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ANTIGENIC CHANGES ACCOMPANYING SPORE FORMATION IN *BACILLUS CEREUS*

by

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SECTION 1

INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. INTRODUCTION

Bacterial endospore formation occurs principally in the genera Bacillus and Clostridium. Under appropriate physiological conditions, a vegetative cell will give rise to a single spore. The spore differs from the parent cell in its increased resistance to heat, physical and chemical agents, and in its optical refractility when seen under the microscope. These unusual characteristics, allied to a potential for rapid growth and metabolism when placed in a suitable environment have aroused the interest of workers in many fields.

The process of spore formation is a complex one involving radical structural changes within the parent cell accompanied by alterations in chemical composition and metabolism. These changes have been studied and described by cytologists and biochemists and much is now known of the physiology and cytology of sporulation (Halvorson, 1957, 1961, 1962; Jacobs & Clegg, 1957; Robinow, 1960). Little, however, is known of the alterations in antigenic constitution which must accompany the change in chemical composition. Much of the early work on spore antigens was mainly of taxonomic significance and it is only in more recent times that serology has been used as a tool in studying cellular architecture.

The following review attempts to give a picture of the present state of knowledge of the antigenic structure of the genus Bacillus.

Closely related to antigenic structure and to antigenic changes occurring during sporulation is the physiology of sporulation. The object of the work described in this thesis is to attack the problem of spore formation from a new angle and to utilise new techniques. In so doing, some attention was paid to enzyme changes accompanying sporulation and a brief review of the literature relevant to the enzymes studied is included.

1.2. REVIEW OF THE LITERATURE

1.2.1. Spore Antigens

Published observations on the serology of the spore give a confused picture. This is partly due to the fact that the type of antigenic material used has varied from worker to worker and partly to a failure to realise and avoid the technical difficulties liable to be encountered in work of this kind. Most of these problems arise from the nature of the spore itself. Viable spores used for injection and agglutination may give misleading results for several reasons. Defalle (1902) in the earliest paper on spore antigens suggested that living spores might germinate in the animal body. This was later shown to be the case by Teale and Bach (1919). Spores of B. anthracis, B. mycoides, and B. subtilis germinated on injection into animals. Although the resulting vegetative cells did not multiply or produce toxaemia, there existed a possibility of a specific antibody response to antigens of vegetative cell origin. This must be taken into account when interpreting the results of immunological experiments. A similar problem arises when living spores are used as the antigen preparation for agglutination. This process normally involves some 2 or more hours incubation at elevated temperatures. In these circumstances the spores might well germinate, thus confusing the results obtained. By using concentrated antigen, shaking the reactants and then diluting them, Noble (1927)

decreased the reaction time substantially. Testing this method with a number of different species he obtained results comparable to those found by standard techniques. Modifications of Noble's method have been used by many workers (Lamanna, 1940a, 1940b; Lamanna & Eisler, 1960; Lamanna & Jones, 1961; Norris & Wolf, 1961).

Another difficulty noted by Defalle (1902) is that both the vegetative cells and spores of many of the genus Bacillus tend to undergo spontaneous agglutination. Noble (1919) agitated a vegetative cell suspension of B. anthracis for 12 hours and allowed it to stand overnight, when coarse aggregates of organisms settled out leaving a homogeneous suspension suitable for agglutination. Perhaps the greatest source of error in the serological study of the spore has been the use of impure material for tests. Apart from the possibility of germination of the spore, it may be contaminated with residual vegetative cell material from the sporangium. Complete sporulation seldom occurs on normal laboratory media, and old cultures normally contain a variable quantity of vegetative cell debris. Injection of such material inevitably gives rise to an antiserum containing antibodies to both spores and vegetative cells. Howie and Cruickshank (1940) described an asparaginate agar which gave complete sporulation of a B. cereus-like organism and of B. mesentericus. Suspensions of these cultures gave rise to antisera containing antibody specific

for the spore stage on injection into rabbits. Davies (1951) reported complete sporulation of B. polymyxa grown on nutrient agar. An alternative approach is the active removal of vegetative remnants. Antiformin was used by Mellon and Anderson (1919) for this purpose. Krauskopf and McCoy (1957) favoured the use of KOH. These agents are deleterious, causing drastic chemical effects which must certainly be reflected in the antigenicity of the spores. Norris and Wolf (1961) used autolysis in the presence of thiomersalate to clean the spores of several species of the genus Bacillus (B. cereus, B. subtilis, B. megaterium, B. licheniformis, B. polymyxa, B. laterosporus, and B. coagulans), but this was not effective with B. alvei, B. circulans, B. brevis, and B. sphaericus. Norris (1957) described a bacteriolytic principle from B. cereus which effectively lysed the cells of aerobic sporeformers, leaving an antigenically clean preparation of spores. B. megaterium is readily lysed by lysosyme and this enzyme was used by Tomcsik and Baumann-Grace (1959) to remove cellular debris from spore suspensions of this organism. Walker (1959) also used lysozyme to remove vegetative debris from spore suspensions of the thermophilic members of the genus Bacillus. Moussa (1959) treated spores of Cl. septicum and Cl. chauvoei with pancreatic extract to remove vegetative cell remnants, while Meisel and Rymkiewicz (1957) used purified lysozyme, deoxyribonuclease, and trypsin to clean

spores of Cl. tetani. The latter authors found that their treatment did not entirely remove vegetative debris as judged microscopically, in spite of the fact that spore suspensions were not agglutinated by antiserum to vegetative cells.

1.2.2. Antigenic Relationships Between Spores and Vegetative Cells

Defalle (1902) made the first observations on the antigenicity of bacterial spores. Using B. mycoides, B. mesentericus-vulgatus, B. alvei, and 2 attenuated strains of B. anthracis, he injected old cultures into rabbits and detected an antibody response by slide agglutination. Antiserum to viable spores agglutinated both viable and autoclaved spores, and similar results were found with antiserum to autoclaved spores. Mellon and Anderson (1919) using a formolised suspension of an old agar culture of B. subtilis for injection into rabbits obtained an antiserum which agglutinated both spores and bacilli. Likewise, antiserum to a formolised vegetative suspension gave agglutination with both spores and vegetative cells. If the spore suspension was treated with antiformin to remove vegetative remnants and washed, the resulting preparation was agglutinated only with anti-spore serum. This suggested that the cross-reaction was due to bacillary remnants in the spore preparation and the antigenic distinction between spore and vegetative phases was confirmed by agglutinin-absorption tests.

Krauskopf and McCoy (1937) examined B. niger using as antigen preparations whole vegetative cells and a spore suspension treated with KOH. Both antisera reacted with bacillary O antigen, intact vegetative cells, untreated spores and KOH-treated spores. The complex results of

absorption experiments were difficult to interpret. KOH-treated spores absorbed their specific antibodies from both antisera, and intact vegetative cells appeared to remove all antibody from spore antiserum. This led to the conclusion that spores contained no specific antigens but unfortunately the authors did not study antiserum prepared against untreated spores. Organisms still remain antigenic although dissolved in antiformin or NaOH (Ferry & Fisher, 1924). Thus some of the results described in Krauskopf and McCoy's paper might be due to inadequate washing when removing dissolved vegetative remnants.

Howie and Cruickshank (1940) described an asparaginate agar which gave complete sporulation of B. mesentericus and an organism resembling B. cereus. Suspensions of such cultures, on injection into rabbits, elicited formation of antibody specific for the spore and it was concluded that the spore antigen was separate and distinct from those of the vegetative cell. Several species of aerobic sporeformers were studied serologically by Lamanna (1940a, 1940b). Agglutinin-absorption experiments demonstrated the presence of a spore antigen which was not found in the vegetative cell. By acid extraction, Lamanna (1942) obtained a precipitinogen from spores which he used to investigate the relationships between B. subtilis and and B. vulgatus. With the organisms B. anthracis, B. mesentericus and B. ubiquitarius, Bekker (1944) prepared

antisera to the H and O vegetative antigens, living and autoclaved spores. In 2 cases the spore antisera showed high titre for the homologous antigen and low titres for the heterologous. With B. mesentericus the living spores stimulated the production of vegetative H antibody, but this was attributed to either impure injection material or germination inside the animal body. An autoclaved spore suspension did not give rise to this H antibody response. Studying B. cereus, B. subtilis, B. vulgatus, B. agri, B. brevis, and B. sphaericus, Doak and Lamanna (1948) found that KOH treatment of spores changed their antigenic properties. Antisera to viable spores of B. cereus gave a high anti-spore agglutinin titre but also a low anti-bacillary titre - a fact easily explained by the presence of residual vegetative material. A similar antigen preparation treated with KOH gave spore antibodies but also an anti-bacillary agglutinin titre of comparable value. This led to the conclusion that spores contained some of the vegetative cell antigens, but that these were detectable only by the harsh treatment of the spore. On prolonged injection of vegetative cells, small amounts of spore agglutinins were detected in the antiserum. The authors concluded that this indicated the presence of spore antigens in the vegetative cell, but this is not necessarily the case. The possibility that spores were present in small amounts in the material used for injection cannot be overlooked.

Precipitinogens were found in both spores and vegetative cells. Results of absorption experiments suggested that the spore had not one but many antigens, and one strain of B. cereus studied proved to possess at least 3 surface antigens.

