock and freshly isolated, including many from Trinidad. The fluorescent staining, which had proved so sensitive and successful originally, was used as a screening test on 140 group G and 10 group C strains. Thirty-seven of the group G and three of the group C strains, all isolated in Trinidad, were also extracted with acid and tested by the capillary precipitin test against all the available group A anti-M sera. No other strains were found which showed any relationship with group A organisms.

Table 3. *The ability of streptococcus group A, type 12, antiserum and antiserum made against a streptococcus group G, type 12, strain (2440) to protect mice challenged with a virulent group A, type 12, streptococcus*

Challenge doses. One-half ml. of 16 hr broth culture diluted in saline + 10% (v/v) broth. One-half ml. of a 10^{-5} dilution of culture contained approximately 750 organisms.

Serum used for protection	Dose of challenging strain 41448 type 12 (dilution)	Time after challenge (days) Deaths in each batch of 20 mice										Total deaths
		1	2	3	4	5	6	7	8	9	10	
Normal rabbit	10^{-5}	1	1	2	4	0	0	0	1	0	0	24/40
	10^{-3}	11	3	—	1	—	—	—	—	—	—	
Group G, type 12 (strain 2440)	10^{-5}	0	0	0	1	0	0	0	0	0	0	4/40
	10^{-3}	0	0	3	0	0	0	0	0	0	0	
Group A, type 12 (strain 1077)	10^{-5}	0	0	0	0	0	0	0	0	0	0	3/40
	10^{-3}	0	0	1	1	0	0	0	0	0	1	

Experiments were made to see whether the G12 strains were in fact group A strains with the unusual ability of synthesizing group G cell-wall or were true group G strains which formed type 12 M-protein.

(1) A cell-wall analysis of the three G12 strains, kindly done by Mr G. Colman by the techniques applied by him to a large variety of streptococci (see Colman & Williams, 1965) showed the presence of galactosamine and galactose, both characteristic of the group G streptococcal cell-wall but not found in group A streptococci.

(2) The phage-associated lysin (Maxted, 1957; Krause, 1958) which attacks the cell-wall of viable group A streptococci did not lyse the three G12 strains.

(3) Group A strains resistant to bacitracin in the 'differentiation' disc test (Maxted, 1953) are, in our experience, very rare. All three of the G12 strains were resistant to bacitracin.

(4) In an extensive experience of routine serological typing of *Streptococcus pyogenes*, we have found that organisms with the M12 antigen are nearly always agglutinated by antisera prepared against the T antigen of type 12 or type 10. Suspensions of the G12 strains were not agglutinated by antisera containing the T antibodies of type 12 or type 10.

(5) Virulent and temperate phages which had been propagated on group A, type 12, streptococci and shown to be active on the majority of group A strains of type 12, did not lyse any of the three group G, type 12, strains.

DISCUSSION

The evidence presented here suggests that strains 2832, 2439 and 2440 belong to Lancefield group G, but have the ability to synthesize type 12 M-antigen. It now seems

possible that an infection with these or similar group G streptococci might result in the production of protective antibody against certain members of group A, a possibility which had not previously seemed to need consideration. The ability of these group G strains to establish an infection is, however, uncertain. Two of the strains were isolated from skin lesions, but there is no certainty that they were the cause of them, since the presence of two or more different streptococci in the skin lesion of the same patient is not uncommon (Barrow, 1961; Dr D. J. C. Bassett, personal communication; Anthony, Perlman & Wannamaker, 1967). The third strain was isolated from the throat of a patient suffering from glomerulonephritis, but a group A streptococcus was isolated concurrently from a skin lesion. There is no conclusive evidence that the M-antigen of the group A, type 12, streptococcus is responsible for its ability to cause glomerulonephritis. The possibility cannot be excluded, however, that the nephritogenic factor might be associated closely with the type 12 antigen either in *Streptococcus pyogenes* or in some other group of streptococci. The significance of these findings will remain uncertain until more is known about the ability of the various strains of *S. pyogenes* isolated in Trinidad to cause glomerulonephritis. It is certain, however, that group G streptococci with the M 12 antigen form only a tiny minority of the streptococcal strains to be found in skin lesions and in the respiratory tract in Trinidad, and it is therefore unlikely that they are a common cause of either impetigo or glomerulonephritis. Their ability to multiply in whole human blood suggests, however, that they may be potentially virulent for man.

The G 12 organisms may prove useful in genetic studies of streptococci, but it is not yet possible to speculate about their origin. In general, it is our experience that strains of *Streptococcus pyogenes* showing undoubted evidence of M 12 antigen are rarely isolated in Trinidad. At the Colindale laboratory we have recently examined serologically over 1000 cultures of *S. pyogenes* from Trinidad and did not find the M 12 antigen in any of them. Several were agglutinated by the T 12 antiserum, but they formed only a small minority of the strains isolated from cases of glomerulonephritis. Although we made a search for M-antigens among group G strains, the survey was a small one and was mainly for type 12 M-antigen, but there is no reason why M-antigens of other types should not be found. A more extensive search for M-antigens in streptococci of other Lancefield groups might be rewarding.

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The Accumulation of Extracellular Macromolecules by *Staphylococcus aureus* Grown in the Presence of Sodium Chloride and Glucose

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SUMMARY

When grown in broth cultures containing sodium chloride and glucose, *Staphylococcus aureus* JHM produced protein, RNA and DNA in the culture fluid. Maximum yields were obtained with about 4% (w/v) sodium chloride and 0·1% (w/v) glucose. Accumulation of macromolecules began at the end of exponential growth and was accompanied by a decrease in culture turbidity. Electron microscopy revealed considerable cellular lysis, arising from rupture of newly formed septa. Before lysis occurred, the cocci exhibited many morphological abnormalities. After incubation for about 15 hr the culture showed evidence of fresh growth and the resulting cocci were characterized by abormally thick and uneven walls.

The results suggest that there was interference with the regulation of cell-wall synthesis, perhaps due to loss of mesosomes, which were never seen in the cytoplasm of organisms grown in sodium chloride glucose broth.

INTRODUCTION

Cultures of *Staphylococcus aureus* have been shown to produce both extracellular deoxyribonucleic acid (DNA) (Catlin & Cunningham, 1958) and an extracellular deoxyribonuclease (Micrococcal Nuclease, EC 3.1.4.7; Cunningham, Catlin & Privat de Garilhe, 1956). Accumulation of extracellular DNA occurred only under conditions where the nuclease was inactivated, this being achieved by adding sodium chloride to the culture medium. The nuclease has also been reported to be active against ribonucleic acid (RNA) (Reddi, 1961; Anfinsen, Taniuchi, Heins & Suriano, 1965). Catlin & Cunningham (1953) described extracellular DNA mainly as a slime-layer, obtainable by treatment of the sedimented organisms with detergent, but found that a DNA-containing precipitate was produced by adding ethanol to the culture supernatant fluid. The slime-layer DNA fraction was designated extracellular-1 and the DNA fraction which was free in the culture supernatant fluid was designated extracellular-2.

The present work was undertaken to investigate further the time course and mechanism of excretion of DNA and other macromolecules by the strain JHM of *Staphylococcus aureus*. The fractions, prepared as described by Catlin & Cunningham (1958), were found to contain RNA and protein, in addition to DNA. Electron microscopy of cocci grown in sodium chloride + glucose broth showed gross morphological abnormalities which might provide a clue to the mechanism of so-called 'excretion'.

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METHODS

Organism. Staphylococcus aureus strain JHM was obtained from Dr P. J. White: it originated from the laboratory of the late Professor D. D. Woods. Stock cultures were maintained on nutrient agar slopes, stored at 2° and subcultured monthly.

Culture medium. The organism was grown in Oxoid nutrient broth No. 2, which contains 0·5 % (w/v) sodium chloride, with glucose and extra sodium chloride added as indicated. Media were inoculated with organisms grown for 17 hr in nutrient broth No. 2 without any addition. Cultures were grown at 37° in 25 ml. or 250 ml. media in 250 ml. or 2 l. vessels, respectively, being shaken on a gyro-rotary shaker.

Extraction of extracellular-1 and extracellular-2 fractions. Organisms were harvested by centrifugation and resuspended in 4 % (w/v) aqueous NaCl solution. To obtain the extracellular-1 fraction, sodium dodecylsulphate was added to the coccal suspension to a concentration of 0·25 % (w/v). The mixture was then stirred at room temperature for 3 hr, centrifuged, and the cell pellet discarded. The supernatant solution was cooled in ice and two volumes of industrial ethanol at 2° added. The resulting fibrous precipitate was recovered by centrifugation and dissolved in distilled water. The extracellular-2 fraction was obtained by addition of 2 volumes of industrial ethanol to the original culture supernatant fluid, before addition of dodecyl sulphate, both liquids being at 2°. The precipitate was collected by centrifugation and dissolved in distilled water. The two fractions were analysed without further purification. When nutrient broth itself was treated with ethanol, a precipitate was produced, which, on analysis, gave positive results for DNA, RNA and protein, and presumably consisted of ethanol-insoluble oligonucleotides and peptides. The amounts of precipitable materials varied from batch to batch, and were corrected for where indicated.

Analytical methods. DNA was determined by the method of Burton (1956), protein by the method of Lowry, Rosebrough, Farr & Randall (1951) and RNA by the orcinol method as described by Morse & Carter (1949). Aqueous solutions of highly polymerized calf thymus DNA and yeast RNA (from The British Drug Houses Ltd., Poole, England) and ovalbumin (from Koch–Light Laboratories Ltd., Colnbrook, Buckinghamshire), were used as references in the chemical analyses. No correction was made for the participation of DNA in the RNA estimation since the amount of RNA present in the fractions was always much greater than that of DNA. The activity of the extracellular deoxyribonuclease (EC 3.1.4.7) in the extracellular-2 fraction was determined as described by Alexander, Heppel & Hurwitz (1961) except that the glycine buffer used was at pH 10·0.

Cell walls Cell walls were prepared and analysed as described by Allsop & Work (1963) with the additional step of heating the capsule for 15 min. at 60° immediately after breaking the organisms.

Optical measurements. Extinctions were determined in SP500 or SP600 Unicam spectrophotometers. The turbidity of cultures diluted 1/5 with water was determined by measuring the extinction at 600 mμ, only slightly higher values being obtained when the culture was diluted with sodium chloride solutions of the same concentration as in the culture medium.

Electron microscopy. Organisms to be studied with the electron microscope were fixed by centrifugation from the culture in the presence of 2 % (v/v) glutaraldehyde, washed for 30 min. in 0·1 M-phosphate buffer (pH 7), and finally fixed overnight in

1 % (w/v) osmium tetroxide in 0·1 M-phosphate buffer (pH 7). Samples were dehydrated and embedded in Epon 812 as described by Luft (1961). Ultrathin sections were cut with a Huxley Microtome and were stained with lanthanum nitrate and, sometimes, lead citrate. Specimens were examined with a Philips 10c electron microscope working at 60 kV.

RESULTS

Relative amounts of extracellular-1 and extracellular-2 fractions

Staphylococcus aureus JHM was grown for 48 hr in nutrient broth No. 2 to which 1 % (w/v) glucose + 4 % (w/v) NaCl had been added (total NaCl concentration 4·5 % w/v), and the compositions of the two extracellular fractions were compared (Table 1). Both fractions contained DNA, RNA and protein, DNA being in the lowest concentration. The amount of materials free in the culture supernatant fluid was at least 10 times greater than that attached to the cocci. It would appear that in this strain, the slime-layer, if one exists in the accepted sense, contributed relatively little to the total extracellular material. In all subsequent experiments, macromolecules were estimated only in the extracellular-2 fraction.

Table 1. *Amounts of DNA, RNA and protein in the extracellular fractions from a culture of Staphylococcus aureus strain* JHM *grown for 48 hr in nutrient broth No. 2 + 1 % (w/v) glucose + 4 % (w/v) NaCl*

	DNA	RNA	Protein
	mg./l. culture fluid		
Extracellular 1	5·4	22·2	17·4
Extracellular 2*	42·4	274	190

* Corrected for the amounts of reacting materials precipitated from broth before inoculation.

Effect of sodium chloride and glucose on accumulation of macromolecules in cultures

Organisms were grown for 24 hr in nutrient broth containing 1 % (w/v) glucose and different concentrations (0·5 to 6·5 %, w/v) of NaCl. Immediately before removing the organisms, solid NACl was added to the cultures to bring the concentration in all flasks to 6·5 % (w/v). Extracellular-2 fractions were then prepared from the culture supernatant fluids and analysed. The results (Fig. 1) indicated that, for all three macromolecules, maximum extracellular accumulation occurred at 2·5–4·5 % (w/v) NaCl. In the absence of added NaCl, the concentrations of nucleic acids were very low, but a sharp increase occurred when the NaCl concentration was increased to 2·5 % (w/v). A marked decrease in extracellular nucleic acids was noted on increasing the NaCl concentration from 4·5 % to 6·5 % (w/v). Protein accumulation showed a similar NaCl dependence, but the concentration with no added NaCl was considerably higher than the nucleic acid concentrations. After incubation for 24 hr, the protein concentration was greater than the RNA concentration, but, on prolonged incubation, loss of protein occurred and at 48 hr less protein than RNA was present.

The effect of glucose concentration on accumulation was investigated by growing the organisms for 48 hr in nutrient broth containing 4·5 % NaCl to which sterile glucose

was added to give various final concentrations (0–0·3 %, w/v). Analysis of the extracellular-2 fractions showed (Fig. 2) that accumulation of all three macromolecules in the culture increased with increasing glucose concentrations up to 0·1 % (w/v), after which the concentrations stayed essentially constant. Some accumulation occurred even in the absence of glucose. Increasing glucose concentrations had less effect on DNA accumulation than on RNA or protein.

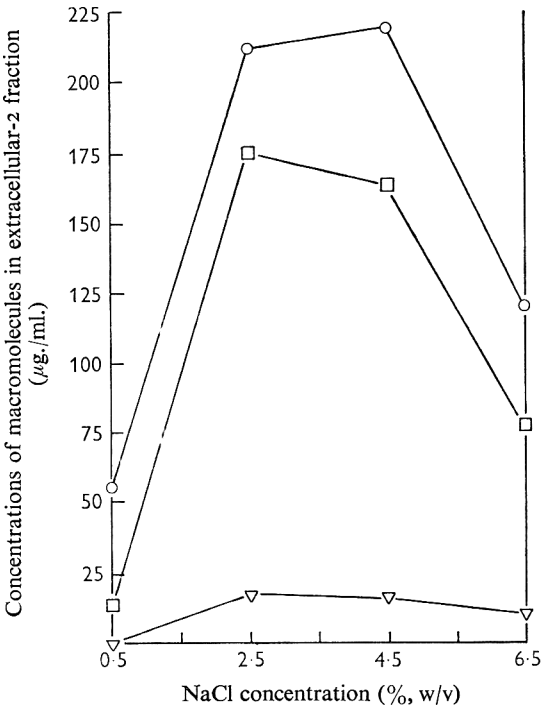

Fig. 1

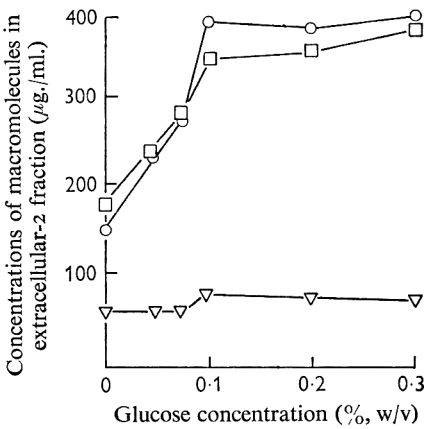

Fig. 2

Fig. 1. The effect of NaCl concentration in the culture medium on the concentrations of DNA (▽—▽), RNA (□—□) and protein (○—○) found in the extracellular-2 fraction after incubation for 24 hr. Glucose constant in all cultures at 1 % (w/v). All values are corrected for the amounts of reacting materials precipitated from broth before inoculation.

Fig. 2. The effect of glucose concentration in the culture medium on the concentrations of DNA (▽—▽), RNA (□—□) and protein (○—○) found in the extracellular-2 fraction after incubation for 48 hr. NaCl constant in all cultures at 4·5 % (w/v). Values not corrected for the amounts of reacting materials precipitated from broth before inoculation.

Growth of the organism and kinetics of excretion of macromolecules

Preliminary experiments indicated unusual growth characteristics of this organism when it was grown in nutrient broth containing glucose and sodium chloride. To exclude the possibility that this might have been due to a mixed population, nutrient broth, containing 4·5 % (w/v) NaCl and 1 % (w/v) glucose, was inoculated with a culture grown up from a single colony. At intervals during the incubation, samples of culture were taken, the extinction determined (Fig. 3) and extracellular-2 fraction isolated and analysed (Fig. 4).

The turbidity curve showed several unusual features. After a short lag period of

about 2 hr, followed by 2·5 hr of exponential growth, the turbidity decreased until 8 hr. As soon as exponential growth ceased (4·5 hr), the concentration of RNA in the extracellular-2 fraction started to increase. Protein began to accumulate at 4 hr, earlier than the nucleic acids and before the cessation of exponential growth, but most of it appeared during the phase of turbidity decrease. From 8 hr to about 15 hr the turbidity remained constant. The cultures, during this time, usually showed visible clumps in an almost clear solution and it was impossible to relate turbidity to colony counts. From 8 hr onwards the concentrations of nucleic acids in the culture supernatant fluid remained constant, whereas the protein concentration decreased, presumably due to the action of excreted proteolytic enzymes. After about 15 hr the turbidity increased again, probably as a consequence of the onset of renewed multiplication. Unexplained variations of this pattern were sometimes observed, mainly in the rate and time of onset of decrease in turbidity (4–7 hr). There was little variation in the amounts of macromolecules which accumulated.

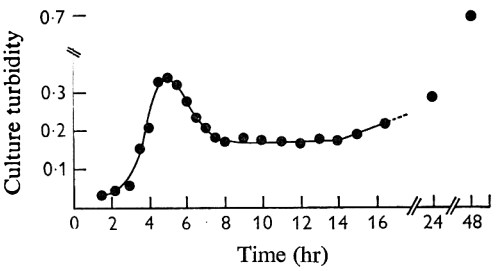

Fig. 3

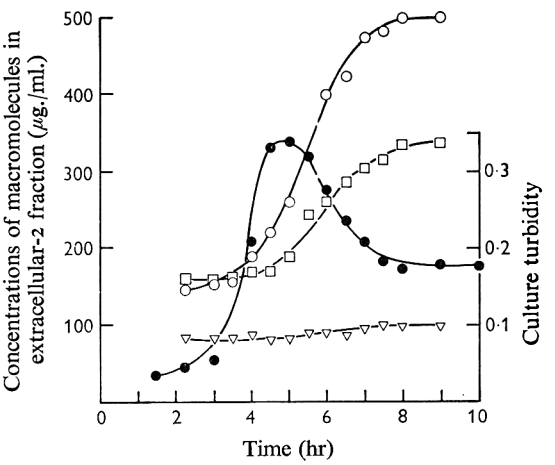

Fig. 4

Fig. 3. Turbidity curve of *Staphylococcus aureus* JHM growing in nutrient broth, to which 4 % (w/v) NaCl and 1 % (w/v) glucose had been added.