Davies (1951) investigated the serological relationships between vegetative cells and spores of B. polymyxa. Antisera to the H and O vegetative antigens were prepared. The spores used were a suspension of an old nutrient agar culture and were found to be free of bacillary forms. Injection of viable spores, however, resulted in production of antiserum containing H agglutinins as well as those of the spore. The material was heated to destroy the H antigen. The author found that vegetative H and O and spore antigens were quite separate and distinct and did not cross-react. Thermophilic members of the genus Bacillus were divided into 3 groups on a basis of physiological and biochemical findings, group 1 being further sub-divided into 1a and 1b (Walker, 1959). Study of spore agglutinogens showed that group 1a possessed a group-specific spore antigen, while group 1b was more complex, and appeared to have at least 2 spore antigens. As with group 1a, spore agglutinogens of group 2 appeared to be group-specific. Injection of spores of group 3 led to death of the rabbits, apparently from some shock reaction and hence spores of this group were not included in the antigenic study. Although both acid and

formamide extraction methods were used, Walker failed to extract precipitinogens from spores of any of the thermophils, nor could he demonstrate any serological relationship between them and the mesophilic members of the genus.

In the spores of each of 12 aerobic sporeformers investigated, Norris and Wolf (1961) detected heat resistant agglutinogens and precipitinogens quite distinct from vegetative cell antigens. They studied the relationship between spore and vegetative antigens in 4 cases - B. cereus, B. subtilis, B. licheniformis, and B. alvei. With the first 3 organisms the spores had no vegetative antigens as surface components. B. alvei spores on the other hand contained H antigen, but this may be explained by the fact that part of the cell wall forms an exterior membrane round the spore of this organism. When viable spores of B. cereus and B. subtilis were injected, H. anti-bodies were detected in the antisera produced, but spore suspensions were not agglutinated by H specific antisera produced to vegetative cells, nor could spores absorb H agglutinins from vegetative cell antisera. No vegetative agglutinins were found when autoclaved spores were injected. The resulting antisera gave a strong precipitin reaction with formamide extracts of homologous spores.

More recently Cavallo, Falcone, and Imperato (1963) have followed antigenic changes during germination of B. subtilis spores using immunoelectrophoresis of ultrasonic

disintegrates of spores and cells. 3 antigens were present in resting spores, an additional 4 in alanine-germinated spores and 9 in vegetative rods. 3 of the latter had the same electrophoretic behaviour as those antigens in germinated spores. None of the antigens of resting spores were detected in vegetative rods.

Publications on the serology of the anaerobic sporeformers have been much less numerous than on the aerobes. Working with Cl. botulinum and Cl. sporogenes, Starin and Dack (1923) found that antisera prepared by the injection of rabbits with spore suspensions of the organisms gave rise to antiserum which failed to agglutinate vegetative cells. They did not test the serum against a spore suspension. Mandia (1955) demonstrated a heat stable antigen common to Cl. tetani, Cl. sporogenes, Cl. histolyticum, and Cl. parabotulinum. The antigen preparations used may well have contained spores and Sussman (1959) has suggested that this heat stable antigen might have been associated with the spores. Studying the relationship between Cl. histolyticum and Cl. sporogenes, Sussman (1959) showed that the organisms shared a single spore antigen while differing in vegetative antigens. Antisera to vegetative cells did not agglutinate spores, while antisera against autoclaved spores contained vegetative cell O agglutinin as well as spore agglutinin. Meisel and Rymkiewicz (1957) showed a specific Cl. tetani spore antigen, but found that antisera to spore suspensions

contained H and O agglutinins in spite of the fact that steps had been taken to remove vegetative debris. In a later paper (Meisel & Rymkiewicz, 1958a) it was found that Cl. sporogenes spores also contained H and O agglutinogens in addition to the spore antigen. As with Cl. tetani, the spores had been freed from vegetative remnants by the use of enzymes and then autoclaved to prevent germination in the animal body. Similar results were obtained with Cl. botulinum and Cl. histolyticum (Meisel & Rymkiewicz, 1958b). The authors concluded that although anti-H and anti-O sera failed to agglutinate enzyme-treated spore suspensions, these suspensions still gave rise to antibodies to spore antigen, and to vegetative H and O antigens on injection into rabbits.

Walker (1963) found that spores of all strains of Cl. sporogenes possessed common agglutinogens and precipitinogens, the 2 antigens appearing to be closely related. Cl. bifermentans and Cl. sordellii could be differentiated by spore agglutinogens, but not by spore precipitinogens.

Bacterial spore antigens are the subject of a review by Norris (1962).

1.2.3. Antigens in Relation to Cell Structure

The work recounted above is concerned mainly with agglutination reactions of intact cells, or precipitin reactions with chemical extracts of cells. While such techniques are valuable in taxonomy, the results yield little information on the nature or the site of the antigens involved. In recent years, attempts have been made to correlate immunological and cytological findings. Tomcsik and Guex-Holzer (1954a) described a specific cell wall reaction in Bacillus M, an organism resembling B. megaterium. The cell wall of the organism reacted specifically with antiserum to intact cells. Digestion of the cytoplasm of the organism with trypsin, pepsin, or papain did not affect the reaction. Homologous antibody also reacted with the capsule forming transverse septa and polar bodies visible under phase-contrast. The active substances in the cell wall and capsule appeared to be polysaccharide in nature. The remainder of the capsule reacted with D-glutamic acid polypeptide antibody, prepared by inoculation of rabbits with suspensions of B. anthracis. In another paper, Tomcsik and Guex-Holzer (1954b) prepared protoplasts of Bacillus M by treatment with lysozyme. Antibody to protoplasts agglutinated the homologous antigen, and unheated whole cells, but did not agglutinate cell wall suspension. Cell wall antiserum on the other hand, precipitated whole cells and whole cell polysaccharide

extract, and agglutinated cells walls, heated and unheated whole cells.

The reaction of anti-protoplast serum with unheated whole cells suggests that there may be some protoplasmic constituents on the cell surface. Flagella may remain attached to the protoplasts and this cross reaction may have been caused by the presence of H antigens in the protoplast preparation used for injection. The reader is referred to a comprehensive review of the use of antibodies as indicators of bacterial surface structures (Tomcsik, 1956).

Fowler and Harrison (1953), studying spores of B. subtilis by phase-contrast microscopy, noted that in reaction with specific agglutinating sera at dilutions where pre-zoning occurred, an 'exudate' was seen around the periphery of spores.

Tomcsik and Baumann-Grace (1959) freed spores of B. megaterium from vegetative debris by lysozyme treatment followed by thorough washing. Using phase-contrast microscopy, specific antigen-antibody reactions were observed at the surface of spores suspended in anti-spore serum. Wet Indian ink preparations demonstrated a capsule-like structure surrounding the spores and it appeared that antigens connected with this layer were responsible for the reaction. The reaction did not occur with antiserum to vegetative cells. The authors

designated this slimy layer the "exosporium" and the reaction the "specific exosporium reaction". The term "exosporium" refers to the discrete membrane surrounding the spores of certain species and should not be used loosely to identify layers of slime surrounding the spore. The structures observed by Fowler and Harrison (1953) in B. subtilis and Tomcsik and Baumann-Grace (1959) appear to be capsule-like areas of antigenic material around the spore rather than the membrane known as the exosporium. Vennes and Gerhardt (1959) isolated the major structural components of B. megaterium, including capsular polypeptide, flagella, cell walls, cell wall polypeptide and polysaccharide, protoplasts, protoplasmic membranes, intracellular granules and nuclear cores. Quantitative complement fixation tests were used for antigenic analysis as the method provided greater sensitivity and quantitative accuracy than the usual agglutination and precipitation tests. Injection of intact cells was shown to stimulate formation of antibody to each of the component structures. The isolated structures were found to be antigenically distinct with a few exceptions. Cell walls and capsules were thought to have a common polysaccharide antigen, and a low cross reaction between flagella and protoplasts or protoplast membranes was attributed to the possibility that small amounts of flagellar material might remain in the protoplast preparation.

The fluorescent antibody technique has recently been employed to study antigenic changes on the surface of Cl. sporogenes during sporulation and germination (Walker & Batty, 1963). Spore antisera were coupled with fluorescein isothiocyanate and vegetative cell antisera with Lissamine Rhodamine B 200. During germination, some areas of spore antigen were lost, presumably due to the splitting of the spore coat. Subsequently vegetative antigens emerged, spore antigens gradually disappearing until only vegetative antigens could be detected. On sporulation, spore antigens appeared on the surface of the vegetative cells only after they had become fully permeable to spore stains. The same authors used fluorescent antibodies to discriminate between various types of Cl. botulinum and in another communication (Batty & Walker, 1963) to differentiate between Cl. septicum and Cl. chauvoei. Using agar gel diffusion, Ellner and Green (1963) investigated the serology of soluble antigens of the pathogenic clostridia. Cell-free supernatant fluid from liquid cultures was saturated to 70% with ammonium sulphate to precipitate soluble antigens. Strains of Cl. sporogenes had from 2 to 4 antigens, there being 5 antigens in all. In 2 strains of Cl. novyi type B 4 antigens were detected, 3 being common to both strains. 4 strains of Cl. bifermentans had a total of 7 antigens, 5 being shared by all. Similarly with the 9 strains of Cl. sordellii, 7 antigens were demonstrated,

5 of which were common. No antigenic difference was detected between pathogenic and non-pathogenic strains of this organism. Of the 6 antigens of Cl. histolyticum 4 were shared by all 4 strains. In the 6 strains of Cl. tetani studied, 12 antigens were demonstrated, none of which was common to all strains. 2 strains of Cl. fallax possessed 7 shared antigens. 9 antigens were found in 4 strains of Cl. septicum, 3 being present in all cases. The same authors prepared soluble antigens from 4 Bacillus spp. and tested them against clostridial antisera. B. anthracis reacted with antisera to Cl. befermentans, Cl. sordellii, Cl. fallax, and Cl. septicum. B. cereus var. mycoides reacted with these 4 antisera and also with antiserum to Cl. novyi type A. B. cereus on the other hand gave a reaction with antiserum to Cl. septicum only and B. subtilis failed to react with any of the sera tested. These cross reactions were very faint, precipitin lines appearing after prolonged incubation. Further studies will be necessary before it is known whether these reactions indicate antigens common to both genera.