Fig. 4. The variation, as a function of time, of culture turbidity (●—●) and levels of DNA (▽—▽), RNA (□—□) and protein (○—○) in the extracellular-2 fraction when 4·5 % (w/v) NaCl is present in the culture medium.

A similar growth curve was observed in cultures grown with 6·5 % (w/v) NaCl. In this case, however, the initial lag phase was longer and the turbidity decrease less. In cultures grown in 2·5 % (w/v) NaCl, the turbidity increased to a higher value than was observed in cultures grown in higher concentrations of NaCl (Fig. 5). Exponential growth lasted only until 3 hr, after which the turbidity increased in a non-exponential manner, reaching a maximum at 6 hr, and then fell slowly. Accumulation of extracellular macromolecules began within 4 hr and continued until the turbidity stopped decreasing. The accumulation of DNA showed a similar pattern to that of RNA and protein, but the increase was, as usual, much less.

In the absence of added NaCl the growth curve was normal. Little or no extracellular nucleic acids were detected (Fig. 1), but protein, estimated in this case as

deoxyribonuclease activity in the extracellular-2 fraction, occurred in measurable amounts in the culture supernatant fluid soon after the onset of growth and increased until the end of growth, after which it decreased slowly.

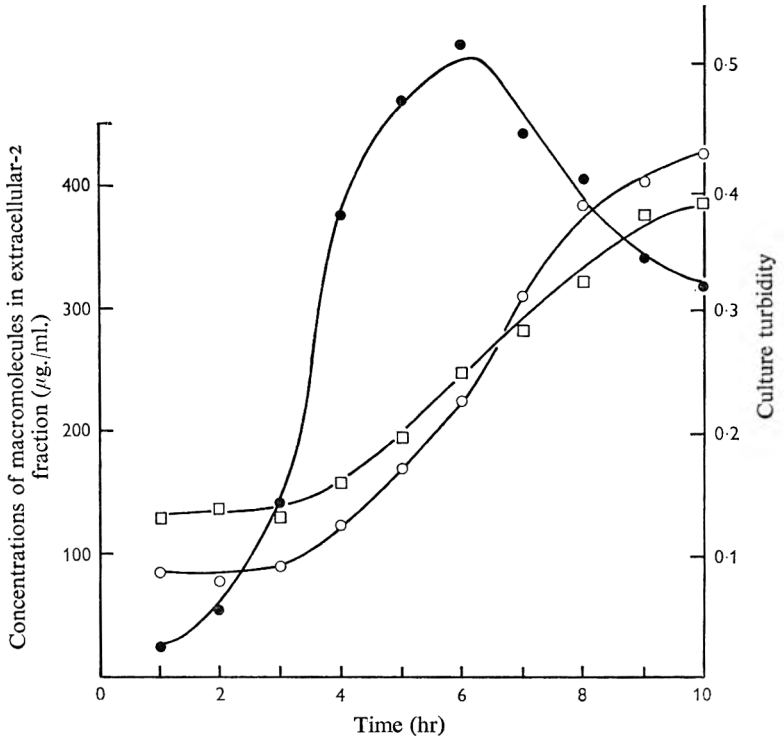

Fig. 5. The variation, as a function of time, of culture turbidity (●—●) and levels of RNA (□—□) and protein (○—○) in the extracellular-2 fraction when 2·5% (w/v) NaCl is present in the culture medium.

Morphology of bacteria grown in the presence of sodium chloride

Cocci growing in broth with 4·5% (w/v) NaCl + 1% (w/v) glucose were examined at intervals with the aid of an electron microscope. They were compared with normal cocci grown to late exponential phase without added NaCl but with glucose.

Normal cocci, sometimes seen in pairs (Pl. 1, fig. 1), were roughly spherical and approximately 1 μ in diameter. Intracytoplasmic membranous structures (mesosomes) were nearly always present. The walls were of constant thickness and the septa were well defined and regular. The addition of NaCl to this culture medium caused considerable changes in cellular morphology. Organisms harvested after 4·5 hr (at the end of exponential growth, when extracellular materials were beginning to accumulate, Fig. 4) had walls of irregular thickness (Pl. 1, figs 2, 3). Septum formation was abnormal, some organisms showing two incipient septa developing at the same time (Pl. 1, fig. 2). Also at this stage of growth, the cytoplasmic membrane of some organisms was seen to have contracted away from the cell wall (Pl. 1, fig. 3), the space so formed containing membrane-enclosed bodies of various sizes: some of the organisms themselves had irregular shapes. There were some cell structures nearly devoid of cytoplasm, but which

still contained numerous membrane-enclosed bodies, mostly aligned round the cytoplasmic membrane.

Cultures incubated for 5 hr (to the onset of the turbidity decrease) exhibited further unusual features (Pl. 2; Pl. 3, figs 9–11). There were very few organisms of normal size and shape and those that were observed had exceptionally electron-dense cytoplasm (Pl. 2, fig. 4). The walls were usually characterized by an irregular and somewhat diffuse appearance. There was much debris, often of a fibrous nature. Many organisms had a break in the wall through which cell contents were being lost in various ways. Most common was the emergence of membrane-bounded cytoplasm (Pl. 2, figs 5, 6) but some structures showed leakage of unbounded cytoplasmic material (Pl. 2, fig. 7). Liberation of membranous structures was also seen (Pl. 2, fig. 8). In all cases, extrusion of cell contents occurred through a break in the wall at a point on the plane of division. At this stage many more cocci were seen with the cytoplasmic membrane contracted from the wall (Pl. 2, figs 4, 7; Pl. 3, figs 9–11) and with the small membrane-enclosed bodies present between cytoplasmic membrane and wall as in Pl. 1, fig. 3; Pl. 3, fig. 9 shows these bodies escaping from the cell through the ruptured septum. Many organisms showed an abnormal feature in having more than one developing septum per cell (Pl. 3, fig. 10). In others, septum formation was complete but an additional 'half' septum was visible in one of the cells (Pl. 3, fig. 11). Also, there were cell-wall structures containing various membranous fragments, but no cytoplasm (Pl. 2, fig. 4).

After incubation for 7·5 hr when the turbidity had almost stopped decreasing, much of the material present consisted of fragments of cell-wall, membranes and fibrous debris (Pl. 4, fig. 13). Several structures of abnormal morphology were also seen. Some protoplast-like bodies were observed (Pl. 3, fig. 12; Pl. 4, fig. 13); their diameters varied between 0·8 and 2 μ and some of them contained vacuoles.

After 48 hr, when the second growth phase was nearing completion, the cocci were still not normal. They were all roughly spherical but their diameters varied from 0·7 to 1·5 μ. The walls were, in many cases, remarkably thick and uneven on the inside surface (Pl. 4, fig. 14). In the organisms undergoing division, the septa were often distorted and enlarged. Fragments of wall and other cellular debris were still visible. Another feature was the occurrence of electron-dense particles, about 0·1 μ in diameter, often aligned on the insides of fragments of membrane or surrounded by fibrous material (Pl. 5).

Cell-wall analyses

Cell walls were prepared from cocci grown for 48 hr in broth containing 1 % (w/v) glucose + 4·5 % (w/v) NaCl and were compared with those of normal cocci grown in broth with glucose (1 %, w/v) for 24 hr. No significant differences in amino acid or amino end-group analyses were found. The molar ratios of glutamic acid, alanine, lysine, glycine, glucosamine and muramic acid were approximately 1:2:1:5:1·2:1, similar to those reported for other Staphylococcus strains. The walls had similar phosphorus contents, but those from normal cocci had less carbohydrate (0·72 %, w/w) and more total hexosamines (24·2 %, w/w) than walls from NaCl-grown cocci (1·90 and 18·4 %, w/w, respectively). This suggests that the walls of the cocci grown in NaCl + glucose medium had a subnormal content of mucopeptide.

DISCUSSION

Many types of bacteria accumulate extracellularly macromolecules which would normally be expected to occur only inside the cell. For example, extracellular transforming DNA has been found in cultures of *Neisseria meningitidis* (Catlin, 1960) and of a pneumococcus (Ottolenghi & Hotchkiss, 1962). An extracellular material, containing mainly DNA but also some protein and RNA, has been obtained from *Micrococcus sodonensis* (Campbell, Evans, Perry & Niven, 1961), and excretion of all three macromolecules by *Bacillus subtilis* has also been reported (Demain, Burg & Hendlin, 1965). Slime-layers containing DNA are produced by *Micrococcus halodenitrificans* and *Vibrio costicolus* (Smithies & Gibbons, 1955). The mode of excretion of these macromolecules has not always been obvious, but it has often been assumed that lysis was responsible. In the present investigation on *Staphylococcus aureus* JHM growing in NaCl + glucose broth, observations of growth characteristics and cellular morphology showed that lysis took place at the time when extracellular accumulation of macromolecules was occurring. It is thus evident that, for this strain, a gross cellular lysis resulted in apparent excretion of these macromolecules. In view of this intracellular origin, the relatively low concentrations of DNA in the culture supernatant fluid as compared with RNA and protein are understandable, since normal cocci contain less DNA than RNA and protein.

It is probable that not all the protein observed in the culture supernatant fluids originated from lysis. It accumulated in the absence of NaCl when little or no nucleic acids were detectable (Fig. 1) and, in the presence of NaCl, it appeared earlier in the growth cycle than did DNA or RNA (Fig. 4). In cultures containing no added NaCl, part, at least, of the protein was micrococcal nuclease, the concentration of which increased as growth proceeded, and since under these growth conditions electron microscopy revealed no lysis, some other excretion process must have been operating.

Since staphylococci are known to be NaCl-tolerant, the lytic effect of NaCl needs explanation. NaCl is known to decrease the rate of hydrolysis of native DNA by extracellular deoxyribonuclease (EC 3.1.4.7; von Hippel & Felsenfeld, 1964; Dirksen & Dekker, 1960). This effect probably only aided the accumulation of precipitable nucleic acid in the culture supernatant fluid, but cannot have caused the lysis. The enzyme was still produced in the presence of NaCl, when it could be detected if the culture supernatant fluid was diluted.

Electron microscopy revealed that lysis involved extrusion of intracellular material from a point on the plane of cell division. At this point the developing septum evidently parted before it had been completed, causing rupture and subsequent loss of cell contents. Whether this was due to an interference with cell-wall synthesis, causing a break in the wall at the position where synthesis was proceeding at its maximum rate, is not known. The localized rupture might have resulted from an inhibition by NaCl of one of the many enzymes involved in cell-wall synthesis; however, no data are available on the NaCl sensitivity of these enzymes. Another possible effect of NaCl is the stimulation of an autolytic enzyme associated with the cell wall. Certain lytic enzymes which attack staphylococcal walls are known to require increased ionic concentrations for maximum activity and some of them have acid pH optima (Schindler & Schuhardt, 1965; Ghuysen & Strominger, 1963; Mitchell & Moyle, 1957); while a phage lysin, active against streptococcal walls, is able to operate in 1·1 M-NaCl

(Barkulis, Smith, Boltralik & Heymann, 1964). It is possible that NaCl and the low pH value resulting from the growth of the staphylococci in glucose may have stimulated one or more lytic enzymes. One such enzyme may serve to separate daughter cocci after septum formation. Other potentially lytic enzymes may open links in the complete mucopeptide structure to enable fresh mucopeptide subunits to be inserted (Weidel & Pelzer, 1964; Shockman, 1965); their over-activity might result in local wall rupture if synthesis of mucopeptide subunits or their transfer to acceptor sites were not sufficiently rapid to keep pace with degradation. Localization of lytic enzymes is known and is well illustrated by the hemispherical wall fragments produced by autolysis of *Staphylococcus aureus* (Mitchell & Moyle, 1957).

The existence, in the lysing cultures, of whole organisms with irregular walls, bizarre shapes and multiple septa suggests that there may have been interference with regulation of cell-wall synthesis and cell division. It is possible that the missing regulators are the mesosomes, since they were never observed in the cytoplasm of cocci grown in NaCl + glucose broth, but were present in all the normal *Staphylococcus aureus* JHM organisms. Ryter & Landman (1964) suggested that mesosomes play a part in regulating cell-wall and septum formation. Mesosomes are known to be lost from the interior of the cytoplasm during protoplast formation and plasmolysis (Fitz-James, 1964; Ryter & Landman, 1964; Weibull, 1965), this loss being associated with the presence of small membrane-bounded structures between the cytoplasmic membrane and the cell wall. Bodies, very similar to these in size and appearance, were found in a similar position in certain organisms of *S. aureus* JHM, and it is probable that these too were derived from mesosomes which had been extruded from the cytoplasm. It is not possible to say whether mesosome loss caused a disturbance in the processes regulating cell division or whether extrusion was the result of some interference with the relationship between wall and membrane.

Whatever the cause or morphological results of lysis of *Staphylococcus aureus* JHM grown in NaCl + glucose broth, the fact is that cultures grown up from a single colony eventually emerged from this condition and grew again. Subcultures into fresh NaCl + glucose broth, made during the long stationary phase before secondary growth began, did not show the usual turbidity decrease. This suggests that the cocci which survived adapted themselves to the conditions and became NaCl-tolerant. Probably the observed NaCl-tolerance of staphylococci always involves such adaptation, but with *S. aureus* JHM other processes take place during the early phase of incubation before adaptation occurs.

It is a pleasure to acknowledge the skilled assistance of Mrs P. Riches in this work. We also thank Dr M. Vesk for the electron micrographs, and Mr J. Shaw for amino acid analyses.

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EXPLANATION OF PLATES

PLATE 1

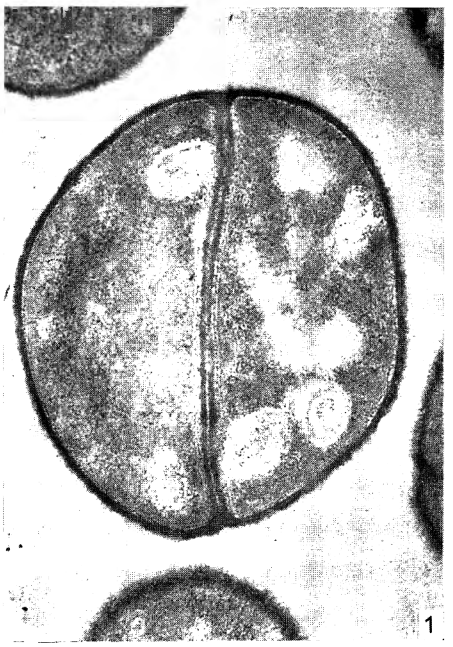
Fig. 1. Section of a normal staphylococcus grown in the presence of glucose but in the absence of added NaCl (i.e. only 0·5 % NaCl). Mesosomes are evident and the wall and septum are well defined. × 80,000.

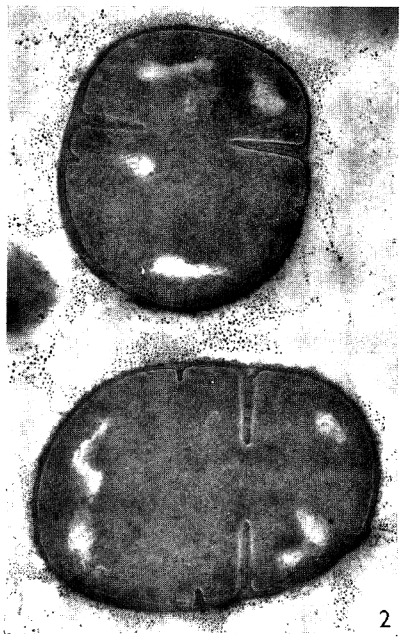
Figs. 2, 3. Staphylococci grown for 4·5 hr in the presence of NaCl (4·5 %)+glucose. Cell walls are of irregular thickness and septum formation is abnormal, there being two incipient septa per cell in some cases (fig 2). In some organisms (fig. 3) the cytoplasmic membrane has contracted away from the wall and membrane-enclosed bodies are visible in the spaces formed. × 40,000.

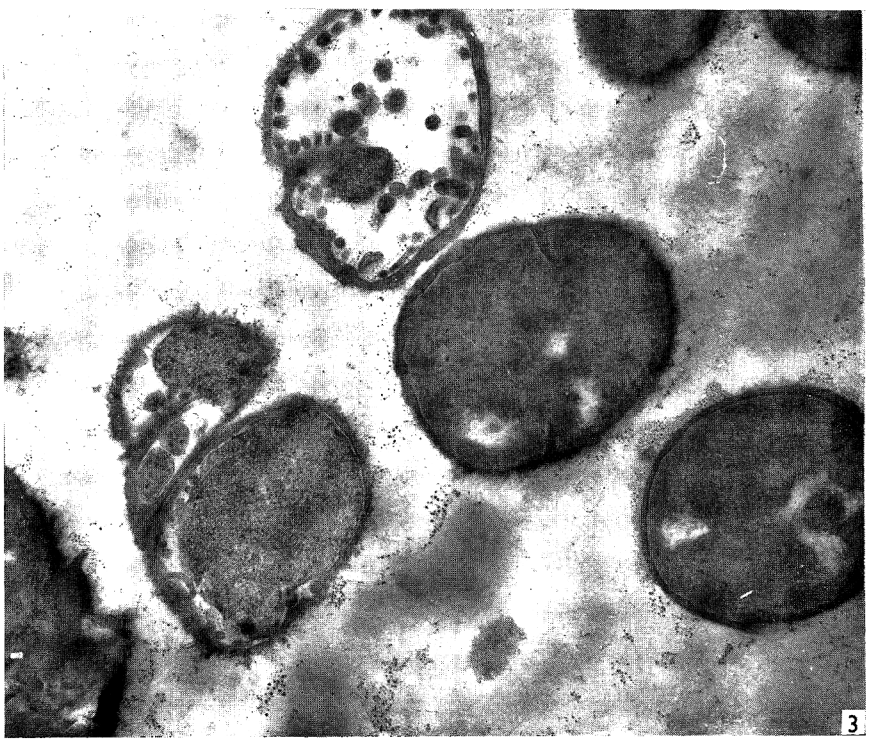
PLATE 2

Staphylococci grown for 5 hr in the presence of NaCl+glucose.

Fig. 4. Field showing organisms with very electron-dense cytoplasm, wall structures containing membrane fragments but no cytoplasm and organisms with contracted cytoplasm. Note the appearance of fibrous debris. × 40,000.







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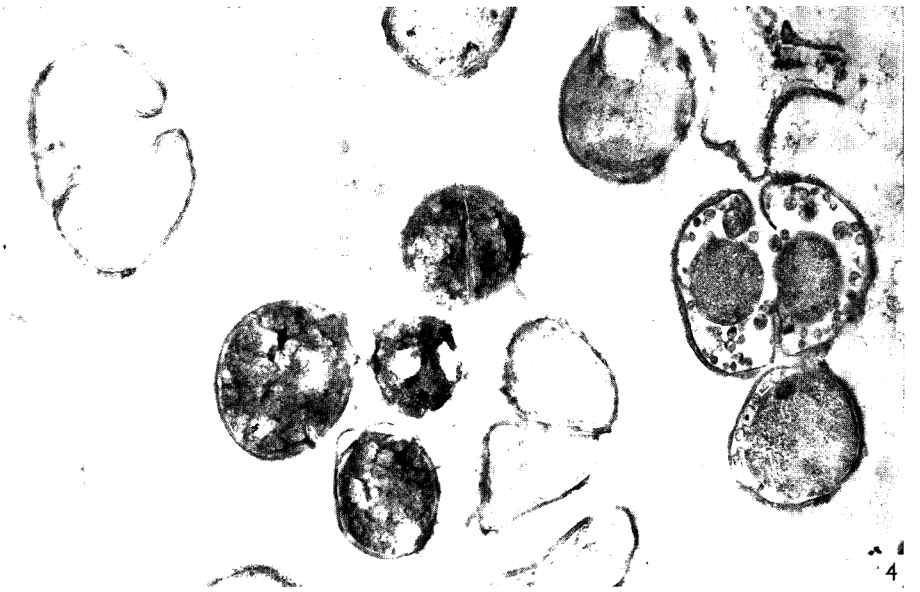
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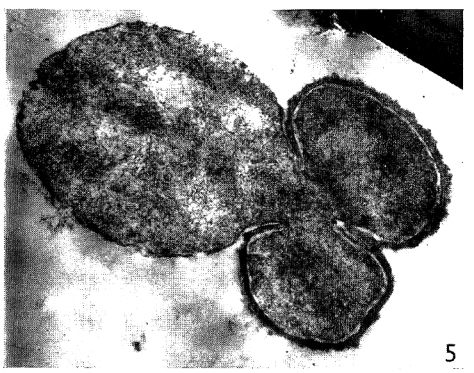
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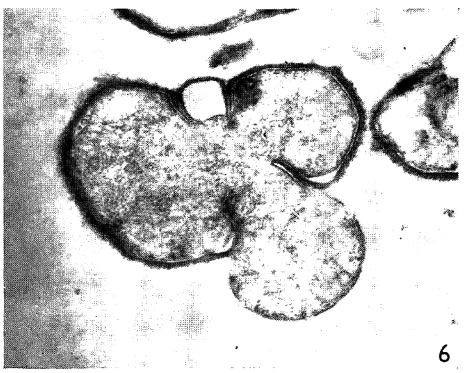
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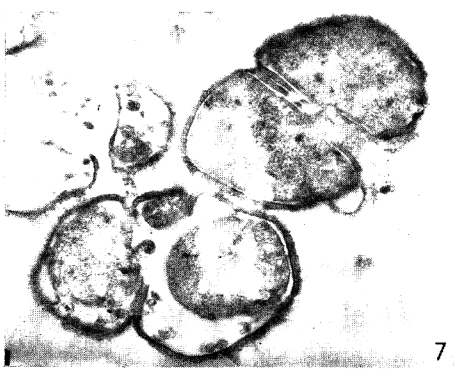
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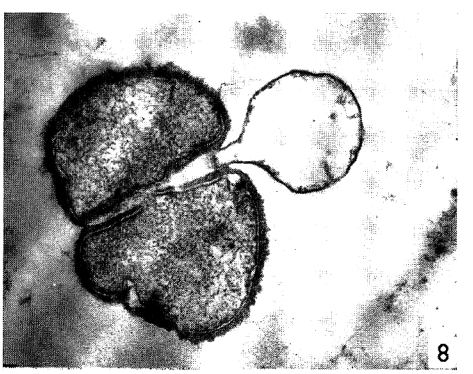
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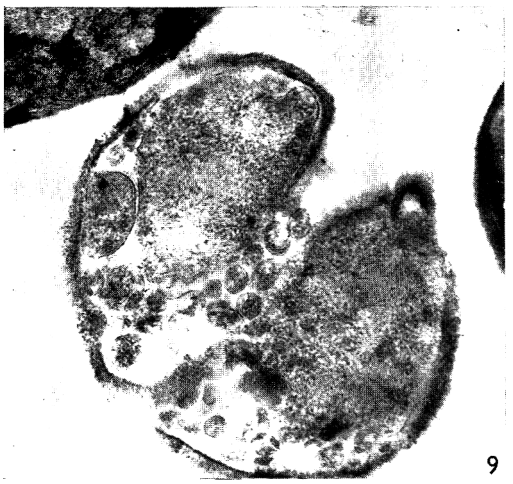

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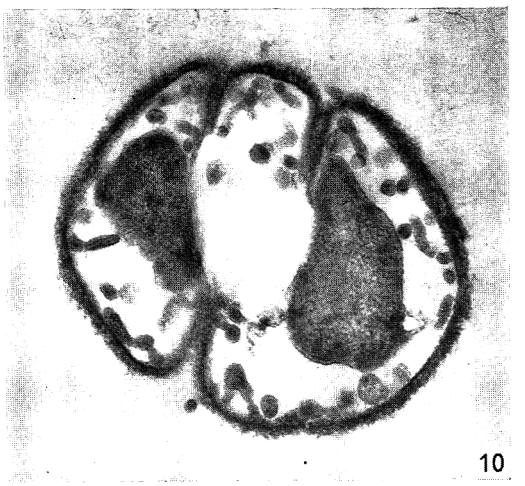

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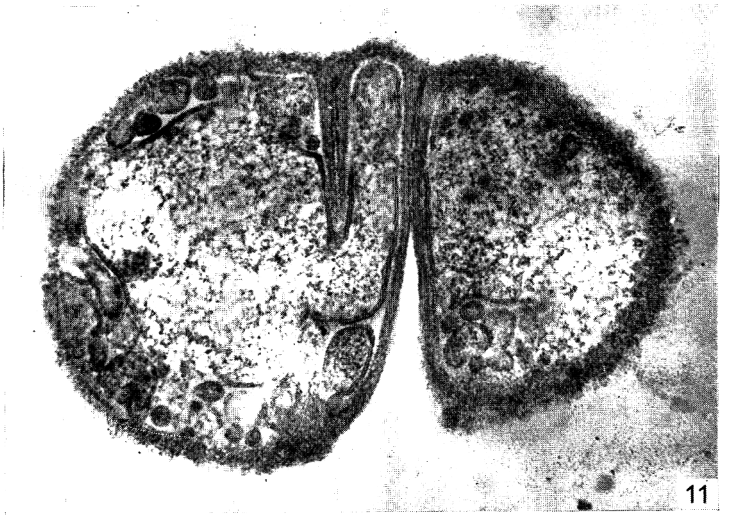

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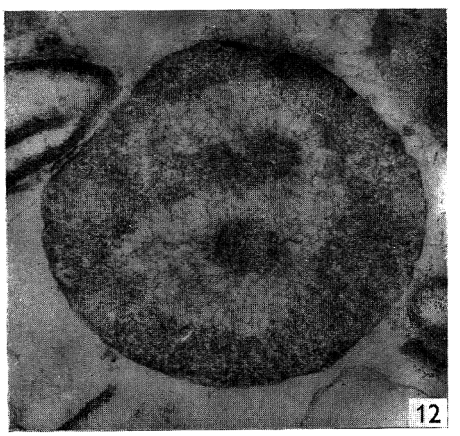

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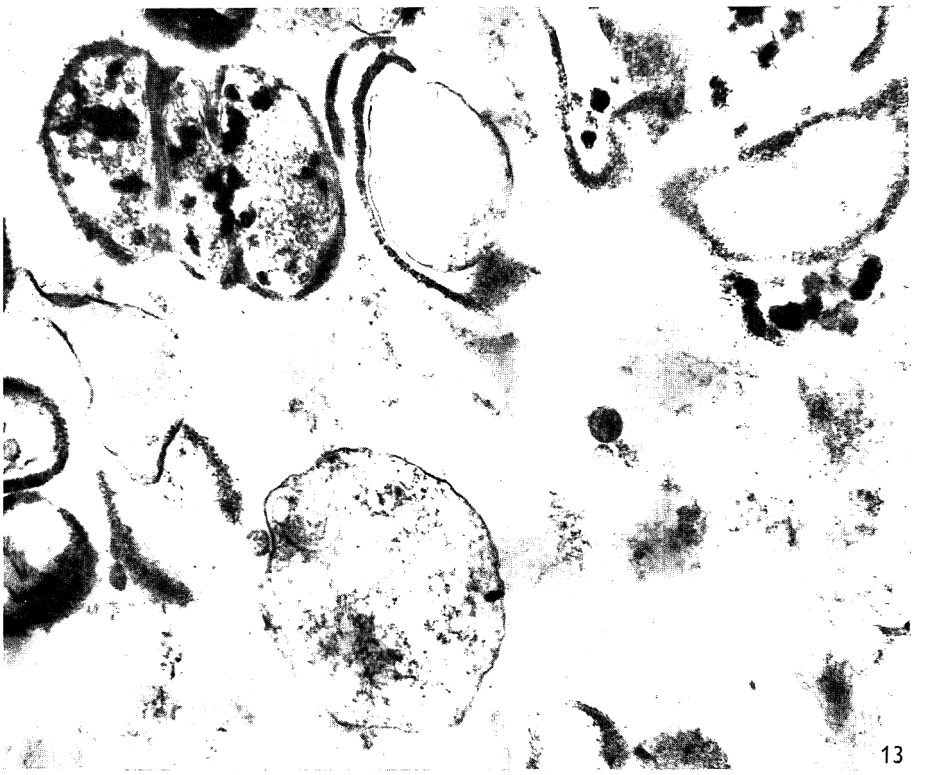

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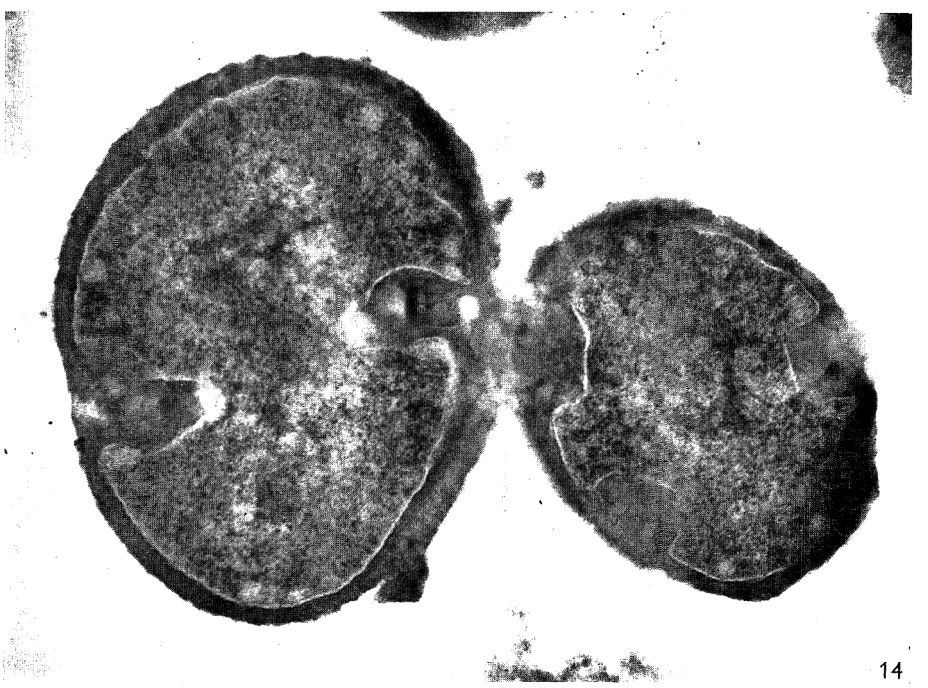

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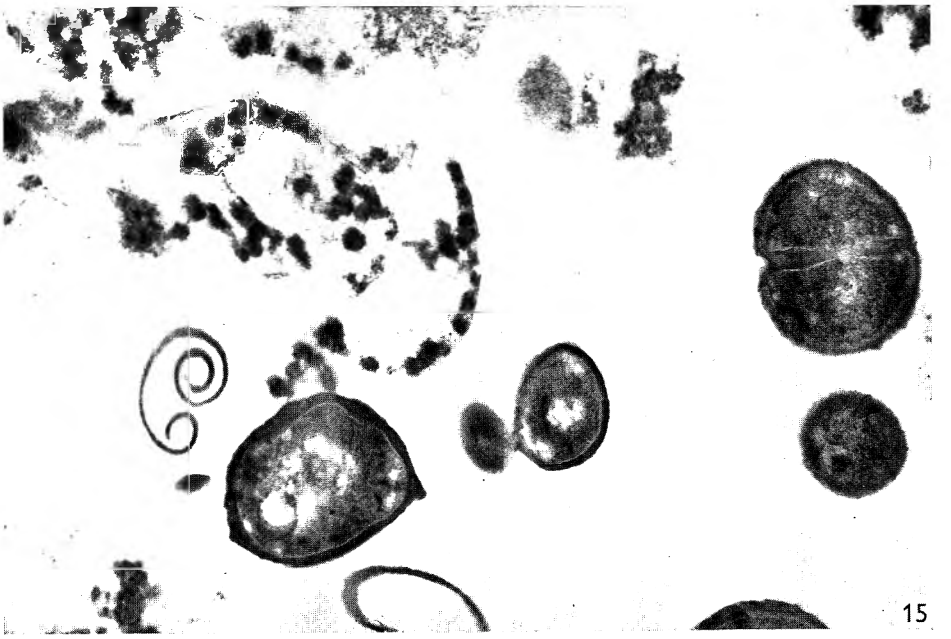

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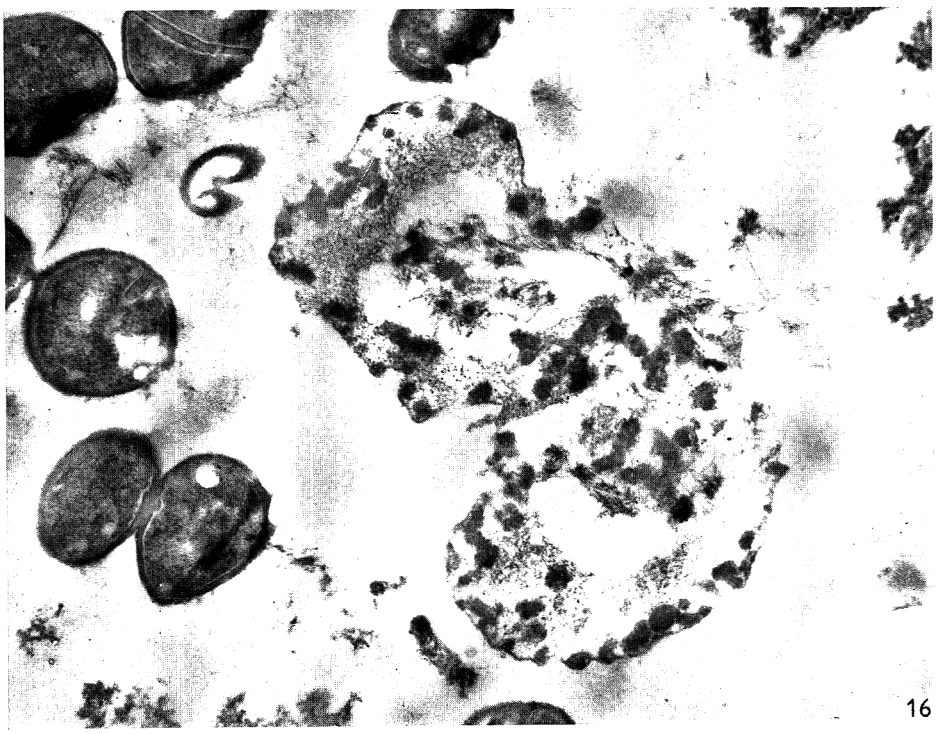

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Figs. 5, 6. The cell contents are being extruded enclosed in the cytoplasmic membrane. The walls have a rough appearance and are irregular in shape. × 40,000.

Fig. 7. Cell contents are being extruded, unbounded by a membrane. Also, contraction of membrane from the wall is apparent. × 40,000.

Fig. 8. Liberation of a membranous structure. × 40,000.

PLATE 3

Figs. 9, 10, 11. Staphylococci grown for 5 hr in the presence of NaCl + glucose.

Fig. 9. Organism with broken wall through which the membrane-enclosed bodies are escaping. × 80,000.

Fig. 10. Organism, of abnormal shape, having more than one septum and a contracted cytoplasm. × 80,000.

Fig. 11. Pair of organisms, one of which contains a 'half' septum. In both structures the membrane has contracted from the wall and membrane-enclosed bodies appear in the spaces formed. × 80,000.

Fig. 12. A large protoplast-like body, 2μ in diameter, produced after 7·5 hr incubation in NaCl + glucose broth. Wall fragments are also visible. × 40,000.

PLATE 4

Fig. 13. Field showing abnormal organisms, protoplast-like bodies, fragments of walls and membranes and much fibrous debris after 7·5 hr incubation in NaCl + glucose broth. × 40,000.

Fig. 14. Cocci, after 48 hr incubation in NaCl + glucose broth, having very thick walls which are very uneven on the inside surface. × 80,000.

PLATE 5

Figs. 15, 16. After 48 hr incubation; fields showing bodies with, in most cases, thick and uneven walls; the septa of dividing organisms often distorted and enlarged; wall fragments visible, also dense particles aligned on membranous structures or surrounded by debris of a fibrous nature. × 40,000.

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Magnesium-limited Growth of *Bacillus subtilis*, in Pure and Mixed Cultures, in a Chemostat

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SUMMARY

The influence of Mg^{2+}-limitation on the growth of a typical Gram-positive organism—*Bacillus subtilis*—was investigated and the data compared with that obtained with *Aerobacter aerogenes* grown under similar conditions. The magnesium contents of both organisms varied with growth rate but were very similar at corresponding growth rates. With Mg^{2+}-limited chemostat cultures of each organism, uptake of Mg^{2+} was almost complete at specific growth rates less than 0·7 × maximum. Cellular Mg^{2+} was tightly bound, none being removed by suspension of the organisms at 20° in 0·85 % (w/v) NaCl. When Mg^{2+}-limited organisms were suspended in environments containing Mg^{2+}, this ion was rapidly adsorbed; the amount adsorbed varied with both the initial extracellular Mg^{2+} concentration and the composition of the diluent. *B. subtilis* had a greater capacity for Mg^{2+} adsorption than *A. aerogenes* but its affinity for this ion was less. The latter difference correlated with the ability of *A. aerogenes* to outgrow *B. subtilis* rapidly in Mg^{2+}-limited chemostat cultures containing both organisms. The significance of these results is discussed in relation to the reports from other laboratories concerning differences between Gram-positive and Gram-negative bacteria in Mg^{2+} content, uptake of Mg^{2+} and ability to grow in media of low Mg^{2+} content.