1.2.4. Enzymic Changes during Sporulation

The mechanism of formation and heat resistance of the bacterial spore is a problem which has been tackled from a biochemical and cytological viewpoint rather than from a serological one (Halvorson, 1957, 1961, 1962; Jacobs & Clegg, 1957). 2 main lines of approach to the problem have been followed - firstly a comparison of the constituents of vegetative cells and spores, and secondly an analytical study of the physiological changes accompanying the formation and maturation of the spore. In the first instance, differences in enzymic constitution of spores and vegetative cells have received much attention. Improvements in techniques for breaking spores have led to a great increase in the number of enzymes detectable in spore extracts. Enzymic constitution of spores and vegetative cells show both quantitative and qualitative differences (see Halvorson, 1962). The catalase system provides an interesting example of enzymic changes during sporulation. Lawrence and Halvorson (1954) demonstrated a heat resistant catalase in intact spores of B. terminalis and Murrell (1955) found a similar enzyme in B. subtilis spores. On extraction from the spores however, both these enzymes lost their heat resistance. Sadoff (1961) reported the appearance of a heat resistant catalase in cells of B. cereus during early stages of sporulation. To ascertain whether the resistant catalase was spore protein or merely

vegetative protein altered in some way to become heat resistant, Sadoff extracted the enzyme from both spores and vegetative cells and prepared antisera to these extracts. Immunodiffusion in cellulose acetate membranes gave a continuous precipitin line indicating that both enzyme preparations contained identical protein. It is uncertain that this precipitin line is in fact the catalase, since it failed to give a positive result with stains used for locating this enzyme. Thermal inactivation and guanidine inhibition studies on partially purified enzymes suggested that there were pronounced differences between spore and vegetative catalase.

Although careful analysis of the vegetative cell and the mature spore is important, it does not furnish a complete picture of the sporulation process. For this, it is necessary to trace the biosynthetic steps between the initial and final product. This has been done, at least in part, for some of the reactions involved (see Halvorson, 1962).

An accompaniment to spore formation is the autolysis of the sporangium and subsequent liberation of the mature spore. Strange and Dark (1957) reported a great increase in the amount of a hexosamine-containing peptide in filtrates of cultures of B. cereus during spore release. Other lytic systems have been demonstrated (Greenberg & Halvorson, 1955; Norris, 1957). Strange and Dark (1957)

studied in detail the lytic activity of culture filtrates of sporulating B. cereus and detected 2 lytic systems, V and S. The enzymes differed in pH optima, V being more active at acid pH values, and S at alkaline pH. Both were active against vegetative cells and cell wall preparations. In contrast, the lytic system of B. cereus described by Norris (1957) did not dissolve the cell wall, but appeared to alter the walls in such a way as to permit the escape of cytoplasmic contents.

From the foregoing review it is apparent that much work has been done on various aspects of spore formation. There has been little correlation between the different lines of research.

In the present work, the main object of study has been the antigenic changes accompanying spore formation. The agglutination reaction and the precipitin ring test yield limited information about the nature and number of antigens involved in the reaction. Double diffusion in agar gels (Ouchterlony, 1949) permits the determination of the number of precipitating systems and also a comparison of antigens present in different preparations; even so, if the number of precipitin lines is large, identification of individual lines can become very difficult. Interpretation is simplified by the technique of immunoelectrophoresis, first described by Grabar and Williams (1953), where the antigen mixture is first separated by electrophoresis in some supporting medium, usually an agar gel. After the electrophoretic run, antiserum is placed in elongated troughs cut in the gel parallel to the axis of migration. Precipitin lines are then formed in the gel due to the process of double diffusion. By this technique it is possible to analyse complex antigen mixtures such as blood serum or extracts of bacterial cells.

Recently other electrophoretic methods have been described by workers in various biological fields. 2 of these -

starch gel electrophoresis (Smithies, 1955) and polyacrylamide gel electrophoresis (Raymond & Weintraub, 1959) - have been used in this work. It is hoped that a serological approach, and the application of techniques which have yielded valuable information in other fields, might help to throw some light on the origin of spore constituents and on the process of spore formation.

The work described in this thesis is essentially exploratory in nature, designed to examine the application of these techniques to the problem of spore formation, to reveal the broad pattern of some of the antigen and enzyme changes which accompany the process, and to provide a basis for further developments in this field.

SECTION 2.

MATERIALS AND METHODS

2.1. ORGANISM

The strain of Bacillus cereus (strain M.8, Mahmoud, 1955) used throughout this work was originally isolated from Egyptian soil and has been maintained by periodic transfer on nutrient agar at 30°C.

2.2. MEDIA

2.2.1. Sporulation Medium

This was essentially the fluid sporulation medium described by Young (1958), in which a well aerated culture of B. cereus shows a useful degree of synchrony during spore formation. It was prepared in 2 parts, a salts solution and a basal medium which were stored separately and mixed just before use. The salts solution was prepared from the following separate solutions (%w/v): KNO_3, 10; Na_2SO_4, 0.142; KH_2PO_4, 6.8; $MgSO_4.7H_2O$, 0.123; $CaCl_2.6H_2O$, 0.219; KOH, 0.33; $MnSO_4.4H_2O$, 0.00223; $Fe_2(SO_4)_3$, 0.02; $ZnSO_4.7H_2O$, 0.0144. These were distributed in 25 ml. amounts in screw-capped bottles and sterilised by autoclaving at 15 lb./sq.in. for 20 Minutes. To make the salts solution, 25 ml. amounts of these sterile solutions were mixed and 25 ml. of sterile distilled water were added, using aseptic technique. Basal medium consisted of 1% (w/v) of Difco proteose peptone added to distilled water containing 40% (v/v) of Oxoid nutrient broth. The medium was sterilised by autoclaving at 15 lb./sq. in. for 20 minutes.

Sporulation medium was prepared by mixing 250 ml. of salts

solution and 80 ml. of basal medium in a sterile 1 litre flask with aseptic technique.

2.2.2. Lab-Lemco Broth

8 g. of Oxoid dehydrated Lab-Lemco broth were dissolved in 1 litre of saline and sterilised by autoclaving at 10 lb./sq. in. for 20 minutes.

2.2.3. Lab-Lemco Agar

23 g. of dehydrated Oxoid Lab-Lemco agar were dissolved in 1 litre of saline and sterilised by autoclaving at 10 lb./sq. in. for 20 minutes.

2.2.4. 0.3% Agar

0.3% (w/v) of Difco agar was added to Lab-Lemco broth and sterilised by autoclaving at 10 lb./sq. in. for 20 minutes.

2.3. CULTURES

2.3.1. Fluid Cultures

1 litre flasks containing approximately 300 ml. of sporulation medium or Lab-Lemco broth were inoculated with 1 ml. of an overnight Lab-Lemco broth culture of *B. cereus*. Flasks were incubated with constant agitation to ensure aeration of the medium.

2.3.2. Agar Cultures

Stock cultures were maintained on Lab-Lemco agar slopes, with periodic streaking on Lab-Lemco agar plates to check purity.

For bulk cultures, Roux bottles containing approximately 150 ml. of Lab-Lemco agar were inoculated with 4 ml. of

an overnight Lab-Lemco broth culture of B. cereus.

All cultures were incubated at 30°C.

2.4. ESTIMATION OF GROWTH

2.4.1. Viable Counts

Numbers of viable organisms present in cultures were estimated by a method based on that described by Miles and Misra (1938). Tenfold dilutions of the cultures were prepared by serial transfer of 1 ml. to 9 ml. amounts of sterile ¼-strength Ringer solution. A range of dilutions, normally between 10^{-2} and 10^{-10} were dropped round the periphery of a Lab-Lemco agar plate, using a calibrated dropping pipette to ensure delivery of a precise volume. Plates were incubated for 24 hours at 30°C, after which time the separate colonies arising from a single drop were counted. From this figure the number of organisms present in the sample was calculated. Each assay was carried out in triplicate.

2.4.2. Heat Resistance Tests

To test the resistance of cells to heat a time-temperature exposure of 10 minutes at 80°C was selected. This resulted in death of vegetative cells but permitted the survival of spores. Dilutions of the cultures were prepared as for viable counts. The tubes were then transferred to a water bath held at 80°C. An exactly similar tube of Ringer solution containing a thermometer was placed in the water

bath beside the samples. After the temperature on this thermometer read 80°C, the samples were given a further 10 minutes in the bath. The time necessary for heat penetration in the tubes used (6" by $\frac{5}{8}$") was 1-2 minutes. After removal from heat, the samples were applied to Lab-Lemco agar plates as described in 2.4.1.

2.4.3. Turbidity Measurements

Undiluted samples of broth cultures were placed in glass cells with a $\frac{1}{2}$ cm. path length. % transmission through the samples was estimated with a Hilger Watt Spekker Photoelectric Absorptiometer. Uninoculated medium was used as a blank.

2.4.4. Phase-Contrast Microscopy

This method of examination of bacterial cultures was found to be more profitable than ordinary light microscopy of fixed and stained preparations. The organisms are seen in a living unfixed state, internal differentiation being due to differences in refractive index of the constituents of the bacterial protoplasm. The phase contrast microscope used was that manufactured by W. Watson and Sons, Ltd. Preparations were examined as wet mounts on glass slides covered with coverslips (no. 1, $\frac{7}{8}$ by $\frac{7}{8}$ in.).

2.5. PREPARATION OF DISINTEGRATES FOR STUDY OF ENZYMES AND ANTIGENS

2.5.1. Vegetative Cell Disintegrates

Cultures were harvested by centrifugation after appropriate

periods of incubation and the cells washed 3 times in distilled water. Pastes of washed vegetative cells and cells at various stages during spore formation were disintegrated by ultrasound using the M.S.E. Mullard 60 W Ultrasonic Disintegrator. Flat-bottomed containers gave more efficient disintegration than the round-bottomed ones normally used with this machine. Control experiments showed that disintegration was complete following exposure to ultrasonic vibration for 5 minutes and prolonged exposure of up to 30 minutes had no observable effect on the antigenic or enzymic composition of the extracts, provided that adequate steps were taken to restrict rise of temperature in the suspensions during disintegration. This was achieved by immersing the sample in a beaker of ice. Treatment of this kind did not disrupt mature spores.

2.5.2. Spore Disintegrates

48 hour cultures were harvested and vegetative debris broken down by 10 minutes exposure to ultrasonic vibration. The intact spores were washed repeatedly until neither spore paste nor washings showed any catalase activity when tested with H_2O_2 (Halvorson & Church, 1957). Ballotini beads (no. 14) were then added to an equal volume of a thick paste of washed spores and the mixture subjected to ultrasonic vibration when 60-70% disintegration occurred after about 15 minutes treatment.

All disintegrates were clarified by centrifugation at 4000 r.p.m. for 30 minutes and stored at -20°C.

2.5.3. Estimation of Protein in Disintegrates

The amount of protein in disintegrates was estimated by the method of Lowry et al. (1951).

Reagents:

Solution A: 2% Na_2CO_3 in 0.10 N. NaOH.

Solution B: 0.5% $CuSO_4.5H_2O$ in 1% NaK tartrate.

Solution C: alkaline Cu solution. 50 ml. A + 1 ml. B.

Solution D: diluted Folin reagent. Folin Ciocalteu reagent (B.D.H.) was titrated to a phenolphthalein end-point with NaOH. On the basis of this titration, the Folin reagent was diluted to make it 1 N in acid.

To 0.2 ml. of protein sample, 1 ml. of reagent C was added. After thorough mixing the sample was allowed to stand for 10 minutes at room temperature. 0.1 ml. of reagent D was then added and mixed immediately. After 30 minutes the intensity of colour produced was measured using the Hilger Watt Spekker Photoelectric Absorptiometer, with cells of ½ cm. path.

2 distinct steps lead to the production of this colour - the reaction of the protein with the copper in alkaline solution followed by the reduction of the phosphomolybdic-phosphotungstic reagent by copper treated protein.

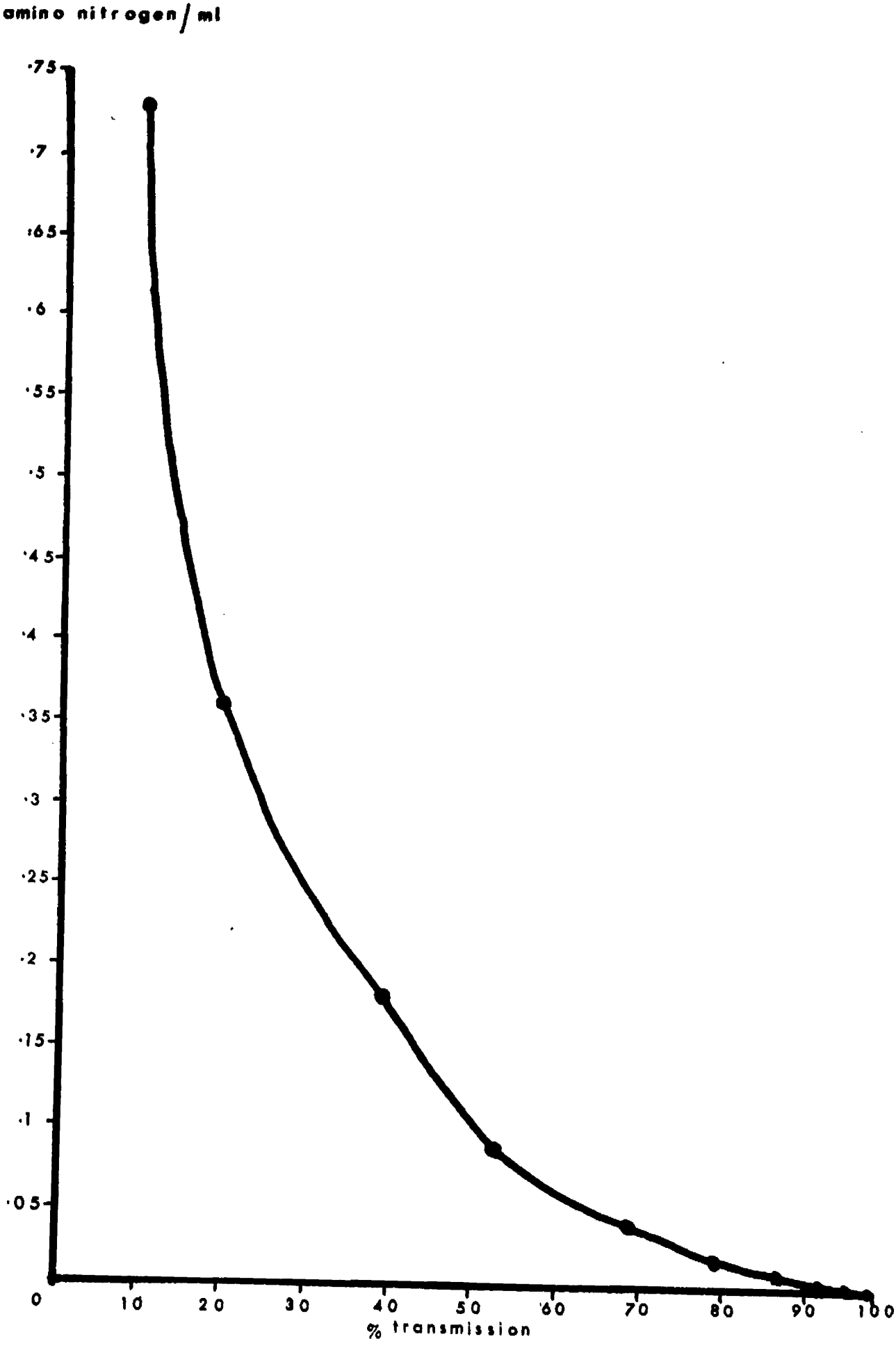
2.5.4. Reference Curve for Protein Estimations

Lactalbumin hydrolysate (Nutritional Biochemicals Corporation, Cleveland, Ohio) was used to prepare a reference curve. Samples of solutions containing known amounts of the hydrolysates were analysed by the above method. Figure 1 shows the reference curve for mg. amino nitrogen/ml. sample plotted against % transmission. This arbitrary reference curve was used to determine the approximate nitrogen content of cell extracts.

TABLE 1

Lactalbumin hydrolysate (mg./ml.)	Total nitrogen (mg./ml.)	Amino nitrogen (mg./ml.)	% Transmission
10	1.2	0.73	9.4
5	0.6	0.365	19.8
2.5	0.3	0.183	38.6
1.25	0.15	0.091	52.3
0.63	0.08	0.046	68.5
0.31	0.04	0.023	78.6
0.16	0.02	.012	86.1
0.08	0.01	0.006	91.5
0.04	0.005	0.003	94.0
0.02	0.003	0.0015	95.0

FIGURE 1


mg amino nitrogen / ml
·75
·7
·65
·6
·55
·5
·45
·4
·35
·3
·25
·2
·15
·1
·05
0
10
20
30
40
50
60
70
80
90
100
% transmission

2.5.5. Protein Content of Disintegrates

The protein content of all disintegrates was estimated by the above method and the solution adjusted to a level of approximately 0.5 mg. amino nitrogen/ml.

Where necessary, concentration of extracts was achieved by vacuum ultrafiltration against distilled water through a collodion membrane, using the apparatus manufactured by Membranfiltergeselschaft, Gottingen.