INTRODUCTION

It has been reported by Webb (1949) and by Rouf (1964) that the magnesium contents of Gram-positive bacilli (*Bacillus subtilis*, *B. cereus*) are much greater than those of Gram-negative organisms (*Aerobacter aerogenes*, *Escherichia coli*). Furthermore, Webb (1966) concluded that, unlike the Gram-negative organisms, bacilli (*B. megaterium* and *B. mesentericus*, as well as *B. subtilis*) would not grow in simple salts media containing less than 1–2 μg. Mg^{2+}/ml. Recently the magnesium content of *A. aerogenes* has been found to be a function of the growth rate and to be related to the bacterial RNA content (Tempest & Strange, 1966; Tempest, Dicks & Hunter, 1966; Dicks & Tempest, 1966). Since the RNA contents of *A. aerogenes* and *B. megaterium* do not differ significantly when grown under similar conditions (Herbert, 1958), the apparent gross difference in magnesium requirement, and content, is of considerable interest and possible importance. It follows from the observations of Tempest & Strange (1966), and of Dicks & Tempest (1966), that a meaningful comparison of the magnesium contents of Gram-positive and Gram-negative organisms can be made only if they are grown under nearly identical conditions—particularly with regard to temperature and growth rate. Furthermore, because a variable amount of magnesium may be loosely bound to the walls of bacteria grown in media con-

taining an excess of this cation, it is preferable to compare organisms that have been grown in a Mg^{2+}-limited environment since these contain no detectable adsorbed magnesium (Tempest & Strange, 1966). Alternatively, adsorbed magnesium can be removed by washing organisms in 0·85% (w/v) NaCl (Strange & Shon, 1964). This paper compares the magnesium contents of Mg^{2+}-limited *B. subtilis* and *A. aerogenes* grown at corresponding rates, at 35°; no significant differences were found. However, differences were observed in the abilities of the organisms to adsorb Mg^{2+} when suspended in various environments, and these findings correlated with the behaviour of the organisms in Mg^{2+}-limited mixed cultures. It is concluded that the saturation constants (K_s values) for Mg^{2+} of *B. subtilis* and *A. aerogenes* organisms differ substantially. A preliminary report on our findings has been published (Tempest, Dicks & Meers, 1967).

METHODS

Organisms. *Bacillus subtilis* var. *niger* (obtained from Fort Detrick, Md., U.S.A., originally called *Bacillus globigii*; strain ATCC9372 is considered to be from the same source) was maintained by monthly subculture on tryptic meat digest agar slopes containing 0·2% (w/v) glucose; *B. megaterium* KM (obtained from Dr J. F. Wilkinson) was maintained on tryptic meat digest agar containing 0·2 (w/v) mannitol; *Aerobacter aerogenes* (NCTC418) was maintained on tryptic meat digest agar slopes; *Torula utilis* (NCYC321) was maintained on yeast extract, peptone, glucose agar slopes.

Growth conditions. Continuous cultures of organisms were maintained in 0·5 l. chemostats of the type described by Herbert, Phipps & Tempest (1965). The temperature was controlled at 35°; the pH value was regulated at 6·5 and 7·0, respectively, for the *Aerobacter aerogenes* and *Bacillus subtilis* cultures. The growth medium, which was limited with respect to its Mg^{2+} content, had the following composition: Na_2HPO_4, $5{\cdot}0\times10^{-3}$M; $NH_4H_2PO_4$, $4{\cdot}5\times10^{-2}$M; $(NH_4)_2SO_4$, $2{\cdot}5\times10^{-2}$M; K_2SO_4, $3{\cdot}0\times10^{-3}$M; citric acid, $1{\cdot}0\times10^{-3}$M; $MgCl_2$, $2{\cdot}5\times10^{-4}$M; $CaCl_2$ and $FeCl_3$, each $1{\cdot}0\times10^{-4}$M; $MnCl_2$ and $ZnCl_2$, each $2{\cdot}5\times10^{-5}$M; $CuCl_2$, $CoCl_2$ and Na_2MoO_4 each 5×10^{-6}M; a sterile glucose solution, in water, was added to a final concentration of 30 g. glucose/l. after sterilization (121°, 30 min.) of the bulk medium. Batch cultures were grown in a similar medium, differing only in the Mg^{2+} concentration.

Analytical procedures. Culture bacterial concentrations (equiv. mg. dried bacteria/ml. culture) and the macromolecular compositions of organisms were determined by methods described previously (Tempest, Hunter & Sykes, 1965). Bacterial magnesium contents were determined by atomic absorption (using an EEL Model 140 Spectrophotometer) on $HClO_4$ extracts, prepared as described by Tempest & Strange (1966).

Mixed culture experiments. Cultures of *Bacillus subtilis*, *Aerobacter aerogenes* and *Torula utilis* were grown in 0·25 l. chemostats (designed by Dr D. Herbert) without pH control. The growth medium was as follows: Na_2HPO_4, $2{\cdot}5\times10^{-2}$M; $NH_4H_2PO_4$, $3{\cdot}75\times10^{-2}$M; K_2SO_4, $2{\cdot}0\times10^{-3}$M; citric acid, $5{\cdot}0\times10^{-4}$M; $MgCl_2$, $3{\cdot}75\times10^{-5}$M; $CaCl_2$ and $FeCl_3$, each $2{\cdot}5\times10^{-5}$M; trace amounts of Mn^{2+}, Cu^{2+}, Co^{2+}, Zn^{2+} and Na_2MoO_4; glucose to a final concentration of 10 g./l. The pH value was adjusted to 6·4 before sterilization; after sterilization (autoclaving at 121° for 30 min.) the pH value was 6·5–6·6. Continuous cultures of each organism were established, in separate chemostats, at a dilution rate of 0·3 hr^{-1} and with the temperature regulated at 33°. The steady-state pH values were found to be 6·4, 6·3 and 5·5, respectively, for

cultures of *B. subtilis*, *T. utilis* and *A. aerogenes*. Mixed cultures were produced by pipetting small volumes (1.0 ml. unless stated otherwise) from one chemostat culture into another chemostat culture. The flow rates were maintained at 0·3 hr^{-1} and samples taken at convenient times thereafter for determination of microbial content and distribution. Total numbers of bacteria and yeast were determined by direct observation and counting, using a Thoma chamber of 0·02 mm depth, and phase-contrast microscopy. The proportions of *A. aerogenes* organisms in mixed cultures containing *B. subtilis* were assessed by microscopic examination of Gram-stained preparations.

RESULTS

Influence of dilution rate on the yield value and macromolecular composition of Mg^{2+}-limited Bacillus subtilis

With Mg^{2+}-limited chemostat cultures of *Aerobacter aerogenes*, the yield (g. bacteria formed/g. Mg^{2+} consumed) varied with the dilution rate (Tempest, Hunter & Sykes, 1965). This indicated a change in cellular Mg^{2+} content with growth rate which

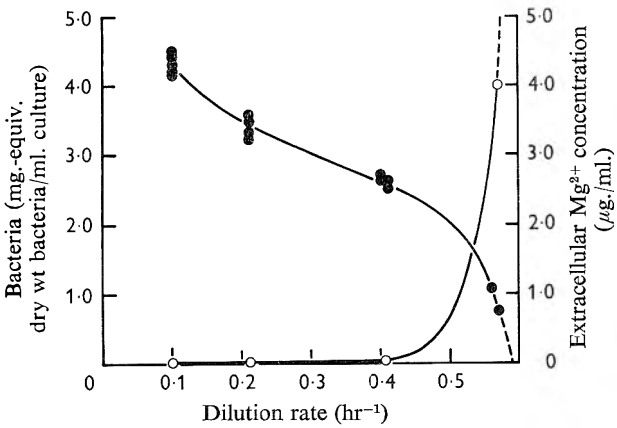

Fig. 1

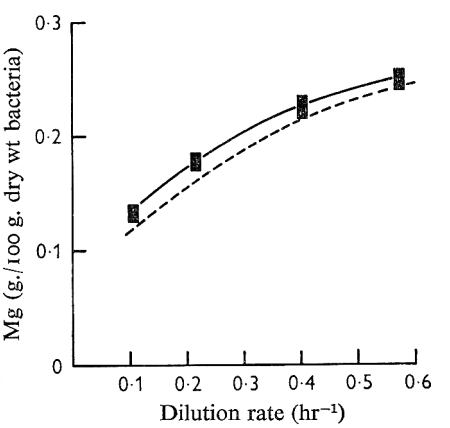

Fig. 2

Fig. 1. Steady-state concentrations of *Bacillus subtilis* and extracellular Mg^{2+} as functions of dilution rate (35°; pH 7·0) in a Mg^{2+}-limited chemostat culture. Values at each dilution rate were determined over a 2- to 3-day period, after initial equilibration of the culture for a period of 2 days. ●, mg. dry wt bacteria/ml. culture; ○, μg. extracellular Mg^{2+}/ml. culture.

Fig. 2. The magnesium content of Mg^{2+}-limited *Bacillus subtilis* and *Aerobacter aerogenes* as a function of growth rate. Mean magnesium content (% of bacterial dry weight) of *B. subtilis* (solid line) and the corresponding magnesium content of Mg^{2+}-limited *A. aerogenes* (broken line).

paralleled the change in RNA content. Figure 1 shows the effect of dilution rate on the steady-state concentration of *Bacillus subtilis* organisms in a Mg^{2+}-limited chemostat culture. Like the Mg^{2+}-limited *A. aerogenes* culture, bacterial concentration varied with growth rate; an increase in dilution rate caused a decrease in yield. Over 90% of the Mg^{2+} added to the culture was present in the organisms at all dilution rates below 0·5 hr^{-1} (Fig. 1); thus, the magnesium content of *B. subtilis* varied with the growth rate (Fig. 2). Also plotted in Fig. 2 are data on the variation in magnesium content of *A. aerogenes* (Tempest & Strange, 1966); the two sets of data are strikingly similar.

The macromolecular composition of Mg^{2+}-limited *Bacillus subtilis* at various growth rates is shown in Table 1. There was a progressive increase in cellular RNA

and carbohydrate contents with increasing growth rates, whereas the DNA and protein contents decreased or varied irregularly. An interrelationship between RNA and magnesium was suggested by the relatively constant RNA/Mg^{2+} ratio (Table 1); this was quantitatively similar to that reported with *Aerobacter aerogenes* (Tempest *et al.* 1966) and with *Pseudomonas fluorescens* (Sykes & Tempest, 1965).

Binding of Mg^{2+} to Bacillus subtilis

It was reported by Strange & Shon (1964) that *Aerobacter aerogenes* organisms which had been separated from cultures containing Mg^{2+} in excess of the growth requirement, and which had been washed in distilled water, had an amount of Mg^{2+}

Table 1. *Macromolecular composition of Mg^{2+}-limited Bacillus subtilis grown at different dilution rates*

All the data in this table are average values, obtained from at least two samples (collected and processed on different days) grown at each rate.

Diln rate (hr^{-1})	Dry wt bacteria (mg./ml. culture)	g./100 g. dry bacteria				Molar ratio RNA/Mg^{2+}
		Protein	Carbohydrate	DNA	RNA	
0·10	4·35	62·1	12·5	1·9	9·4	4·6
0·21	3·40	53·1	12·5	1·9	12·2	4·6
0·41	2·56	56·7	13·4	1·8	13·9	4·2
0·56	0·93	55·3	15·0	1·8	15·5	4·5

Table 2. *Comparison of magnesium contents of water- and saline-washed Bacillus subtilis organisms, grown at various dilution rates (35°, pH 7·1)*

Samples of bacteria, separated from chemostat cultures, were washed with distilled water or 0·85% (w/v) NaCl and extracted with N-$HClO_4$ for Mg^{2+} assay (see Methods).

Diln rate (hr^{-1})	g. Mg^{2+}/100 g. dry bacteria		Ratio water-washed: NaCl-washed
	Water-washed	NaCl-washed	
0·10	0·140	0·140	1·00
0·21	0·169	0·169	1·00
0·21	0·184	0·188	0·98
0·40	0·238	0·233	1·02
0·41	0·212	0·216	0·98
0·57	0·247	0·237	1·04
0·57	0·257	0·252	1·02

bound loosely at their surface; this adsorbed Mg^{2+} could be removed by suspension of the organisms in 0·85% (w/v) NaCl solution. Mg^{2+}-limited *A. aerogenes* organisms were devoid of surface-bound Mg^{2+} (Tempest & Strange, 1966); similarly, no significant difference was found between the magnesium contents of water- and saline-washed Mg^{2+}-limited *Bacillus subtilis* organisms (Table 2). However, both Mg^{2+}-limited *A. aerogenes* and *B. subtilis* organisms would take up Mg^{2+} when exposed to dilute solutions of $MgCl_2$ for brief periods of time (2 min. or less). Over 80% of this Mg^{2+} could be recovered from the organisms by subsequent treatment with 0·85% saline, suggesting that it was largely adsorbed. *B. subtilis* had a greater capacity for Mg^{2+} adsorption than *A. aerogenes*. Thus, with organisms grown at a dilution rate of 0·2 hr^{-1} and washed once, in 2·5 mM-$MgCl_2$ solution, *B. subtilis* adsorbed 5·6μg.

Mg^{2+}/mg. equiv. dry bacteria whereas *A. aerogenes* adsorbed 3·6μg. Mg^{2+}/mg. equiv. dry bacteria ($3{\cdot}05 \times 10^{-15}$ g. Mg^{2+}/organism and $1{\cdot}29 \times 10^{-15}$ g. Mg^{2+}/organism, respectively).

The greater capacity for Mg^{2+} adsorption of *Bacilus subtilis*, compared with *Aerobacter aerogenes*, indicated a greater number of cation binding sites per organism (about 8×10^7, compared with about 3×10^7); it provided no information on strength of binding (Mg^{2+}-affinity) or ion specificity. This was sought by studying the adsorption of Mg^{2+} in the presence of a competing ion. Figure 4 shows the results of an experiment in which Mg^{2+}-limited *A. aerogenes* and *B. subtilis* organisms were suspended (for 2 min.) in saline containing graded concentrations of Mg^{2+}, then washed with water and analysed for Mg^{2+}. Although *B. subtilis* had the greater Mg^{2+} binding capacity at high Mg^{2+} concentrations, at low Mg^{2+} concentrations (in the presence of saline) these organisms adsorbed less Mg^{2+} than *A. aerogenes* organisms (Fig. 3); this indicated a difference in affinity for this cation. Similarly, the presence of growth-medium constituents, or of EDTA, markedly influenced the uptake of Mg^{2+} by *B. subtilis*, whereas they had substantially less effect on Mg^{2+} adsorption by *A. aerogenes* organisms (Fig. 3).

Growth of Bacillus subtilis in Mg^{2+}-limited mixed cultures

Webb (1966) concluded that Gram-positive bacilli, unlike Gram-negative organisms, were unable to utilize Mg^{2+} when the external concentration of this cation was low. Whereas it is clear (Fig. 1) that *Bacillus subtilis* would grow in a chemostat under conditions where the extracellular Mg^{2+} concentration was 10%, or less, of the minimum value reported to support the growth of other bacilli in a batch culture (see Webb (1966), his table 1), the differences in Mg^{2+} adsorption observed between *B. subtilis* and *Aerobacter aerogenes* accorded, qualitatively, with Webb's conclusion. However, the ability of organisms to assimilate Mg^{2+} may not be related directly to their surface adsorption properties. Therefore mixed population experiments were carried out, in Mg^{2+}-limited chemostat cultures, to assess the relative efficiencies of Mg^{2+} utilization (i.e. to compare the saturation constants for Mg^{2+} of the various organisms).

Mixed culture experiments were carried out as described in the Methods section and the results of these are contained in Figs 4 and 5. In each of three experiments in which Mg^{2+}-limited *Aerobacter aerogenes* organisms were added to growing Mg^{2+}-limited *Bacillus subtilis* cultures (initial *B. subtilis* concentration, $4{\cdot}5 \times 10^8$ organisms/ml.; initial *A. aerogenes* concentration, less than 2×10^6 organisms/ml.), the Gram-negative organism rapidly outgrew the bacillus; after 24 hr the *A. aerogenes* concentration had increased to $6{\cdot}7$–$8{\cdot}5 \times 10^8$ organisms/ml. and the *B. subtilis* concentration had diminished to less than 6×10^6 organisms/ml. (Fig. 4).

Previous observations on the growth of a contaminant bacillus (probably *Bacillus subtilis*) in K^+- and Mg^{2+}-limited chemostat cultures of *Torula utilis* suggested that bacilli may not be able to outgrow *T. utilis* in environments where growth rate is limited by the availability of Mg^{2+}. Therefore experiments were carried out to study the growth of *B. subtilis* in mixed Mg^{2+}-limited cultures containing *T. utilis*. When 10 ml. of a Mg^{2+}-limited *B. subtilis* culture ($4{\cdot}5 \times 10^8$ organisms/ml.) were added to 90 ml. of a similarly limited culture of *T. utilis*, containing 6×10^7 organisms/ml., the bacterial concentration in the mixed chemostat culture progressively diminished over

24 hr from 5×10^7 organisms/ml. to 1×10^6 organisms/ml. (Fig. 5). However, contrary to our expectations when small amounts of *T. utilis* (equivalent, after mixing, to about 6×10^6 organisms/ml.) were added to a Mg^{2+}-limited *B. subtilis* culture ($4{\cdot}5 \times 10^8$ bacteria/ml.) the yeast failed to grow. Even *T. utilis* organisms at an initial concentration of $2{\cdot}8 \times 10^7$ organisms/ml. (obtained by adding 50 ml. of yeast culture to 50 ml. of the bacterial culture) failed to outgrow the bacillus (Fig. 5). This suggested the presence, in the Mg^{2+}-limited *B. subtilis* culture, of either a mycostatic substance or a bacterial product which enhanced Mg^{2+} uptake by the bacillus. Since *T. utilis* would grow, without lag and at an undiminished rate, in a bacteria-free Mg^{2+}-limited *B. subtilis* culture fluid (supplemented with Mg^{2+}) it seemed unlikely that a mycostatic substance was synthesized by *B. subtilis*. Therefore the possible involvement of extracellular bacterial products in Mg^{2+} uptake by the bacillus was investigated.

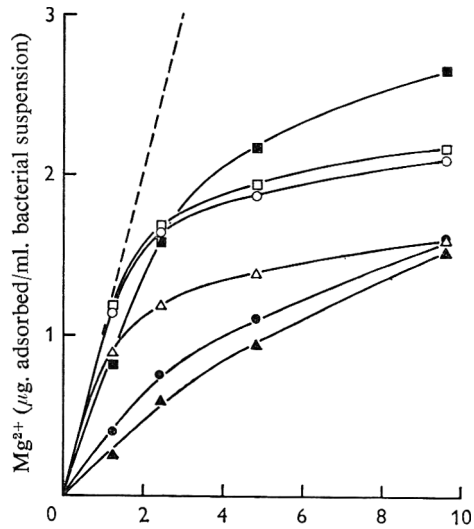

Fig. 3

Fig. 3. Adsorption of Mg^{2+} by *Bacillus subtilis* and *Aerobacter aerogenes* organisms. The organisms were grown at a dilution rate of 0·2 hr^{-1} (35°; pH 7·0 and 6·5, respectively), centrifuged and resuspended in various solutions containing 0·05–0·50 mM-$MgCl_2$. Suspensions were centrifuged (within 2 min. of mixing), the supernatant solutions discarded and the bacteria washed once with distilled water. The bacterial magnesium was extracted with 1 N-$HClO_4$ (Tempest & Strange, 1966) and determined by atomic adsorption spectrophotometry. Adsorbed Mg^{2+} was calculated as that in excess of magnesium present in untreated organisms. ■, □, Mg^{2+} adsorbed from solutions containing 0·85% (w/v) NaCl; ●, ○, Mg^{2+} adsorbed from solutions containing 0·85% (w/v) NaCl plus 1·5 mM-EDTA; ▲, △, Mg^{2+} adsorbed from the growth medium salts solution (each containing the graded concentrations of $MgCl_2$ as detailed above). The closed symbols refer to adsorption by *B. subtilis*, and the open symbols to adsorption by *A. aerogenes*. The broken line indicates Mg^{2+} adsorption by each organism in the absence of competing ions.

Webb (1966) had shown that addition of amino-acid mixtures to a simple basal medium increased Mg^{2+} uptake by *Bacillus megaterium* and decreased the value of the minimum Mg^{2+} concentration necessary for initiation of growth. When the experiment detailed in Fig. 5 was repeated using a medium modified by the addition of 1% (w/v) Casamino Acids (Difco Laboratories, Detroit, U.S.A.) plus tryptophan

(0·02 %, w/v), the bacillus now outgrew the yeast. In subsequent experiments the initial *B. subtilis* concentration was decreased to 2×10^6 organisms/ml.; the bacilli still outgrew the yeast, though requiring 48 hr to replace the yeast completely (cf. Fig. 4).

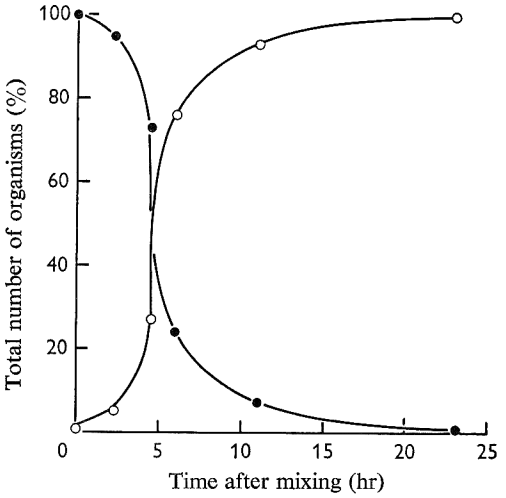

Fig. 4

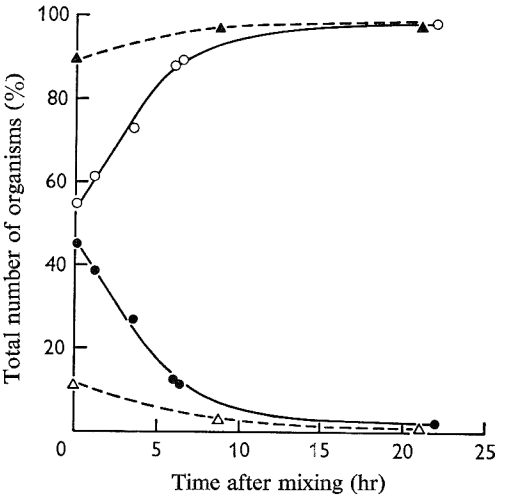

Fig. 5

Fig. 4. Growth of *Bacilus subtilis* (●) and *Aerobacter aerogenes* (○) in a Mg^{2+}-limited simple salts medium in a chemostat. The initial concentration of *B. subtilis* was $4{\cdot}5 \times 10^6$ organisms/ml. and of *A. aerogenes*, 2×10^3 organisms/ml. (99·5 % and 0·5 %, respectively, of the initial population). The dilution rate was 0·3 hr^{-1} and the temperature 33°; the initial pH value was 6·4 but decreased to 5·5 with the progressive increase in the concentration of *A. aerogenes* organisms. Identical results were obtained when 1 % (w/v) Casamino Acids was present in the medium.

Fig. 5. Growth of *Bacillus subtilis* and *Torula utilis* in a Mg^{2+}-limited simple salts medium in a chemostat. In the experiment represented by the solid lines, the initial concentration of *B. subtilis* (●) was $4{\cdot}5 \times 10^7$ organisms/ml. and of *T. utilis* (○) $5{\cdot}4 \times 10^7$ organisms/ml., i.e. 45·5 % and 54·5 % of the initial population, respectively. Also recorded (broken lines) are the results of an experiment in which the initial concentration of *B. subtilis* was increased to $2{\cdot}2 \times 10^8$ organisms/ml. (▲) and that of *T. utilis* reduced to $2{\cdot}8 \times 10^7$ organisms/ml. (△), i.e. 89·5 % and 10·5 % of the initial population, respectively. In both experiments the dilution rate was 0·3 hr^{-1} and the temperature 33°. The pH value (6·4) did not vary during the course of each experiment.

DISCUSSION

Since the Mg^{2+} content of bacteria may vary with growth rate—which, in a batch culture, would depend on factors such as medium composition and temperature—then a meaningful comparison of the Mg^{2+} contents of various species would be possible only if the growth conditions were rigidly controlled and standardized. Clearly the differences in Mg^{2+} content between Gram-positive and Gram-negative organisms, reported by Webb (1949) and Rouf (1964), are of no significance, since widely different conditions were used in growing the various organisms.

The gross differences, reported by Webb (1966), between the abilities of Gram-positive bacilli and Gram-negative bacteria to concentrate Mg^{2+} (presumably intracellularly) from simple chemically defined media are not confirmed here. *Bacillus subtilis* var. *niger* grew at steady-state Mg^{2+} concentrations far below the minimum values reported to be necessary for the growth of other bacilli (*B. subtilis* F 3, *B. subtilis* MARBURG, *B. mesentericus*, *B. megaterium*). Whereas it is possible that products of

metabolism, excreted into the medium, may have stimulated Mg^{2+} uptake by Mg^{2+}-limited *B. subtilis* organisms, small numbers (about 5×10^6 organisms/ml., final concentration) of continuously grown Mg^{2+}-limited *B. subtilis* would readily grow when inoculated into a simple salts medium containing 0·3 μg. Mg^{2+}/ml.; after 24 hr, more than 95% of the total Mg^{2+} could be recovered from the organisms whose concentration had increased to about 3×10^8 bacteria/ml.

It can be argued that, among the bacilli, *Bacillus subtilis* var. *niger* may be unique in requiring much lower concentrations of Mg^{2+} for growth. However, *B. megaterium* also would grow in a Mg^{2+}-limited chemostat culture and take up almost all the Mg^{2+} from the environment at dilution rates less than 0·3 hr^{-1}. We conclude, therefore, that the observations of Webb (1966) must reflect physiological factors other than the relative ability of bacilli and Gram-negative bacteria to take up from their environment, and utilize, low concentrations of Mg^{2+}. In this connexion the rapid death-rate of vegetative bacilli in aqueous suspension (Mr R. E. Strange, personal communication) and the known effects of Mg^{2+} on the survival of micro-organisms in aqueous environments (Postgate & Hunter, 1962, 1964; Tempest & Strange, 1966) may be factors contributing to Webb's findings.

Despite the quantitative differences between the results reported here and those of Webb (1966), the broad conclusions are similar. In the presence of competing ions *Bacillus subtilis* var. *niger* has a demonstrably lower affinity for Mg^{2+} than *Aerobacter aerogenes* (Fig. 3) and at low extracellular Mg^{2+} concentrations (imposed, in the chemostat, by maintaining the dilution rate at a value substantially less than the critical rate—D_c) the bacillus grows more slowly than the Gram-negative organism (Fig. 4). Furthermore, the rapid rate at which *A. aerogenes* outgrew *B. subtilis* in the Mg^{2+}-limited culture suggests that the respective saturation constants for Mg^{2+} are substantially different. In contrast, the rates of take-over of *B. subtilis* and *Torula utilis*, respectively, when inoculated together into a Mg^{2+}-limited simple salts medium with and without amino acids, indicates that the Mg^{2+} saturation constants of these organisms are less different. If, in our experiments, amino acids influenced Mg^{2+} uptake as Webb (1966) indicated, then it is clear that they had a greater effect on *B. subtilis* than on *T. utilis*, since they reversed the take-over patterns in mixed culture experiments. But addition of amino acids did not influence the rate at which *A. aerogenes* outgrew *B. subtilis* in a Mg^{2+}-limited mixed culture (Fig. 4), suggesting that these compounds have an equivalent effect on Mg^{2+} uptake by *A. aerogenes* and *B. subtilis*.

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Lethal Mutations and Balanced Lethal Systems in *Aspergillus nidulans*

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SUMMARY

The frequency of spontaneous and ultraviolet-induced, recessive, lethal mutations was estimated in two diploid strains of *Aspergillus nidulans*. The parent diploids were heterozygous for recessive and semi-dominant markers; each recessive lethal resulted in failure to recover haploid strains carrying the allele linked in coupling to that lethal. Diploids 1 and 2 carried markers permitting direct assay of lethals on 4 and 8 chromosomes, respectively, out of the total diploid complement of 16. No lethals were detected in 100 isolates from each untreated diploid. One hundred isolates from each treated parent yielded 6 and 16 lethals, respectively, suggesting a frequency for the whole genome, at 5% survival, of about 28% lethals. The lethals were nutritionally irreparable and were not temperature-sensitive. Several of them have been located roughly by mitotic crossing-over.

One isolate was heterozygous for two unlinked lethals. It segregated to give a stable heterokaryon bearing two classes of non-viable haploid conidia. Meiotic analysis, via a three-component heterokaryon, showed that the stable heterokaryon was balanced by the non-allelic lethals, one in each haploid component. Another isolate had a balanced lethal system resulting from linked, non-allelic lethals in *trans*.

Six isolates from treatment were stable diploids which produced no haploid sectors.

INTRODUCTION

Recessive lethal mutations have been used extensively in higher organisms as objective measures of mutation rates, as a tool in developmental genetics and as a means of maintaining balanced lethal systems. Most micro-organisms have haploid nuclei and this has restricted the study of irreparable mutations except in the case of temperature-sensitive or other environment-dependent lethal mutants. Atwood (1949) circumvented this difficulty in an elegant fashion through the use of heterokaryotic multinucleate conidia of Neurospora. The conidia of Aspergillus are uninucleate, but the availability of strains with diploid nuclei permits the study of recessive lethals. Tector & Käfer (1962) and Käfer & Chen (1964) have already exploited this system. The present work was undertaken to measure the frequency of spontaneous and induced irreparable lethals and to explore the possibility of their use to stabilize heterokaryons and strains with diploid nuclei.

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METHODS

The general techniques used throughout this work were those of Pontecorvo *et al.* (1953). Incubation was at 37° except where otherwise stated.

Media. Minimal medium (MM) was Czapek-Dox medium with 1% glucose. Complete medium (CM) was a complex medium, containing yeast extract, casein hydrolysate, hydrolysed nucleic acid, vitamins, etc. Solid media contained 2% agar.

Inhibitors. DL-*p*-fluorophenylalanine (*p*FA; from Koch-Light and Co. Ltd., Poyle Trading Estate, Colnbrook, Buckinghamshire) was used in CM and supplemented MM at 20 and 40 μg./ml., respectively. Acriflavine (British Drug Houses, Ltd., Poole, Dorset) was used at 25 μg./ml.

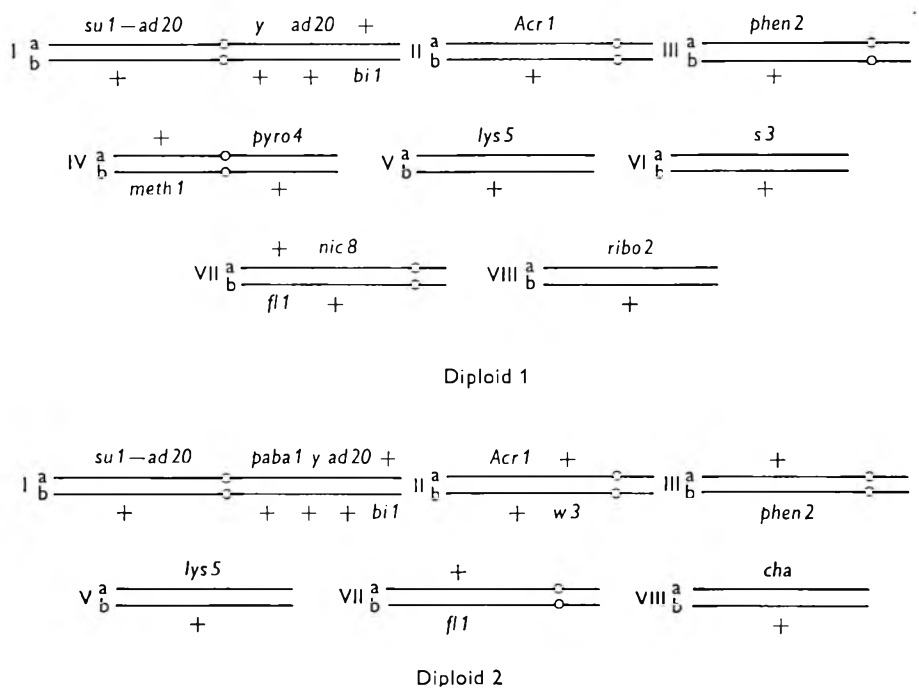

Fig. 1. *Aspergillus nidulans*. Genotypes of diploids 1 and 2.

Strains. Genotypes of the two diploid strains used in this work are shown in Fig. 1. Mutant alleles, and their locations, were given by Pontecorvo *et al.* (1953), Käfer (1958), Pontecorvo (1963), Ball & Azevedo (1964) and Barratt, Johnson & Ogata (1965). Alleles used in this work were: *y*, *w3*, *cha* determining, respectively, yellow, white, chartreuse conidia; *fl1*, fluffy; *ad20*, *bi1*, *lys5*, *meth1* and *meth2*, *nic8*, *paba1*, *phen2*, *pyro4*, *ribo2*, *s12* and *s3* determining, respectively, requirement for adenine, biotin, lysine, methionine, nicotinic acid, *p*-aminobenzoic acid, phenylalanine, pyridoxin, riboflavine, thiosulphate; *su1-ad20*, suppressor of *ad20*; *Acr1*, resistance to acriflavine; *mal1*, inability to use maltose.

Diploids 1 and 2 were free from chromosomal translocations; they were synthesized by the method of Roper (1952) and kept on MM.

Ultraviolet (u.v.) irradiation. Saline suspensions of conidia were irradiated by a mercury vapour lamp to give about 5% survival.

Induction and detection of recessive lethal mutations. Conidia, untreated or irradiated to 5% survival, were plated on MM and incubated 3–4 days. For each diploid 200 colonies were taken, 100 control and 100 following treatment; colonies which showed morphological abnormality were excluded. Each such isolate was purified by selection of a single colony from a plating of conidia on MM.

Each isolate was tested for newly arisen recessive mutations by the following steps. First, by plating conidia, about 10 per dish, on MM+*p*FA+all nutritional requirements of the haploid parents. Diploid strains grow poorly on *p*FA but the haploid nuclei formed during this stunted growth gave vigorous sectors (Morpurgo, 1961; Lhoas, 1961). Mitotic haploidization does not involve crossing-over (Pontecorvo, Tarr Gloor & Forbes, 1954); in the absence of a lethal mutation all the markers of the parent haploids, apart from the exception discussed below, are recovered among the haploid segregants. Absence of any allele from among these segregants suggested a lethal linked in coupling with that allele. Scoring was limited to sectors phenotypically distinct from the parent diploids, which were non-fluffy and had green conidia. With this limitation, diploid 1 isolates were expected to yield yellow sectors, fluffy and non-fluffy. Diploid 2 was expected to yield yellow, white and chartreuse, fluffy and non-fluffy in each case. This permitted scoring of mutations on 3 and 7 chromosomes, respectively. *p*FA selects against haploids carrying *phen 2*; a lethal in coupling with *phen*$^+$ would give a non-sectoring isolate. This, subject to later reservations, assayed lethals on a further chromosome in each diploid.

The *p*FA test on supplemented MM would record as lethal any newly arisen nutritional requirement not satisfied by the supplements. Furthermore, *p*FA might select against haploids carrying certain new but non-lethal mutations and so overestimate the frequency of lethals. Conidia of each isolate, suspected on the *p*FA test of carrying a lethal, were plated on CM and incubated at 37° and 25°. Segregants differing from the parent diploid in morphology or conidial colour were isolated and tested for ploidy by their conidial size (Roper, 1952). Classification of haploids produced at 37° provided a cross-check with the *p*FA selection; haploids produced at 25° checked the possible temperature sensitivity of each lethal. Diploid segregants arose mainly from mitotic crossing-over; in appropriate cases they were used for an approximate location of the lethal mutants.

Genetic analysis. The techniques were those of Pontecorvo *et al.* (1953), with a modified technique for ascus dissection (Bainbridge, 1964). The methods of Roper & Käfer (1957) and Pritchard (1955) were used for the selection of mitotic segregants by resistance to acriflavine and by suppression of adenine requirement, respectively.

RESULTS

Lethal mutations in diploid 1

One hundred control isolates carried no lethals on the chromosomes tested. In summary, the 100 isolates from treatment were as follows: 92 sectored normally in respect of the four tested chromosomes; one isolate carried a mutation, located on chromosome I, which determined requirement for *p*-aminobenzoic acid; three carried single lethals; one carried two unlinked lethals and gave the balanced heterokaryon

described below; one carried two non-allelic, linked, recessive lethals in *trans* arrangement; the remaining two isolates failed to sector.