2.5.6. Heat Resistance of Disintegrates

To study heat resistance of enzyme and antigen constituents of the disintegrates, a sample was placed in a freeze-drying ampoule (4" x ½"). Ampoules were placed in a water bath at the required temperature. Penetration of heat throughout these ampoules took only a few seconds. Temperatures used were 60°C, 80°C, and 100°C for a period of 10 minutes.

2.6. PREPARATION OF ANTISERA

Suspensions of the different antigens were injected either intravenously or intramuscularly into young adult rabbits. A primary series of 6 injections at 3 day intervals was followed by booster series at approximately monthly intervals. The animals were bled from the ear 7 days after the last injection of a series. Sera were stored at -20°C without added preservative.

At least 2 rabbits were used for each antigen preparation.

Before the commencement of the primary series of injections, a sample of normal blood was withdrawn from each rabbit to act as a control.

2.7. ELECTROPHORETIC METHODS

2.7.1. INTRODUCTION

Zone electrophoresis is the separation of materials in an electric field in the presence of some supporting medium, in addition to the electrolyte used to carry the current. Its principle advantage over free electrophoresis is that it permits a higher resolution of components. A wide variety of supporting media have been employed, including glass beads, resins, filter paper and gels. Gel electrophoresis has become increasingly popular and at least 3 gelling agents have been used.

An invaluable technique, immunoelectrophoresis in agar gels, originally described by Grabar and Williams (1953, 1955) depends on double diffusion (Ouchterlony, 1949) in conjunction with the prior electrophoretic separation of one of the reactants. A micro-modification evolved by Scheidegger (1955), less wasteful in materials than the original technique was used for the study of antigen changes in B. cereus described in this thesis.

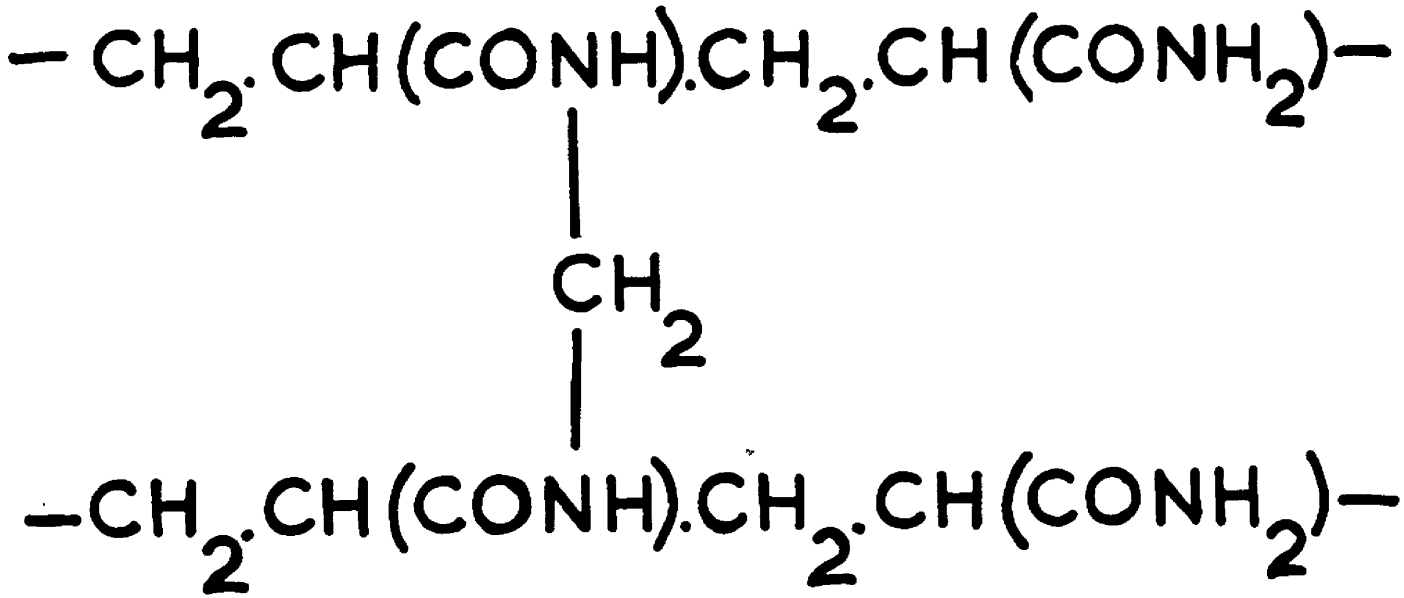
Separation in agar gels is similar to that obtained in filter paper, surface charge of the components being the major factor involved in their separation. Much higher resolution can be achieved in starch gel electrophoresis where the gel mesh provides a molecular "sieving" effect, sorting substances by molecular size, in addition to the separation due to differences in surface

charge. Initially used in the study of serum proteins (Smithies, 1955), electrophoresis in starch gels has been applied to the study of serum enzymes (Lawrence, Melnick, & Weimer, 1960), fungal (Clare, 1963), and bacterial (Fowler et al., 1963) protein.

A new synthetic polymer, polyacrylamide has been used as a supporting medium for zone electrophoresis. Cyanogum 41 (B.D.H.) contains a mixture of acrylamide monomer with N:N'-methylene-bis-acrylamide in such proportions that stiff gels are formed from dilute aqueous solutions in the presence of a suitable catalyst. These gels are linear polymers of acrylamide consisting of long 3-dimensional hydrocarbon chains, cross-linked at intervals by methylene bridges (Figure 2).

Such gels have several advantages over starch: greater transparency resulting in greater sensitivity, a higher degree of reproducibility and improved resolution in protein separations. Aqueous solutions of the monomer will form useful gels between 4.5 and 10% (w/v) concentration, average pore size being inversely proportional to the square root of monomer concentration (B.D.H.). By adjusting the latter, it is therefore possible to control pore size and hence the migration rates of the components analysed. Several catalyst systems may be used and gelling time can be varied from several minutes to several hours. Gellation will not take place in the presence of oxygen. Raymond and

FIGURE 2



POLYACRYLAMIDE

Weintraub (1959) first suggested the use of this synthetic polymer as a supporting medium in zone electrophoresis, and in later communications (Raymond & Wang, 1960: Murray, 1962), apparatus for the preparation of gels has been described.

Both acrylamide and starch gels have been employed in the study of changes in protein and enzyme constituents of cells during sporulation reported in this thesis.

Polyacrylamide gels at a 4-5% concentration have been used for immunoelectrophoresis (Crowle, 1961, p.199; Antoine, 1962). Resolution in gels at this concentration is little improved on that obtained in agar. Higher concentrations of the monomer, with a resultant decrease in pore size gives better resolution, but the antibody molecule is too large to diffuse effectively into such a gel.

As resolution of the constituents of extracts of B. cereus was found to be better in a 7% polyacrylamide gel than in starch or agar, an attempt was made to correlate the protein bands observed in this medium with antigens detected by immunoelectrophoresis.

Full details of the electrophoretic techniques used are given in the following pages.

2.7.2. IMMUNOELECTROPHORESIS

2.7.2.1. Materials

Electrophoresis agar; Barbitone-acetate buffer (Oxoid), 8.25g.; thiomersalate, 0.1g.; Ionagar No. 2 (Oxoid), 10g.; distilled water to 1 litre.

The agar was distributed in 25 ml. amounts in universal containers. Sterilisation was unnecessary due to the presence of thiomersalate and the agar was only re-heated once before use. Excessive heating was found to cause changes in gel characteristics.

Tank buffer: Barbitone-acetate buffer (Oxoid), 8.25g.; distilled water to 1 litre.

Indicator dye: Bromocresol purple, 1g.; distilled water to 100 ml.

Agar for coating slides: Ionagar No. 2 (Oxoid), 0.2g.; distilled water to 100 ml.

Slides: Lantern slide cover glasses, 3¼ in. by 3¼ in.

2.7.2.2. Preparation, Electrophoresis, and Development of Agar Gels

3¼ in. by 3¼ in. lantern slide cover glasses were thoroughly cleaned, coated with 0.2% agar and allowed to dry in air. They were then placed on a horizontal surface and flooded with 12.5 ml. of molten electrophoresis agar which was allowed to set and cool for at least 2 hours before use.

Serum troughs and antigen wells were cut to a standard pattern using an agar cutter (Buchler Instruments, Fort

Lee, New Jersey, U.S.A.).

Antigen reservoirs were filled and a drop of indicator dye applied to the gel at the side of these reservoirs using a fine capillary pipette. Contact was established between the ends of the gel and the tank buffer with absorbent lint wicks. Gels were run at constant voltage, with a potential drop of 10 volts/cm., taking an initial current of approximately 20 milliamps. Electrophoresis took about an hour, and was completed when the spot of indicator dye was ½ cm. from the anode wick. On completion of electrophoresis the agar was removed from the antiserum trenches which were then filled with antiserum. Gels were placed in a moist chamber at room temperature. Lines of antigen-antibody precipitate developed over a period of 24-48 hours and were recorded photographically after 48 hours.

2.7.2.3. Staining of Agar Gels

Precipitin lines in agar gels may be stained with protein, polysaccharides or for the detection of enzymes. Prior to any staining procedure, the gels were washed for 48 hours in frequent changes of tap water. Antiserum trenches were then filled with 1% Ionagar No. 2 in distilled water, and the gels placed in an incubator at 30°C to allow theagar to dry on to the glass.