The three isolates carrying single mutations were analysed readily by their sectoring behaviour on CM, CM+*p*FA and CM+acriflavine. The two isolates which carried two lethal mutations each required more intensive investigation as they were included initially in the series failing to give any haploid sectors.

One of these isolates carried a lethal in *cis* arrangement with *phen 2*$^+$ (chromosome III) and was therefore expected to give no sectors on *p*FA media. The other lethal was shown to be carried in *cis* arrangement with *fl 1*$^+$ (chromosome VII) since all haploid sectors on CM carried *phen 2* and *fl 1*. In fact the isolate yielded a single sector on *p*FA. The yellow conidia of this sector, plated on CM or supplemented MM, gave micro-colonies about 60 μ in diameter but did not grow further. The sector, which had a growth requirement for pyridoxin and riboflavine, could be maintained only by subculture of mycelium. It seemed likely that the sector was a balanced heterokaryon produced by the breakdown of a diploid heterozygous for two non-allelic lethals; the heterokaryon carried one lethal in each of its haploid components, which were designated *Al 1* and *Bl 2*. This was confirmed by meiotic analysis. The heterokaryon was combined with the strain *meth 2 phen 2*; *nic 8 mal 1 fl 1* (designated *C*) in a three-component heterokaryon maintained in balance on MM. With relatively rare exceptions, each perithecium of *Aspergillus nidulans* derives from two nuclei only (Hemmons, Pontecorvo & Bufton, 1953). From a three-component heterokaryon six classes of perithecia are to be expected, three hybrid and three selfed. Five distinguishable classes were found, including all three hybrid classes and the selfed non-lethal class. Some perithecia yielded only non-viable ascospores; since the lethals could not be distinguished on their germination pattern, these perithecia may have included both selfed lethal types. Genotypes of nuclei *A* and *B* were determined by analysis of $A \times C$ and $B \times C$ perithecia; they were—*A*: *su 1–ad 20 y ad 20*; *l 1*; *pyro 4*; *fl 1*; *ribo 2*; and *B su 1—ad 20 y ad 20*; *Acr 1*; *phen 2*; *pyro 4*; *l 2 nic 8*; *ribo 2*. *l 2* showed free recombination with *mal 1*, *nic 8* and *fl 1*; *l 1* was located 16 ± 3 units from *meth 2*, tentatively between *meth 2* and *phen 2*.

The diploid isolate carrying non-allelic lethals in *trans* also did not sector normally. It produced a single poorly growing sector on CM+*p*FA. Plating of the sparse yellow conidia of this sector gave abnormal colonies which, on further incubation, yielded vigorous stable sectors. Both sectors and centres required adenine, pyridoxin and nicotinic acid and were acriflavine-sensitive. The sectors carried conidia of diploid size; conidia from the colony centres were variable in size but mainly near-haploid. The stable diploid sectors, arising from the poorly growing centres, did not sector haploids on *p*FA. The single poorly growing sector was probably an aneuploid which arose following two types of mitotic event: (i) mitotic crossing-over between *y* and its centromere which gave homozygosis for *y* and *ad 20*; (ii) loss of one member from certain chromosome pairs which gave an aneuploid (Käfer, 1961) disomic for chromosome I, monosomic for II, IV and VII and, perhaps, monosomic for the remaining chromosomes. The aneuploid was unable to produce viable haploid nuclei; by non-disjunction it could produce diploid nuclei which gave stable diploid sectors. The original isolate did not produce diploid *su 1–ad 20*/*su 1–ad 20* or haploid *su 1–ad 20* sectors by Pritchard's (1955) technique and this implied a lethal distal to and in *cis* arrangement with *su 1–ad 20*. It seemed probable that the isolate, and the

aneuploid derived from it, carried non-allelic lethals in *trans* on chromosome I. The lethal on Ib is excluded from calculations on the total lethal frequency since chromosome Ib was not generally assayed.

Lethal mutations in diploid 2

One hundred control untreated isolates sectored normally on *p*FA. Treatment gave 80 isolates with no lethals on the 8 chromosomes tested, and 16 with one lethal each. The remaining 4 isolates, still diploid, produced no haploid sectors on any media. The 16 isolates carrying lethals sectored on *p*FA and permitted chromosomal location of all the lethals.

Table 1. *Aspergillus nidulans. Chromosomal location of ultraviolet-induced lethals*

	Chromosome*	Number of lethals
Diploid 1	Ia	3
	IIIb	2
	VIIa	1
	VIIb	0
Diploid 2	Ia	3
	IIa	2
	IIb	3
	IIIa	4
	VIIa	0
	VIIb	3
	VIIIa	1
	VIIIb	0

* See Fig. 1.

Table 2. *Aspergillus nidulans. Decreased frequency of certain diploid segregants from lethal-bearing isolates of diploid 2*

The number of diploid segregants was estimated from 44 point inoculations of each strain on CM.

		Segregants				
		Yellow	White	Fluffy	Char-treuse	
	Chromosomal location of	Class and number				Implied location of
Isolate	lethal					lethal
118	IIb	20	10*	32	9	Proximal to *w3*
119	IIb	19	0*	30	8	Distal to *w3*
143	VIIb	25	38	0*	11	Distal to *fl1*
179	VIIb	25	25	11*	11	Proximal to *fl1*
196	Ia	8*†	37	34	20	Between *paba* and centromere
Control	—	22	33	30	14	

* $P \leqslant 0{\cdot}05$ by χ^2 test, on comparison with control.
† Segregants requiring, and others independent of, *p*-aminobenzoic acid were obtained.

Location of lethals

Each lethal was located to a particular chromosome, mainly on the basis of haploidization (Table 1). More precise location of some lethals induced in diploid 1 has been described above.

It was sometimes possible to attempt more precise location of a lethal by estimating the frequency with which a lethal-bearing isolate produced certain classes of diploid segregants by mitotic crossing-over. For example, an isolate with a lethal distal to *y* and in *cis* arrangement would produce no homozygous *y* sectors; a lethal proximal to *y* would decrease the frequency of such sectors; a lethal on the left arm of I should not affect the frequency of diploid yellow sectors. In appropriate cases the frequency of diploid sectors produced by lethal-bearing isolates was compared with the frequency of such sectors from the diploid parent. The significant results are summarized in Table 2.

DISCUSSION

The present study has yielded a total of 22 irreparable lethals following ultraviolet treatment to 5% survival; 6 came from 100 isolates of a diploid assaying 4 chromosomes and 15 from a diploid assaying 8 chromosomes out of the diploid complement of 16. Similar numbers of isolates from untreated diploids gave no lethals. On the assumption that the frequency of lethals is the same for all chromosomes—an assumption unlikely to be fully justified—the lethal frequency for the whole genome was between 24% and 32%. This agrees well with the 25% found by Käfer & Chen (1964) under similar conditions.

Despite an intensive investigation, 6 of the 200 u.v. isolates gave no haploid sectors, though they did produce diploid segregants resulting from mitotic crossing-over. Two other isolates produced only very rare sectors and they were shown to carry two lethal mutations each. It is possible, then, that the refractory six isolates also carried multiple lethals. Lethal mutations are likely to be genetically heterogenous and to include, for example, translocations of a type permitting no viable haploid segregants. Several of the six isolates produced diploid segregants at a frequency significantly less than the control and this would tend to support the idea of multiple lethals. Käfer (1963) described similar cases of stable diploids and Tector & Käfer (1962) observed such strains after gamma-irradiation.

Discovery of the parasexual cycle (Pontecorvo & Roper, 1952) opened the way to the planned breeding of certain asexual fungi used in industrial fermentations. Sermonti (1959), Ishitani, Ikeda & Sakaguchi (1956), Ikeda, Nakamura, Uchida & Ishitani (1957) and Macdonald (1964) have discussed the possible value of diploid strains of fungi for industrial purposes, since such diploids would be buffered against selection of undesirable spontaneous recessive mutations; their main disadvantage might lie in the production of haploid segregants and diploid mitotic cross-over types. The present study has shown the relative ease with which balanced lethal systems can be produced and used to maintain stable diploids or heterokaryons. The use of such systems would prevent or decrease the frequency of viable haploid segregants and, in certain cases, would decrease the effective frequency of mitotic crossing-over.

Of the 22 lethal mutants detected, none was temperature-sensitive and the lethals could be maintained only in heterozygous, or in one case heterokaryotic, condition. Haploid conidia, from a heterokaryon with a different lethal in each component, gave micro-colonies about 60 μ in diameter. It would be of interest to know whether lethal mutations in Aspergillus, like those of other organisms, have different times of action (*see* Hadorn, 1951; Epstein *et al.* 1963). This is now being studied in strains carrying temperature-sensitive lethals which permit genetical and biochemical study of the

mutant lesion in haploid as well as in heterokaryotic and heterozygous diploid condition.

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The Pigments of *Sarcina flava*: a new series of C_{50} Carotenoids

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SUMMARY

The carotenoids of *Sarcina flava* were examined; four main fractions were separated. These were hydrocarbons, mono- and di-hydroxylated compounds and a very polar fraction. Further separation of the hydrocarbon fraction showed the presence of nine compounds and of the very polar fraction which showed an all-*trans* form and three *cis* isomers. Data for these fractions is presented; at least two of the fractions are C_{50} carotenoids.

INTRODUCTION

After showing that *Sarcina flava* and *S. lutea* apparently synthesize identical pigments (Thirkell & Strang, 1967), the following work was undertaken to examine the chemical nature of these carotenoids. Most carotenoids which have been reported to occur in bacteria have 40 carbon atoms, but recently a novel C_{50} carotenoid has been reported in *Flavobacterium dehydrogenans* (Jensen & Weeks, 1966). The work reported here shows the existence of C_{50} carotenoids in another Gram-positive organism, *S. flava*.

METHODS

Bacteria. Large quantities of *Sarcina flava* (NTCC 7503) were grown on nutrient agar in large oblong aluminium dishes. The bacteria were harvested and stored at $-20°$ until used.

Chemicals. All solvents used were Analar grade and were dried and redistilled before use. For spectrophotometric work, spectral quality solvents were used.

Pigment extraction and purification. Method (*a*) of Thirkell & Strang (1967) was used and the total pigment obtained was first purified by the lipid precipitation technique of Blessin (1962). The ether of the resultant supernatant fluid was removed on a rotary evaporator under reduced pressure at 35°. The pigment was dissolved in methanol, made 10% (w/v) with respect to KOH and saponified overnight at room temperature in the dark and in an atmosphere of nitrogen. The unsaponifiable material was extracted in the usual way.

Isolation of the individual pigment fractions. Preparative thin-layer chromatography on silica gel G (Merck) was used: 0·5 mm. layers on 200 × 200 mm. plates were strip-loaded by using the mechanical applicator produced by Desaga (Camlab (Glass) Ltd.). The plates were developed in chloroform + methanol (95 + 5, v/v); this separated four main fractions. The solvent used in the previous work (Thirkell & Strang, 1967) was not used in case the presence of a small concentration of acetic acid should induce the formation of isomers of certain fractions.

Tests used for characterization of fractions

Acetylation (Kuhn & Sorensen, 1938; Jensen, 1962). A sample of chromatographically pure material was taken to dryness on a rotary evaporator, and further dried under high vacuum for 30 min. The fraction was dissolved in 1 ml. pyridine which had been dried over NaOH pellets and redistilled; 0·1–0·2 ml. acetic anhydride was added and the reaction allowed to proceed at room temperature in the dark under an atmosphere of nitrogen. Samples were withdrawn at regular intervals in order to follow the course of the reaction chromatographically over a period of 24 hr, by which time it was complete. The chromatography was done on 18·5 cm. diameter kieselguhr-filled circular Schleicher & Schüll chromatography papers (Jensen & Jensen, 1959), using acetone and light petroleum (b.p. 60–80°) in various ratios as solvent. Thus the initial compound, the formation of intermediates during the reaction, and the formation of the final ester was followed. The ester formed was recovered into ether, and the ether thoroughly washed with water to remove pyridine. Spectrophotometric examination was then used to test that no degradation had occurred and to estimate the yield.

Test for tertiary hydroxyl groups after acetylation (S. L. Jensen, private communication). A sample of dry ester from acetylation was dissolved in 0·5 ml. dry pyridine and 0·2 ml. hexamethyl-disilane + 0·1 ml. trimethylchlorosilane added. The reaction proceeded at room temperature in an atmosphere of nitrogen. After 1 hr, carbon tetrachloride was added and the solution taken to dryness which removed the excess silane added. The product was dissolved in methanol and examined on kieselguhr-filled papers as before. The presence of a compound other than the substrate would be indicative of the formation of a silane from a tertiary hydroxyl group.

Reduction of esters with $LiAlH_4$ (Goodwin, 1956). A small quantity of $LiAlH_4$ was suspended in dry ether and the suspension filtered through glass wool to remove the larger particles. The filtered suspension was added to an ethereal solution of the ester in a separating funnel. After a short time, the reaction was terminated by adding wet ether. The products were removed, and excess $LiAlH_4$ destroyed by adding water. Chromatography on kieselguhr-filled papers was again used to follow the reaction. The appearance of products other than the ester or original fraction would indicate that reducible groups were present.

Oxidation with nickel peroxide (S. L. Jensen, private communication). The fraction was dissolved in dry ether and nickel peroxide added in the ratio 5 mg. nickel peroxide to 1 mg. sample. The reaction occurs spontaneously at room temperature under nitrogen. Samples were taken at intervals, and the course of the reaction followed chromatographically as before. The test is specific for the presence of allylic hydroxyl groups.

Iodine isomerization (Zechmeister & Polgár, 1943). To a solution of the fraction in acetone, a drop of iodine solution in hexane (10 μg./ml.) was added. The mixture was exposed to weak sunlight for 2–3 hr, samples were taken at intervals and the course of the reaction followed as before. When the pseudo-equilibrium was reached, the positions of the isomers were cut from the papers which were eluted with methanol, and the spectra and relative proportons determined.

Methylation of carboxyl group (Metcalfe & Schmitz, 1961). A sample of the fraction under test was dried as for acetylation and dissolved in moisture-free methanol.

0·5 ml. methanolic boron trifluoride was then added and the reaction mixture refluxed for 2–3 min. The product was recovered into ether and examined chromatographically and spectrophotometrically.

Partition ratio (Petracek & Zechmeister, 1956). A mixture of methanol+water (95+5, v/v) and hexane was equilibrated and the sample was taken up in a known volume of one of them and transferred to a 10 ml. measuring cylinder. The sample was partitioned between equal volumes of both solvents and the two layers collected. The concentration of pigment in each layer was estimated spectrophotometrically.

Infrared spectroscopy. This was done with a Unicam SP200G instrument, by using either liquid cells and solutions in carbon tetrachloride or carbon disulphide, or KBr discs (approximately 50 μg. sample/90 mg. dry KBr).

Mass spectrometry. The sample was dissolved in the minimum volume of methylene dichloride and the solution spotted onto a ceramic direct insertion probe which was inserted through a vacuum lock into the ion chamber of an Associated Electrical Industries' MS 9 mass spectrometer. The sample was heated by contact with the walls of the ion chamber to achieve a reasonable rate of evaporation, and the mass spectrum scanned with the resolving power set at 1000. The temperature of the ion chamber was about 250°. Masses of the peaks were measured very accurately at a resolving power of 12,000.

RESULTS

The results of analysis of the four main fractions were as follows.

Fraction 1 (5% of the total pigments). With chloroform+methanol (95+5, v/v) as solvent, this fraction ran with the solvent front. It was rechromatographed on silica-loaded papers (Whatman SG 81) with light petroleum (b.p. 60–80°) as solvent. This resolved the fraction into nine bands as documented in Table 1.

Table 1. *The nature of the carotene fractions resolved from fraction 1 by circular paper chromatography on silica-loaded papers (Whatman SG 81) using light petroleum (b.p. 60–80°) as solvent*

The partition ratio of each fraction between methanol+water (95+5, v/v) and hexane, was 0/100.

Band	Type	R_F value	Colour	$\lambda_{max.}$ in methanol	Comment
F 1 (*a*)	Fluorescent	0·48	Colourless	—	Early carotene precursor?
(*b*)	Fluorescent	0·40	Colourless	—	Early carotene precursor?
(*c*)	Absorbent	0·28	Yellow	392, 424, 448	?
(*d*)	Fluorescent	0·17	Colourless	—	Very low concentration
(*e*)	Absorbent	0·12	Yellow	408, 431, 450	Unknown carotene
(*f*)	Absorbent	0·11	Yellow	406, 428, 450	Isomer of F 1 (*e*)
(*g*)*	Absorbent	0·07	Yellow	395, 415, 438	Unknown carotene
(*h*)*	Absorbent	0·06	Yellow	394, 415, 438	Isomer of F 1 (*g*)
(*i*)†	Absorbent	0·03	Yellow	414, 438, 469	Sarcinene?

* Subfractions (*g*) and (*h*) did not separate completely but gave an elongated egg-shaped spot.

† The $\lambda_{max.}$ of F 1 (*i*) are consistent with the presence of a chromophore containing nine conjugated double bonds. This is probably the parent hydrocarbon of the polar carotenoids in this bacterium.

Fraction 2 (8% of the total pigments). The results of the tests of this fraction are shown in Table 2. Some data obtained for this fraction and its derivatives from kieselguhr-filled circular chromatography papers are shown in Table 3.

Fraction 3 (26% of the total pigments). The results of tests on this fraction are shown in Table 4. Some data obtained for this fraction and its derivatives from kieselguhr-filled circular chromatography papers are shown in Table 5.

Table 2. *The results of chemical tests carried out on fraction 2 in an attempt to gain information as to its nature*

Test	Result	Comment
Acetylation	Max. number of spots detected during reaction—2 (original compound + an ester) Final compound formed—monoester	Single hydroxyl group present
Tertiary hydroxyl group	Negative	No tertiary hydroxyl group present
Reduction with $LiAlH_4$	Negative	No other reducible group present
Oxidation with nickel peroxide	One α-β unsaturated aldehyde formed	One allylic hydroxyl group separated from the polyene chain
Iodine isomerization	One isomer induced (Neo U) (20% of original converted to isomer)	—
Partition ratio	27:73	Polar compound, value consistent with monohydroxyl compound
Infrared spectroscopy	No *cis* peaks; peak at 1050 cm^{-1}	All *trans* form; primary hydroxyl group
Mass spectrometry	Parent ion at m/e 686; mass measurement gave the empirical formula as $C_{50}H_{70}O$	C_{50} carotenoid fraction

Table 3. *Data obtained for fraction 2 and its derivatives from separation on kieselguhr-filled circular chromatography papers*

The solvent used was acetone+light petroleum (b.p. 60–80°; 2+98, v/v).

Sample	R_F value	$\lambda_{max.}$ in methanol (mμ)
All *trans* fraction 2	0·74	—, 415, 439, 469
Neo U isomer	0·61	331, 413, 437, 466
Monoacetate	0·95	—, 415, 439, 469

Fraction 4 (61% of the total pigments). This polar fraction from the chloroform+methanol (95+5, v/v) separation was further separated on kieselguhr-filled circular chromatography papers into four fractions by using 40% acetone in light petroleum (b.p. 60–80°) as solvent. The four subfractions were: fraction 4 (*a*), neo B isomer of fraction 4 (*c*) (6·5% of fraction 4); fraction 4 (*b*), neo A isomer of fraction 4 (*c*) (10·5% of fraction 4); fraction 4 (*c*), all *trans* compound (53·0% of fraction 4); fraction 4 (*d*), neo U isomer of fraction 4 (*c*) (30·0% of fraction 4). Fraction 4 (*c*) was examined in more detail and the results are given in Table 6. Some data obtained for this fraction and its derivatives from kieselguhr-filled circular chromatography papers are given in Table 7.

Table 4. *The results of chemical tests carried out on fraction 3 in an attempt to gain information as to its nature*

Test	Result	Comment
Acetylation	Max. number of spots detected during reaction—3 (original compound, mono- and di-ester). Final compound formed—di-ester	Two hydroxyl groups present
Tertiary hydroxyl group	Negative	No tertiary hydroxyl group present
Reduction with $LiAlH_4$	Negative	No other reducible group present
Oxidation with nickel peroxide	One mono and one di α–β unsaturated aldehyde formed	Two allylic hydroxyl groups separated from polyene chain
Iodine isomerization	One isomer induced (Neo U) (33 % original converted to the isomer)	—
Partition ratio	71:29	Polar compound, value consistent with the presence of two hydroxyl groups
Infrared spectroscopy	No *cis* peaks; peak at 1050 cm^{-1}	All *trans* form; primary hydroxyl groups
Mass spectrometry	Parent ion at m/e 702; mass measurement gave the empirical formula as $C_{50}H_{70}O_2$	C_{50} carotenoid fraction

Table 5. *Data obtained for fraction 3 and its derivatives from separation on kieselguhr-filled circular chromatography papers*

The solvent used was acetone + light petroleum (b.p. 60–80°; 10 + 90, v/v).

Sample	R_F value	$\lambda_{max.}$ in methanol (mμ)
All *trans* fraction 3	0·50	—, 415, 439, 469
Neo U isomer	0·42	331, 412, 436, 466
Monoacetate	0·60	—, 415, 439, 469
Diacetate	0·99	—, 415, 439, 469

Table 6. *The results of chemical tests carried out on fraction 4 (c) in an attempt to gain information as to its nature*

Test	Result	Comment
Acetylation	Max. number of spots detected during reaction—7. Final number of spots detected—1	Three hydroxyl groups present
Tertiary hydroxyl group	Negative	No tertiary hydroxyl group present
Reduction with $LiAlH_4$	Not carried out on this fraction	—
Iodine isomerization	Three isomers were induced:	
	One Neo B isomer	Equivalent to fraction 4 (*a*)
	One Neo A isomer	Equivalent to fraction 4 (*b*)
	One Neo U isomer	Equivalent to fraction 4 (*d*)
Partition ratio	100:0	Very polar compound
Methylation of carboxyl group	Positive	Carboxyl group present?
Infrared spectroscopy	No *cis* peak. No absolute conclusion could be reached from the traces as to the nature of the hydroxyl groups or as to the presence or absence of a carboxyl group	All *trans* compound

Table 7. *Data obtained for fraction 4 and its derivatives from separation on kieselguhr-filled circular chromatography papers*

The solvents used were *acetone+light petroleum (b.p. 60–80°; 40+60, v/v) and †acetone+light petroleum (b.p. 60–80°; 10+90, v/v).

Sample	R_F value	$\lambda_{max.}$ in methanol (mμ)
Neo B isomer	0·99*	331, 414, 437, 468
Neo A isomer	0·83*	331, 414, 438, 468
All *trans* fraction 4 (*c*)	0·77*	—, 416, 439·5, 469·5
Neo U isomer	0·69*	331, 414, 436, 467
Triacetate	0·67†	—, 416, 439·5, 469·5

DISCUSSION

This present work revealed the presence of a new series of C_{50} carotenoids in *Sarcina flava* (and presumably also in *S. lutea*; Thirkell & Strang, 1967). We suggest that a C_{50} carotene (sarcinene ?; Chargaff & Dieryck, 1932) would be the logical parent compound of the series. As fraction 1 (i) has the same chromophore as the other fractions, it is likely that this is the parent hydrocarbon in question. The addition of a further ten carbon atoms to the normal C_{40} skeleton may make the hydrocarbon relatively more polar as suggested by its low R_F value. Examination of the other compounds in fraction 1 by co-chromatography showed that the normal precursors of the C_{40} carotenoids, e.g. phytoene, phytofluene and neurosporene, were absent. This may suggest an alternative biosynthetic pathway for this series of compounds. On the basis of the evidence presented, it would seen that the parent hydrocarbon is first converted to a monohydroxyl compound (fraction 2), then to a dihydroxyl compound (fraction 3), and finally to a more polar compound (fraction 4).

Aged chloroform has a tendency to form a trace of HCl which could dehydrate allylic alcohols or induce isomerism in carotenoids. The chloroform used in this work was redistilled immediately before use, stored in the dark, and the chromatographic separations, which were reproducible, were done in an inert atmosphere. If *cis* isomers are present, appropriate $\lambda_{max.}$ are detected on a spectrophotometer. Further separation attempted on the fractions revealed the presence of *cis* isomers in fractions 1 and 4. Two subfractions of fraction 1 appeared to be *cis* isomers, but the concentration of these fractions was very low. Fraction 4 was resolved into four subfractions on kieselguhr-filled chromatography papers using the solvent system as reported. Three of these subfractions were *cis* isomers and constituted 47% of fraction 4. Using a different solvent system, Thirkell & Strang (1967) also found *cis* isomers present in the pigments of *Sarcina flava*, and whether or not these *cis* isomers are naturally occurring remains to be elucidated. It is extremely difficult to show that a *cis* isomer is naturally occurring and not induced by a preparative procedure.

Under Dr S. L. Jensen's guidance, fraction 3 was compared with compound P439 from *Flavobacterium dehydrogenans* (Jensen & Weeks, 1966), the only previously reported C_{50} carotenoid. The two compounds gave identical results on chemical examination and neither the original compounds nor their derivatives could be separated by co-chromatography. Whereas compound P439 has an empirical formula of $C_{50}H_{72}O_2$, the mono- and dihydroxyl fractions reported here have their most intense peak in mass spectral analysis corresponding to compounds with 70 hydrogen atoms.

Evidence was also obtained which indicates the co-existence of both an H_{72} and an H_{68} series. Other than mass spectral data, no evidence was found which suggested the presence of more than one carotenoid in fractions 2 and 3.

The nature of fraction 4 is obscure. It forms a triple acetate, indicating three hydroxyl groups, but the R_F value of this ester was still relatively polar and much more so than the esters produced from fractions 2 and 3. This polarity was not due to a tertiary hydroxyl group, since no silane derivative was produced. Some other polar group must be present; the methylation reaction indicated the possibility of a carboxyl group. This was not proved or disproved by infrared analysis. If the hydroxyl groups in fractions 2 and 3 are allylic and primary, hydroxylation of carbons 5 and 5′ would be the most likely explanation. It is conceivable that one of these groups could be oxidized to carboxyl and that the three hydroxyl groups of fraction 4 are such that one primary hydroxyl group remains on carbon 5 or 5′, and that two others, presumably secondary hydroxyl groups, are substituted elsewhere on a ring.

It is not possible to arrive at the exact structure of any of the fractions but the following may be said. The work of Jensen & Weeks (1966) has suggested the presence of two α-ionone rings in compound P439. Since P439 gave identical chemical results and co-chromatographs with fraction 3, it is reasonable to assume the presence of two α-ionone rings in this series. Fractions 2 and 3 have primary hydroxyl groups (again similar to compound P439). The absorption spectra indicate that the main chromophore has nine conjugated double bonds and that the conjugated system does not extend into the rings. However, hydroxylation of methyl groups substituted on carbons 5 and 5′ of the rings may cause a spectral shift and thus definite proof of this proposed chromophore is still lacking. One would assume that the ten extra carbon atoms are derived from the inclusion of two extra isoprene units into the skeleton of these molecules, as compared with C_{40} carotenoids. This inclusion would be at some stage during biosynthesis and since many C_{40} carotenoids are mirror images about the central C 15–15′ bond, it is most likely that one C_5 unit is added on each side of this bond. The mass spectra of these compounds are not consistent with the C_5 units being substituted either on the rings or on the central chain of carbon atoms. It may be that the central chain is elongated in some way so that the chromophore of nine conjugated double bonds is not altered.

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Morphological Changes in *Escherichia coli* Strain C Produced by Treatments Affecting Deoxyribonucleic Acid Synthesis

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SUMMARY

Unusual swollen and branched cell forms were produced in a thymineless mutant of *Escherichia coli* strain C by treatment with mitomycin C, and to lesser extents, by incubation without thymine and by exposure to ultraviolet light. In the case of mitomycin C treatment the morphological changes were accompanied at first by degradation of deoxyribonucleic acid (DNA), later by degradation of ribonucleic acid (RNA) and inhibition of protein synthesis, and throughout by inhibition of DNA synthesis and extensive loss in viability. Thymineless incubation prevented DNA synthesis and also resulted in extensive killing. Cultures incubated after exposure to ultraviolet light exhibited a small amount of DNA degradation and a lag in DNA synthesis. Upon prolonged incubation with mitomycin C or without thymine many of the abnormal forms became very enlarged and eventually lysed. No evidence of bacteriophage or bacteriocin could be detected in the treated cells.

INTRODUCTION

Strain C of *Escherichia coli* is quite distinct from others of the species in that its shape is more spherical than rod-like, and its nuclear matter is peripherally distributed in the cell (Lieb, Weigle & Kellenberger, 1955). In experiments with a thymineless auxotroph derived from this strain (Hewitt, Suit & Billen, 1967), it was noticed that prolonged incubation of the organism without thymine resulted in the production of swollen and branched forms different from the filamentous 'snakes' produced by this treatment in other *E. coli* strains (Barner & Cohen, 1954). Upon further investigation it was found that incubation of *E. coli* C with the antibiotic mitomycin C was particularly effective in producing such abnormal forms, again quite different from the long non-septate filaments formed by *E. coli* strains B and K12 during incubation with mitomycin C (Reich, Shatkin & Tatum, 1961). The present report describes the morphological abnormalities and other changes that take place in cultures of *E. coli* C incubated with mitomycin C, without thymine, and following exposure to ultraviolet light.

METHODS

Organisms. Escherichia coli strain C was kindly supplied by Dr R. L. Sinsheimer. Derivation of the mutant designated *thy*-321 has been described by Hewitt, Suit & Billen (1967). The cultures were maintained at room temperature in sealed 0·6% (w/v) nutrient agar stab tubes.

Conditions for growth. Escherichia coli C *thy*-321 was grown in a minimal medium (MM) containing 0·7% K_2HPO_4, 0·3% KH_2PO_4, 0·05% Na citrate. $2H_2O$, 0·01% $MgSO_4.7H_2O$, 0·1% $(NH_4)_2SO_4$, and 1% (w/v) glucose and supplemented with 2 μg./ml. thymine. Two ml. media were loop inoculated from slants or stabs and shaken overnight at 37°. Next day, the cultures were diluted to an optical density (at 660 mμ in a Bausch and Lomb Spectronic '20' spectrophotometer) of 0·05–0·1 (2–4 × 10^7 cells/ml. by viable count) with fresh media and incubated at 37° with forced aeration by sparging to produce either large inoculum cultures or experimental cultures. Inoculum cultures were grown to an optical density of 0·35, harvested by filtration (Schleicher and Schuell membrane filters, 0·45 μ pore size, 47 mm. diam.), washed, concentrated threefold in unsupplemented MM, and stored in the refrigerator for up to 5 days' use. Experimental cultures were grown from diluted overnight or inoculum cultures to an optical density of 0·25–0·3 (about 1 × 10^8 cells/ml.) and subjected to the experimental treatments described below. The normal doubling time for *E. coli* C *thy*-321, as measured by optical density increase, was 58 min. in MM.

Viable counts. Viable counts were made by spreading 0·1 ml. of appropriate dilution of the cultures on the surface of nutrient agar plates [1% (w/v) agar, 1·3% (w/v) tryptone, 0·8% NaCl, 0·2% Na citrate, and 0·13% glucose]. The colonies were counted after overnight incubation at 37°.

Thymineless incubation. Experimental cultures were grown in fully supplemented MM, harvested, washed, resuspended at the same concentration in MM containing no thymine, and returned to aeration at 37°. In some cases, in order to ensure that small amounts of thymine, possibly available from internal pools, would not be utilized by the organism, 50 μg./ml. uridine was added to the culture (Freifelder, 1965).

Incubation with mitomycin C. Mitomycin C (obtained from Calbiochem) was added to experimental cultures growing in fully supplemented MM at final concentrations of 0·1–5 μg./ml.

Irradiation with ultraviolet light (u.v.). Experimental cultures were grown in MM, harvested, washed, and resuspended in unsupplemented MM at a concentration of 1 × 10^8 cells/ml. Layers of the suspension, 2 mm. deep, were exposed while being stirred in sterile glass Petri dishes, to two Westinghouse germicidal u.v. lamps at a distance of 95 cm., giving an average incident dose of 6·4 ergs/mm.^{2}/sec. Exposure for 60 sec. reduced the viable count to 15–20%. The suspensions were then diluted into supplemented MM and returned to aeration at 37°.

Phase microscopy. Cultures were examined microscopically with a Leitz Ortholux Research Microscope equipped with dark phase optics and illuminated by a 60 W light source manufactured by the W. H. Talley Co., Houston, Texas. Coverslip impressions were made from drops of the cultures which had been allowed to soak into a minimal agar plate. The coverslips were placed on microscope slides coated either with 1% (w/v) agar or with a mixture of 1% (w/v) agar and 25% (w/v) gelatin in peptone broth, in a manner similar to that described by Alder & Hardigree (1965). The gelatin preparation enhanced visualization of the nuclear material (Mason & Powelson, 1956). It was filtered through an 0·45 μ Millipore filter immediately before use.

Chemical analyses. Ten-ml. samples were removed from incubating cultures at intervals and the cells pelleted by centrifugation at 12,000 *g* for 15 min. The pellets were extracted by the method of Ogur & Rosen (1950), and the extracts were analysed

for deoxyribonucleic acid (DNA) by the method of Burton (1956) and for ribonucleic acid (RNA) by the method of Visser & Chargaff (1948). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Changes in morphological appearance during treatment. The changes in morphological appearance of *Escherichia coli* C *thy*-321 that occurred during incubation with mitomycin C are shown in Plate 1. Within 30 min. of incubation with mitomycin C the cells began to enlarge, both in length and girth. At early times 'y' or knobbed forms were frequently observed, and later some obviously branched forms appeared. As incubation continued, the cell type most often observed was grossly swollen in the centre with two or three elongated 'arms'. The cultures were never uniform in appearance, however, and cells of all shapes and sizes could be seen throughout the incubation period.

The cells in Pl. 1, *a–c*, were mounted on gelatin-coated slides in order to provide a background with a refractive index near that of the cytoplasm of the cells, allowing better resolution of the nuclear material (Mason & Powelson, 1956). The nuclear material appears white or lighter in the photographs. The nuclear material in the normal cells did not show up distinctly with the gelatin technique; we assume that this was because of its peripheral distribution (Lieb, Weigle & Kellenberger, 1955). Upon mitomycin C treatment the nuclear material first became more visible, as if coalesced, and almost filled the cell; later, it became diffuse or patchy. However, in the cell shown in Plate 1, *f*, a branched form at 4 hr, the nuclear material was still obvious and condensed in one area.

Similar morphological changes were observed in cells incubating without thymine or following u.v. irradiation (Pl. 2, *a–f*). In the case of thymineless incubation the changes developed more slowly than with mitomycin C treatment. For some time most of the cells were more elongated than swollen, but eventually (4–5 hr) huge, swollen and branched forms were frequently observed. The changes seen during the post-irradiation incubation were never as extensive as with the other two treatments.

After 3–4 hr of mitomycin C treatment, and to a lesser extent after 5 or more hr of thymineless incubation, 'ghosts' of the swollen branched forms were seen (Pl. 2, *g–i*) as well as clumped fragments of membrane-like material, suggesting that as the abnormal cell forms enlarged they became fragile and eventually lysed.

Effect of treatments on growth and viability. The treated cultures were examined for other changes which might have been related to the changes in morphological appearance.

Figure 1 shows that incubation with mitomycin C brought about rapid and extensive killing of the organism after about 20 min. After a somewhat longer period extensive killing was also produced by thymineless incubation. The survivors of u.v.-exposure remained viable and began to divide after a lag period of an hour or more.

Even the cultures that were declining in viability increased substantially in mass for a time during the incubation (Fig. 2). This is probably a reflexion of the enlargement of the abnormal forms described above. The addition of mitomycin C or removal of thymine did not appear to affect the rate of increase in optical density significantly for about 30 min. Then the rate slowed and the increase gradually came to a halt

within the subsequent 2–3 hr of incubation. After this, little further change was observed for up to 6 hr of thymineless incubation (not shown in figure). However, in the case of mitomycin C, the optical density of the culture began to decrease after about 3 hr. This was the time when microscopic examination began to reveal ghosts and debris in the culture. The time and extent of lysis produced by mitomycin C were variable from experiment to experiment. Each of four concentrations tested (0·1, 0·5, 1·0 and 5·0 μg./ml.) produced some lysis, but the onset was earliest with 5 μg./ml.

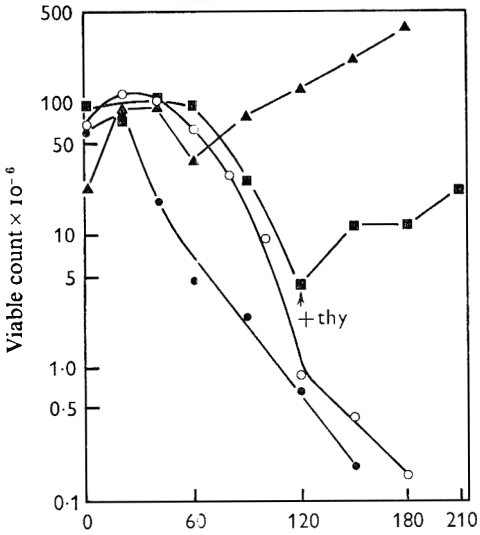

Fig. 1

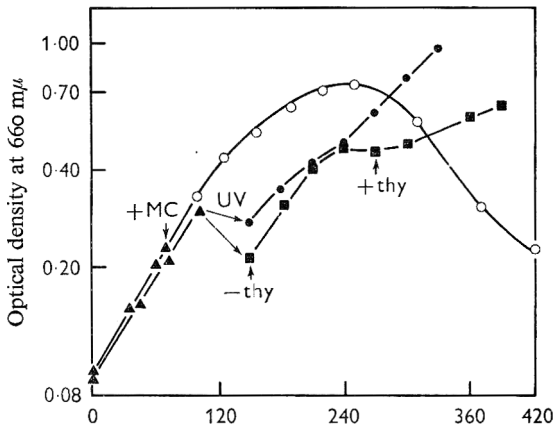

Fig. 2

Fig. 1. Effects of incubation with mitomycin C (MC, 5 μg./ml.) incubation after ultraviolet light (u.v.)-irradiation, and thymineless (−thy) incubation with or without subsequent readdition of thymine (+thy, 2 μg./ml.) on the viability of *Escherichia coli* C *thy*-321. Cultures were grown in minimal salts media and treated as described in Methods. ○—○, −thy; ●—●, +MC; ▲—▲, u.v. exposed; ■—■, −thy, 120 min.