(a) Antigen-antibody precipitin lines were stained for protein by flooding the dried gel with a saturated solution of naphthalene black in 10% aqueous acetic acid. Decolourisatio was effected by repeated washing in 5% acetic acid.

(b) Catalase-anticatalase zones were detected by flooding dried gels with 10% H_2O_2 (100 vol.). Zones with catalase activity were sharply defined by formation of small gas bubbles in the agar film (Uriel, 1963).

(c) Esterase-antiesterase zones were detected by the esterase stain used for starch gels. Full details are given in 2.7.3.3.

(d) Polysaccharide antigens were detected by a modification of the periodic acid-Schiff reaction (Stewart-Tull, 1964). The dried gel was flooded with a 1% aqueous solution of sodium periodate (B.D.H.). After 15 minutes the periodate was replaced with distilled water into which gaseous sulphur dioxide was passed, giving the agar a brown colouration. The gel was left in SO_2 water until this colour disappeared. After a brief rinse in water, the gel was stained with a 1% aqueous solution of pararosaniline hydrochloride (Hopkin & Williams Ltd.) which had been decolourised by passing SO_2 gas through the solution. The stain was allowed to act for 30 minutes, following which the gel was washed in SO_2 water prior to being dried rapidly in a film drying-cabinet. When dry, the gel was sprayed with "Quelspray" (clear, No. 418 A), a vinyl protective coating (Fisons Scientific Apparatus Ltd., Loughborough). This latter procedure prevented the background of the gel from staining with increasing intensity on exposure to the atmosphere.

The final preparation showed the specific reaction a dark

pink against the light bluish-purple background of the gel.

2.7.3. STARCH GEL ELECTROPHORESIS

2.7.3.1. Materials

Starch: Hydrolysed starch (Connaught Laboratories). The gel was prepared with approximately 12% starch. The exact quantity used varied with the batch of starch, the optimal concentration being indicated on the container.

Gel buffer: Tris(hydroxymethyl) aminomethane, 4.59g.; citric acid, 0.53g.; distilled water to 1 litre.

Tank buffer: Boric acid, 18.5g.; NaOH, 2.0g.; distilled water to 1 litre.

Sample inserts: 3 MM Whatman filter paper, 6 MM. wide was cut to the desired length.

Gel moulds: Suitable gel moulds were prepared on $\frac{1}{4}$ in. plate glass sheets (5 in. by 7 in.) by attaching edge strips of $\frac{1}{4}$ in. by $\frac{1}{8}$ in. glass to the upper surface by means of silicone stopcock grease (Edwards High Vacuum Co., Ltd.). Glass sheets were siliconed by flooding with silicone solution and wiping dry before the moulds were assembled.

Gel cover: "Melinex O" film, gauge 50 (polyester film, I.C.I.).

Silicone solution: 5% silicone (I.C.I.) in CCl_4.

2.7.3.2. Preparation and Electrophoresis of Starch Gels

The appropriate quantity of starch was mixed with 120 ml.

tris-citrate buffer in a 500 ml. conical flask and heated gently over a bunsen flame with continuous swirling. The gel first thickened, then liquefied and boiled freely. At this stage heating was discontinued and air bubbles removed under vacuum. The molten gel was poured into the prepared tray, covered with a sheet of Melinex film and allowed to cool at room temperature for 2 hours before use.

The gel was cut across approximately 3 cm. from one end and the short end slipped gently back to expose the cut surface. Cell extracts for analysis were soaked on to 10 mm. lengths of filter paper strip and excess liquid removed by touching on absorbent paper. The strips were applied to the cut surface of the gel, which was closed by replacing the short end and the Melinex cover.

For electrophoresis, contact was made between the tank buffer and the gel by means of wicks of absorbent lint, care being taken to ensure that the wicks were thoroughly soaked with buffer. Electrophoresis was carried out under conditions of constant voltage, the potential drop being 8-10 volts /cm., with an initial current of approximately 20 milliamps.

During electrophoresis, a brown line developed in the gel, moving towards the anode. This artifact marks the interface between the borate tank buffer and the tris-citrate gel buffer. Electrophoresis was continued until the brown line was 10 cm. from the line of sample inserts. At this stage, electrophoresis was terminated and the gel removed

from the tank. After removing the Melinex cover and the glass supports, the short end of the gel was slipped back and the filter paper inserts removed. With 3 perspex guide strips, 1½ mm. thick replacing the original 7 in. glass edge strips, the Melinex cover was put back in position. A second sheet of plate glass 7 in. by 5 in. was placed on top of the gel to maintain an even pressure while slicing. 4 horizontal slices were obtained from the gel which was cut with a fine blade. The guide strips controlled the thickness of the slices. Only the 2 middle slices were used as trailing may occur along both upper and lower surfaces. Hence protein or enzyme bands detected on top or bottom slices are often distorted.

2.7.3.3. Staining of Starch Gel Slices

Starch gel slices may be treated in many ways to detect the presence of proteins, other substances and enzymes (Furness, 1961; Lawrence, Melnick, & Weimer, 1960). In the starch gel electrophoresis described in this thesis, 2 enzyme systems were studied.

Detection of catalase activity: The procedure was based on the method of Paul and Fottrell (1961). The gel slice was covered with 1% (v/v) H_2O_2 (100 vol.) for a few moments and washed thoroughly in running water. It was then immersed in 2% (w/v) KI solution acidified with a few drops of glacial acetic acid. In a few minutes catalase bands were seen as white areas against the blue background of the gel where it was stained with iodine released by the

residual peroxide.

Detection of esterase activity: This was carried out by the method of Lawrence, Melnick, and Weimer (1960). The gel slice was flooded with the following solution: tris-maleate buffer, 0.1M, pH 6.4, 50 ml.; 1% a-naphthyl acetate in 50% acetone, 2 ml.; fast blue B salt, 50 mg. Esterase bands developed as red areas and reached a maximum intensity in about 2 hours at room temperature.

2.7.4. POLYACRYLAMIDE GEL ELECTROPHORESIS

2.7.4.1. Materials

Monomer: Cyanogum 41 (B.D.H.), 7g.; tris-citrate buffer (as described for starch gels) to 100 ml.

Catalysts: A: 10% aqueous solution of ammonium persulphate.

B: 10% aqueous solution of dimethylaminoethyloyanide.

Gel mould: A perspex tray ¼ in. deep with a close-fitting lid which carried sample slot formers. These were also of perspex, 1 mm. thick and 8 mm. long, situated 3 cm. from one end of the gel.

Tank buffer: Borate tank buffer described for starch gel electrophoresis was used.

Soft white paraffin (paraffinum molle album, B.D.H.) was melted in a beaker and pipetted across the sample slots after charging. This solidified and prevented cross-leakage between the slots. "Melinex O" film was

used to cover the gel during the run.

2.7.4.2. Polymerisation and Electrophoresis of Polyacrylamide Gels

1% of each of the catalyst solutions A and B was added to the appropriate amount of the monomer (150 ml.) and the solution evacuated thoroughly to remove dissolved air. It was then poured into the perspex tray and the lid lowered gently into position, care being taken to exclude air bubbles.

Polymerisation took approximately 1 hour and the gel was ready for immediate use.

After the gel had formed, the lid of the tray was removed and the sample slots charged with the material under examination. The row of slots was sealed as described in 2.7.4.1. and the gel covered with Melinex film. Contact between gel and tank buffer was made with absorbent lint wicks. The gel was run under conditions of constant voltage, with a potential drop of 10 volts/cm., taking an initial current of approximately 50 milliamps. As with starch gels run in the discontinuous buffer system, a brown line developed at the interface of the 2 buffers and migrated towards the anode. This was allowed to reach a point 10 cm. from the row of sample slots before the run was terminated. 4 horizontal slices were obtained from the gel in the same manner as from starch gels. A fine suture wire held taut by finger pressure was used in preference to a thin blade.

2.7.4.3. Staining of Polyacrylamide Gel Slices

(a) Protein stain - a saturated solution of naphthalene

2.7.4.3. Staining of Polyacrylamide Gel Slices

black in 10% aqueous acetic acid was applied to the gel slice for 20 minutes. Decolourisation was effected by washing repeatedly in frequent changes of 5% aqueous acetic acid. Protein bands stained dark blue against the clear, colourless background of the gel.

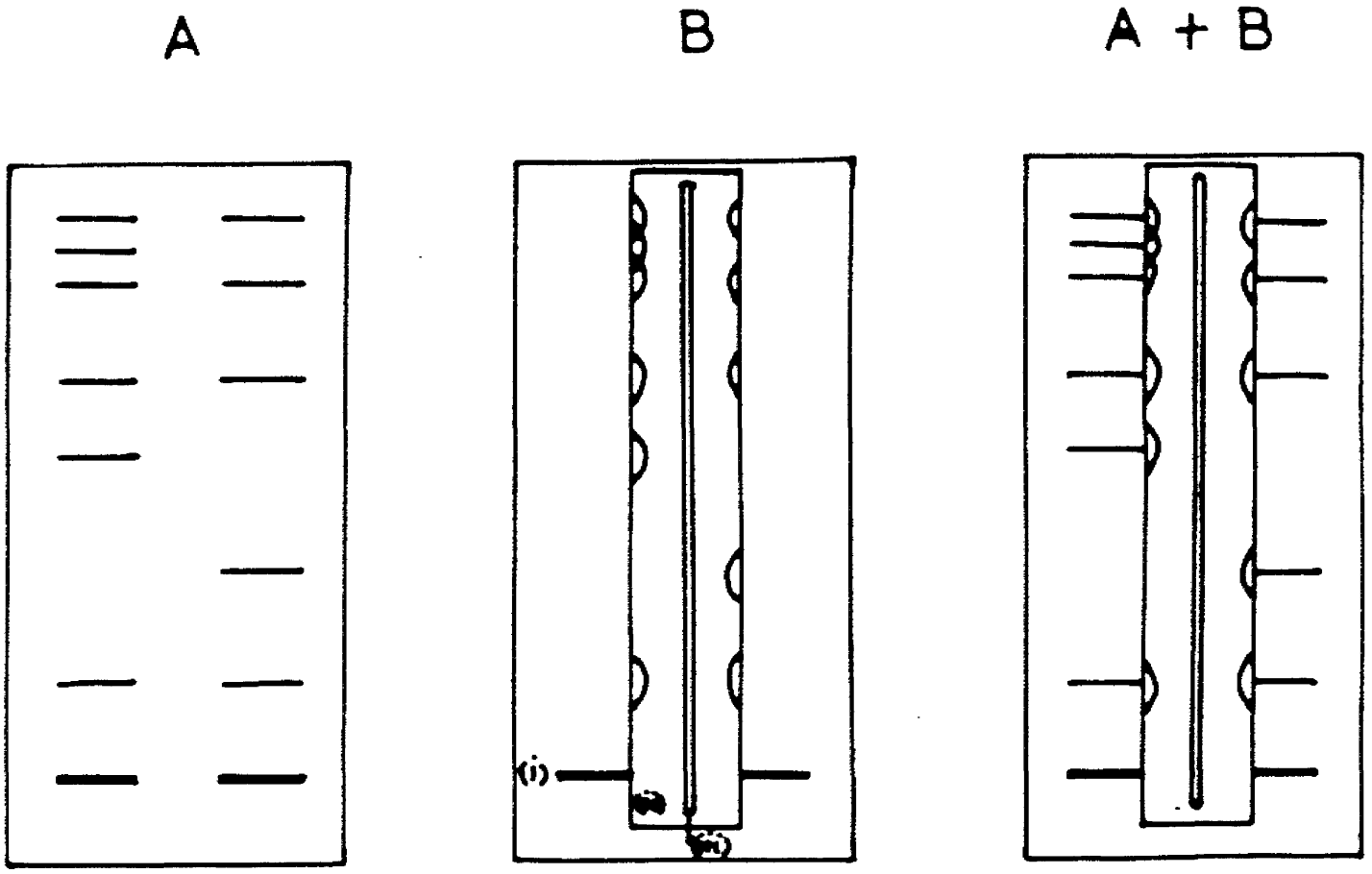
(b) Polysaccharide components were stained by the method for agar described in 2.7.2.3., the gel slice being immersed in 1% aqueous sodium periodate for 45 minutes to permit penetration through the acrylamide. After washing in SO_2 water, the gel was stained with 1% aqueous pararosaniline hydrochloride for 16-24 hours. Polysaccharide bands stained dark pink against a pale pink background.

2.7.4.4. Immuno-analysis in Polyacrylamide Gels

After slicing a polyacrylamide gel as described above, one slice (slice A) was stained for protein in the usual manner. Slice B (the second of the 2 middle slices) was placed on a sheet of perspex. Cuts were made in the polyacrylamide parallel to, and slightly overlapping the electrophoretic run (see Figure 3). This section was removed and the polyacrylamide replaced by pipetting in molten agar (1% Ionagar No. 2, in saline). This was allowed to solidify for 20 minutes before an antiserum trench was cut in the centre of the agar insert. The trench was filled with antiserum and the gel slice incubated in a moist chamber

for 48-72 hours at room temperature to allow precipitin lines to develop. At the end of this period both slices were photographed at the same magnification and the protein and immuno-analysis superimposed upon each other (Figure 3). Thus it was possible to correlate protein bands with antigen-antibody precipitin lines.

FIGURE 3



(i) SAMPLE SLOT (ii) REMOVED SECTION FILLED WITH AGAR (iii) ANTISERUM TRENCH

2.8. CELL FRACTIONATION

In recent years great advances have been made in techniques for obtaining isolated components of the bacterial cell. Flagella, capsules, cell walls, protoplasts, protoplast membranes, cytoplasmic granules and nuclear bodies have been obtained from many microorganisms. A comprehensive review of the many papers in this field is beyond the scope of this thesis and reference is made to 2 excellent surveys of the methods of preparation and the properties of isolated cellular constituents (Spooner & Stocker, 1956; Gunsalus & Stanier, 1960). In an effort to trace the source of some of the antigens detected in ultrasonic extracts of *B. cereus* attempts were made to isolate some cell fractions in a pure state. These were flagella, cell walls, and protoplasts.

2.8.1. FLAGELLA

2.8.1.1. Induction of Motility

As *B. cereus* M.8 was not actively motile and possessed few microscopically detectable flagella, the organism was passed through 0.3% agar in order to stimulate motility. Plates of 0.3% agar were given a single central inoculum. After 24-48 hours incubation, growth permeated the entire plate. A loopful of growth from the periphery was used to inoculate the centre of a second plate. Agar passage was repeated in this way 6 times, by which time swarming to the edge of the plate occurred on overnight incubation. Smears were taken from the edge of the plate and stained with Kirkpatrick's silver stain (Mackie & McCartney, 1959)

when they showed peritrichously flagellate rods. Lab-Lemco broth cultures of the organism were actively motile and showed heavy peritrichous flagellation (Plate 1).

2.8.1.2. Preparation of Flagella

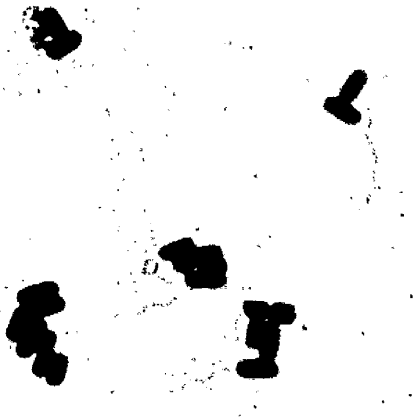
Actively motile cells of B. cereus M.8 obtained by passage through 0.3% agar were inoculated into Lab-Lemco broth and incubated at 30°C for 8 hours. Sterile 1 litre flasks containing 300 ml. of Lab-Lemco broth were inoculated with 1 ml. of this 8 hour culture and incubated for 16 hours at 30°C with continuous agitation.

The method of isolation of flagella was essentially that of Gard (1945). Cells were harvested by centrifugation, washed 3 times in distilled water, and resuspended in a minimum volume of distilled water. The resulting suspension was transferred to a 500 ml. conical flask and shaken vigorously for 2 hours on a Microid Flask Shaker (Griffin & Tatlock). A large flask was used to avoid splashing of the suspension into the plug.

At the end of the 2 hour period, microscopic examination of the preparations treated with Kirkpatrick's stain showed that the majority of the flagella had become detached from the cells. The latter were removed by centrifugation in an angle head centrifuge at 1000 r.p.m. for 1 hour. The clear supernatant was spun at 18,000 r.p.m. (25,000 g) for 1 hour to give a gelatinous precipitate which was resuspended in a small volume of

PLATE I

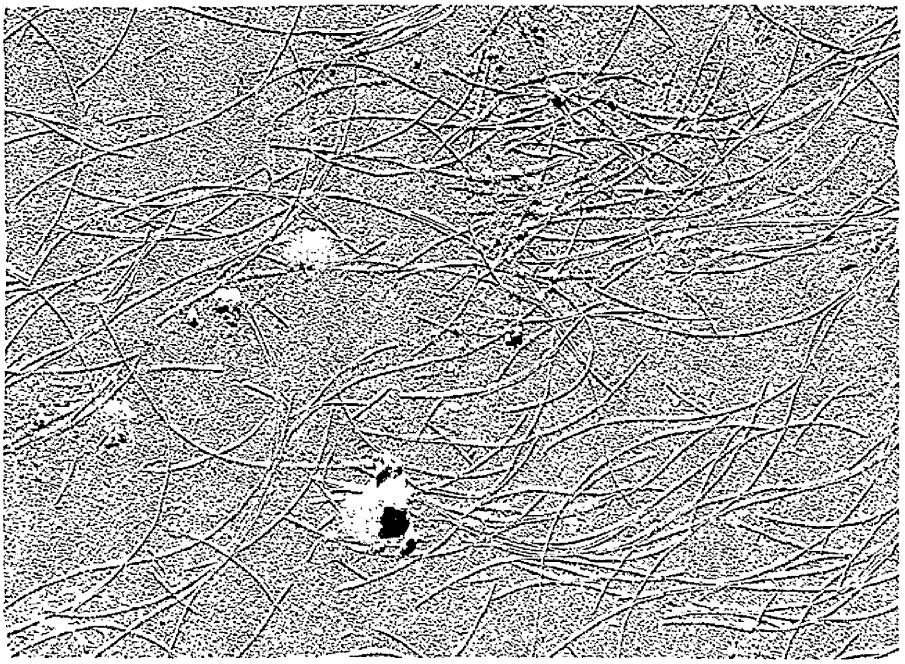
FLAGELLA



B. CEREUS M.8.P

KIRKPATRICK'S STAIN

×1200



DETACHED FLAGELLA

ELECTRONMICROGRAPH ×20,000

distilled water. 3 short runs at 1000 r.p.m. removed the residual cells. The flagella were then washed twice with distilled water, and finally resuspended in a small volume of distilled water. The flagellar preparation was examined by electron microscopy (Plate I).