Fig. 2. Effects of various treatments on the growth of *Escherichia coli* C *thy*-321. The data shown for +MC, u.v.-exposed, and −thy, 120 min., were obtained from the cultures shown in Fig. 1. ▲—▲, Logarithmically growing cultures.

Mitomycin C had the most drastic effect on the synthesis of DNA, RNA, and protein of any of the treatments (Fig. 3). There was no net increase in DNA (diphenylamine-reacting material) in the presence of 5 μg. mitomycin C/ml. and almost 30% of the DNA initially present disappeared from the cold acid-precipitable fraction within the first 30 min. of incubation. During the first 30–45 min. of incubation with mitomycin C, there were 40% net increases in RNA and protein. Subsequently, RNA was degraded, but the protein content of the culture remained constant. The period of time covered by the sampling in these experiments preceded the onset of visible lysis in the culture.

Thymineless incubation, with or without 50 μg. uridine/ml., also prevented net DNA increase, but significant DNA degradation was not observed until after an hour or more. RNA and protein synthesis in cultures incubated without thymine continued at linear rates for almost 90 min. In the case of u.v.-exposed cells, a 12%

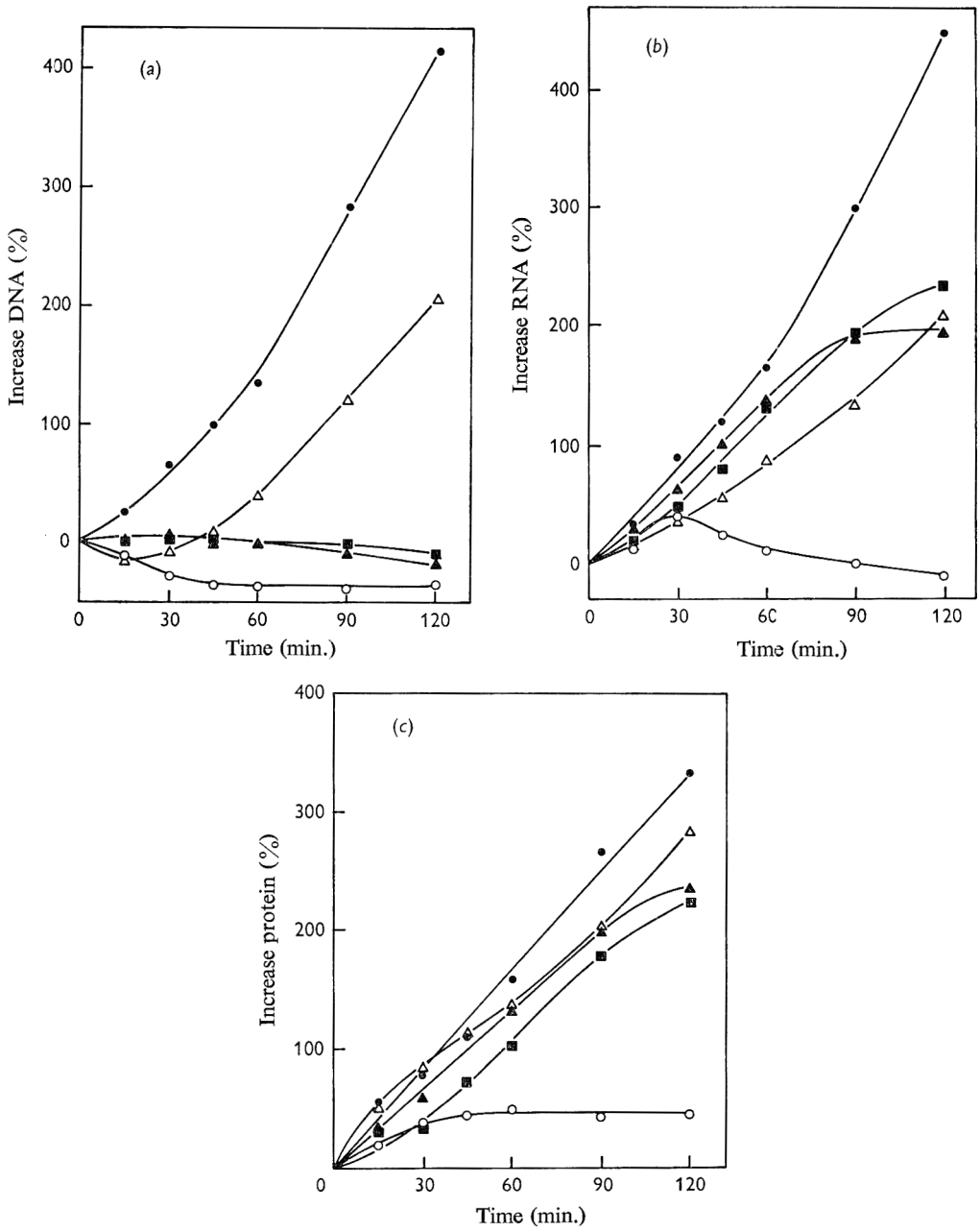

Fig. 3. Effects of various treatments on synthesis of (*a*) DNA, (*b*) RNA, and (*c*) protein; by *Escherichia coli* c *thy*-321. Cultures were grown in minimal salts media and treated as described in Methods. Final concentrations of mitomycin C (MC), thymine (thy), and uridine (uri) were 5, 2 and 50 μg./ml., respectively. The u.v. exposure reduced the numbers of colony formers to 23%. ●—●, +thy; ▲—▲, −thy; ■—■, −thy+uri; ○—○, +thy+MC; △—△, +thy (u.v.-exposed).

loss in DNA was observed during the first 15 min. of post-irradiation incubation. DNA synthesis then commenced and continued throughout the remainder of the incubation. The synthesis of RNA was depressed by u.v. exposure; about 80 min. of the incubation elapsed before the RNA content of the culture doubled. Protein synthesis was less affected, even appearing to be somewhat elevated in rate, during the first 30 min. of incubation.

Search for induction of bacteriophage or bacteriocin. Since incubation with mitomycin C, thymineless incubation and u.v.-irradiation are treatments known to induce prophage carried by lysogenic bacteria (cf. Korn & Weissbach, 1962; Lwoff, Siminovitch & Kjeldgaard, 1950), and since mitomycin C produced obvious lysis of *Escherichia coli* C *thy*-321 (Fig. 2) the treated cultures were examined for evidence of bacteriophage or bacteriocin. Tests of treated 'shift-down' cultures for stimulated messenger RNA synthesis (Frampton & Brinkley, 1965), tests of supernatant fluids and artificial lysates of the treated cultures for lytic activity against various indicator strains, and examination of these materials in the electron microscope for the presence of complete or incomplete phage particles were all negative. Therefore, it appears that no readily inducible agent of this sort is carried by the organism. The lysis that occurs during incubation with mitomycin C can be explained as the result of the breakdown of the abnormal swollen cell forms that are produced by the treatment.

DISCUSSION

In other strains of bacteria mitomycin C, thymineless incubation, and exposure to ultraviolet light usually cause the development of long, non-septate filaments otherwise superficially normal in appearance (Hughes, 1956; Reich *et al.* 1961). Thus, a major effect of the treatments is the prevention of septation and cell division. The effects of the treatments on *Escherichia coli* C *thy*-321 are probably qualitatively the same as that on other organisms, and the differences between the morphological aberrations produced in it and in other organisms may be only extensions of the normal morphological differences between it and other cells; e.g. its spherical rather than rod-like shape.

The inhibition of cell division by the treatments can be explained as a secondary result of their effects on DNA. Each of the treatments affects the integrity of DNA and/or inhibits DNA synthesis (cf. Kelner, 1953; Barner & Cohen, 1954; Shiba, Terawaki, Taguchi & Kawamata, 1959; Iyer & Szybalski, 1963). According to the replicon model (Jacob, Brenner & Cuzin, 1963), septum formation is dependent upon the synthesis of the cell membrane at a site where the DNA molecule is attached, but in each cell generation synthesis of that membrane must await completion of a round of DNA replication. Thus, the inhibition of DNA replication could bring the entire sequence of events to a halt, and might result in lesions in the cell wall and membrane so that the cells would swell and become fragile as they increase in mass. This is the case with cells that are specifically prevented from cell-wall synthesis, e.g. during spheroplast formation (Lederberg & St Clair, 1958). As overall control of the normal sequential steps in growth and cell division is lost, growth may be attempted at several points resulting in the production of branched forms.

The reason that u.v.-irradiation was much less effective than the other treatments in producing extensive morphological changes is probably because the DNA synthetic

capacity of the treated cells soon recovered upon incubation in growth medium (Fig. 3). Greater doses would probably produce more drastic changes. The fact that thymineless incubation, while completely inhibiting DNA synthesis, still resulted in fewer swollen and branched forms than did treatment with mitomycin C suggests that the more extensive degradation of DNA brought about by the drug may be an accelerating factor in producing the hypothetical membrane and wall lesions.

Other than the striking differences in morphological changes that have been discussed, the effects of the treatments on *Escherichia coli* C *thy*-321 that we observed are similar in many respects to those reported by other investigators with other organisms. In the case of incubation with mitomycin C, DNA synthesis was completely inhibited and some DNA was degraded, although the loss was less extensive than that observed by Reich, Shatkin & Tatum (1961) in *E. coli* 15T$^-$. In *E. coli* 15T$^-$, treatment with 0·5–20 μg. mitomycin C/ml. brought about degradation of DNA to acid-soluble fragments at a linear rate for at least 2½ hr. The conditions found necessary for the breakdown, conditions which permit growth, were met in our experiments. However, DNA measurements in our experiments were made only during the first 2 hr of incubation because some lysis usually began shortly thereafter. The visible changes that suggest fragmentation and dispersion of the nuclear material were most obvious after 2 hr and may have been accompanied by further DNA degradation. The changes in the appearance of the nuclear material that we have observed are quite similar to those described by Reich and associates for *E. coli* B.

Sekiguchi & Takagi (1960) found that the effects of mitomycin C treatment on RNA and protein synthesis in *Escherichia coli* B changed with different concentrations of the drug and with different stocks of the organism. The effects that we have observed with *E. coli* C *thy*-321 are similar to those obtained with the more sensitive B, although our use of a different concentration of mitomycin C precludes a strict comparison. With an unspecified strain of *E. coli* Smith-Kielland (1966) observed a stimulation of RNA synthesis, chiefly that of sRNA, during the first 30 min. of treatment with 10 μg./ml. mitomycin C. She reported that longer periods of incubation resulted in RNA degradation, as did Suzuki & Kilgore (1964) in the case of *E. coli* B. The latter investigators found that 50 S ribosomes were especially sensitive and were almost completely degraded after 60 min. incubation with 5 μg./ml. mitomycin C. We have found that RNA is lost from *E. coli* C *thy*-321 after 30 min. incubation with mitomycin C (Fig. 3) but have not determined what kind of RNA is most affected. The fact that our mitomycin C-treated cultures demonstrated no net increases in RNA or protein after the first 30 min. and actually showed some degradation of RNA is surprising, for the extensive enlargement of the treated cells appears to involve a substantial increase in mass (Fig. 2). It is likely that turnover is taking place, with some of the dying cells undergoing lysis and degradation while others are carrying out some synthetic activity.

The extensive thymineless death exhibited by the organism is of interest in view of the suggestion that the major cause of thymineless death is phage or bacteriocin induction (Mennigmann, 1964; Ishibashi & Hirota, 1965). This is not obvious in the present case at least. The lag in death is somewhat longer than that usually observed with *Escherichia coli* 15T$^-$ (Barner & Cohen, 1956). This does not seem to be because thymine from internal pools is available to the organism, for it was found that incubation without thymine and with or without 50 μg. uridine/ml. gave identical

kinetics of thymineless death. Freifelder (1965) showed that ribotides antagonize the utilization of small amounts of thymine by thymineless organisms.

It would appear that the events responsible for thymineless killing in *Escherichia coli* C *thy*-321 remain reversible by thymine for some period of time. Readdition of thymine to a dying culture after 2 hr of starvation stopped death immediately (Fig. 1) and seemed to rescue some of the cells as plating centres because the viable count increased more than fivefold in 90 min. (The normal doubling time of viable count in this media is 54 min.) The rate of increase was erratic. Somewhat different results were obtained by Barner & Cohen (1956) with *E. coli* 15T$^-$. Following readdition of thymine to a starving culture, there was a lag of 30–45 min., and then the survivors began to multiply at the normal rate. Strain 15 may sustain more extensive damage from the attempted repair of single strand breaks in the DNA (Pauling & Hanawalt, 1965) during thymine deprivation than strain C. It would be of interest to examine strain C for the abnormal features of DNA replication that have been observed in *E. coli* 15 derivatives after thymine starvation and have been implicated in the mechanism of thymineless death (Pritchard & Lark, 1964; Pauling & Hanawalt, 1965).

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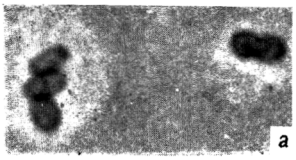
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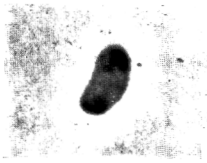
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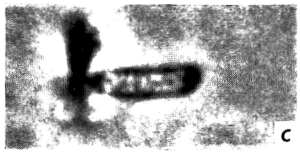
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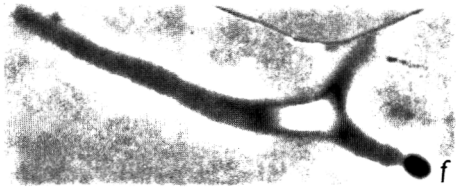
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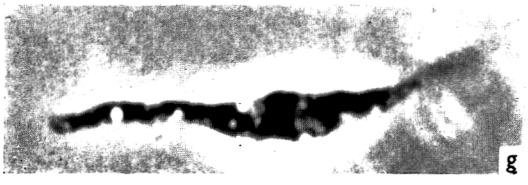
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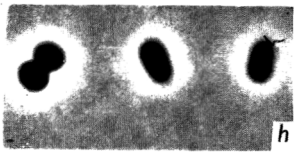
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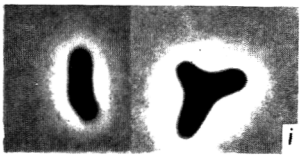
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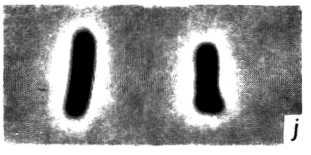
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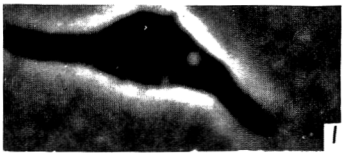
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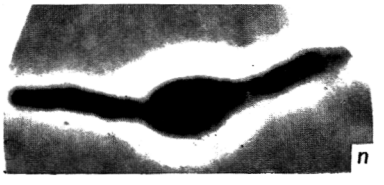
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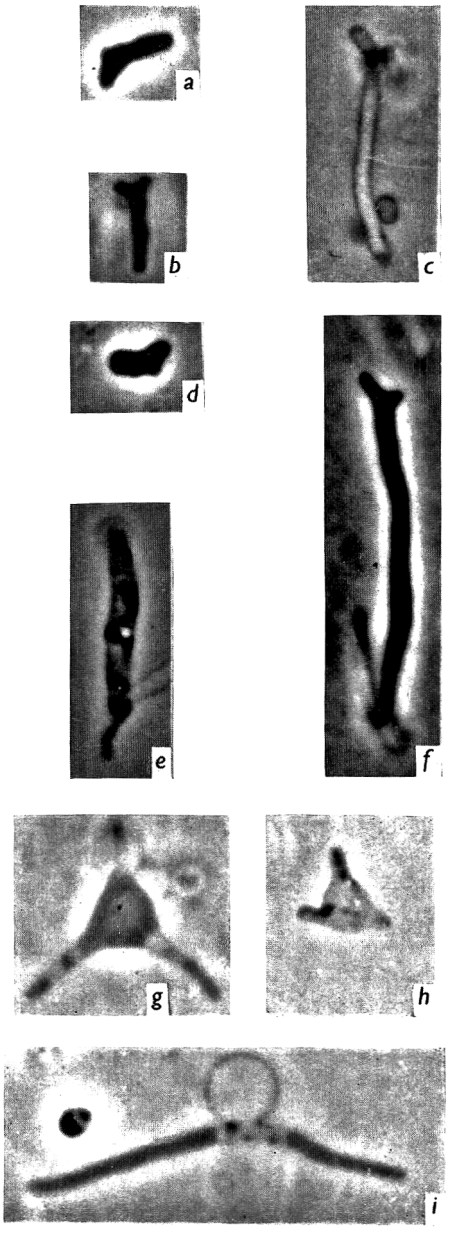
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EXPLANATION OF PLATES

PLATE 1

Escherichia coli C *thy*-321 incubated with 5 μg. mitomycin C/ml.: *a* and *h*, 0 min.; *b* and *i*, 30 min., *c* and *j*, 1 hr; *d* and *k*, 2 hr; *e* and *l*, 3 hr; *f* and *m*, 4 hr; *g* and *n*, 5 hr. Cells were incubated in minimal salts media and mounted on microscopic slides coated with gelatin (*a–g*), or with agar (*h–n*) as described in Methods. × 2400.

PLATE 2

Escherichia coli C *thy*-321 incubated: *a–c*, after u.v. exposure; *d–f*, without thymine; *g–i*, with 5 μg. mitomycin C/ml. *a*, 1 hr, agar mount; *b*, 1½ hr, agar mount; *c*, 2 hr, gelatin mount; *d*, 1 hr, agar mount; *e*, 4 hr, gelatin mount; *f*, 4 hr, agar mount; *g*, 4 hr, agar mount; *h*, 4 hr, gelatin mount; *i*, 5 hr, wet mount. × 2400.