For electrophoretic analysis the suspension was concentrated by vacuum ultrafiltration following a 5 minute exposure to ultrasonic vibration.

2.8.2. VARIATION IN BACILLUS CEREUS M. 8

On several occasions during the induction of motility by passage through 0.3% agar, an interesting and potentially valuable variant of B. cereus M. 8 arose. After some 6 successive platings on sloppy agar, the organisms were found to be very actively motile in contrast to the culture used for the original inoculum. When this motile strain was plated out on Lab-Lemco agar as a routine purity check, a striking variation in colonial morphology was noted. After 16 hours incubation the plate appeared normal, but some 24 hours later many of the colonies had undergone a marked change in appearance. Instead of being whitish-cream in colour, these variant colonies were rapidly becoming transparent (Plate 2). On examination by phase-contrast microscopy it was found that the organisms were undergoing lysis. The colonies consisted mainly of empty walls and a few swollen distorted cells (Plate 3). The variant was isolated in pure culture by successively

PLATE 2

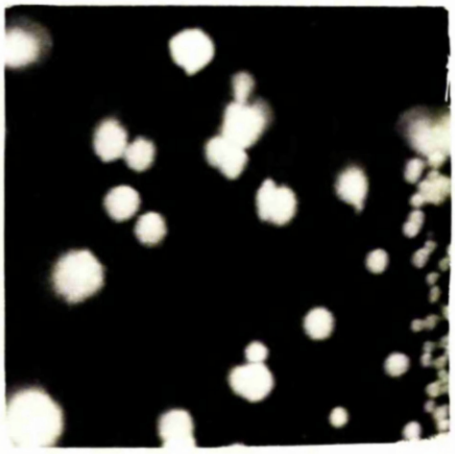
COLONIAL APPEARANCE OF B. CEREUS M.8 P

A

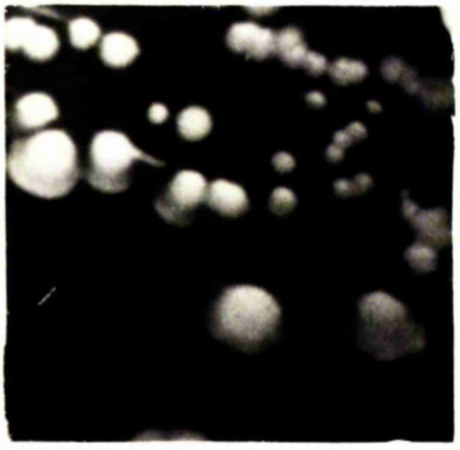
A 24 HOUR CULTURE

B 48 HOUR CULTURE

C 72 HOUR CULTURE



B



C

D

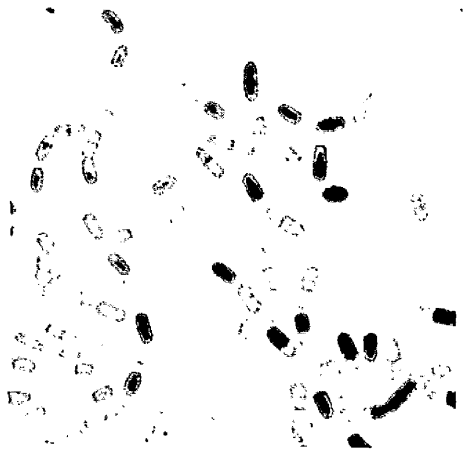
D 24 HOUR CULTURE

B. CEREUS M.8

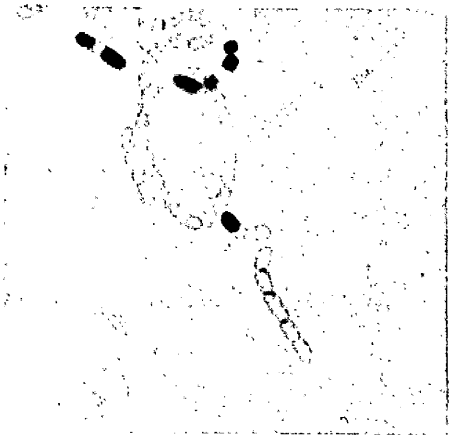
PLATE 3

MICROSCOPIC APPEARANCE OF B. CEREUS M.8.P

24A



48A



72A

PHASE CONTRAST ×1200

re-streaking it on Lab-Lemco agar, and has been maintained by periodic transfer on Lab-Lemco agar. Variation in colonial morphology of Bacillus spp. has been noted by many workers. Soule (1928) studied S - R variation in B. subtilis. During this work he observed a third type of colony which he designated a P or phantom colony. Initial growth of this variant on agar was normal, but when about 8 hours old it appeared to undergo an autolytic process. Instead of increasing in size, the resulting colonies became small, flat, colourless and almost invisible. A pure culture of this phantom variant was obtained by serial transfer on nutrient agar. No details were given of microscopic appearance of the phantoms beyond the fact that they were Gram positive rods.

Nungester (1929) examined dissociation in B. anthracis and isolated 7 different colonial forms, including 2 types of phantom colony, rough and smooth. Colonial morphology closely resembled that of the parent strain in the early stages of growth, but older cultures showed characteristic swollen cells when examined microscopically. Spores were formed by the original variants, but on prolonged sub-culture the phantom forms became practically asporogenic, and did not revert to the original type.

The variant isolated from B. cereus M. 8 has been designated M.8.P. as it appears to be similar to the phantom forms noted by Soule (1928) and Nungester (1929).

Due to inadequate description of the microscopic details of these phantom forms it is impossible to determine if they were in fact the same type of variant as that isolated from M.8.

2.8.3. GROWTH AND MORPHOLOGY OF VARIANT

Lab-Lemco agar. Overnight cultures appeared similar to B. cereus M.8 in colonial morphology. On further incubation (36-72 hours) the colonies collapsed and gradually became transparent and ghost-like. Examination by phase-contrast microscopy showed that young cultures consisted of normal phase-dark rods. Older preparations contained many swollen, almost coccoid forms and many empty shells devoid of cytoplasmic contents. After 5-7 days incubation, colonies consisted almost entirely of cell walls with a few swollen forms. Cultures appeared to be completely asporogenic.

Lab-Lemco broth. 8-16 hour broth cultures contained phase-dark vegetative rods which were very actively motile. Older cultures showed granulation (as the cells of Stage II, B. cereus M.8) but no further development to spore formation took place.

The variant B. cereus M.8.P. was isolated in November, 1963, and has been maintained in a pure state by periodic transfer on Lab-Lemco agar since that time. No tendency to revert to the parent form has been shown and the

organism appears to be a stable variant.

2.8.4. CELL WALLS

2.8.4.1. Preparation of Cell Walls

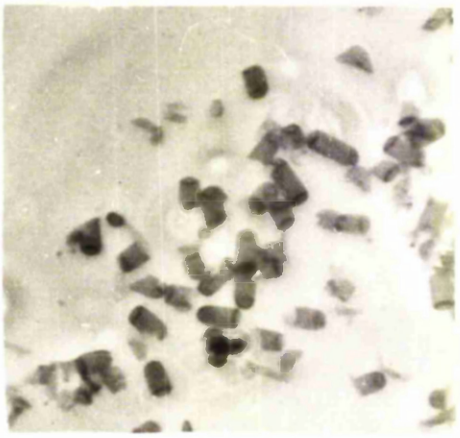
For the preparation of cell walls the organism used was B. cereus M.8.P. Roux bottles containing 200 ml. of Lab-Lemco agar were inoculated with 4 ml. of an overnight broth culture of the organism. Cultures were incubated at 30°C for 5 days, when microscopic examination showed a few swollen cells, but mainly empty cell walls arranged in chains. Growth was harvested in sterile distilled water and cell walls isolated by the method of Salton (1953). The chains of walls and some of the pleomorphic cells were disrupted mechanically by shaking with an equal volume of Ballotini beads (no. 14) in a Mickle Tissue Disintegrator. Residual cells were removed by centrifugation in an angle head centrifuge at 1000 r.p.m. for 20 minutes. Cell walls were washed 4 times in 1 M NaCl, which rapidly removed cytoplasmic debris. This was followed by 15 washes in distilled water.

2.8.4.2. Purity of Cell Walls

Phase-contrast observations showed cell walls free of intracellular granules and no intact cells were seen. The preparation was examined in a Seimens Elmiskop I electron microscope. Droplets of the suspension were applied to formvar grids, allowed to dry in air, and shadow-cast with gold-palladium at an angle of 15°.

PLATE 4

CELL WALLS



a

(a) Phase contrast
×1200

(b) Electronmicrograph
× 15000

b

The single condenser system, with a 200 u condenser aperture, a 50 u objective aperture and an accelerating voltage of 60 kV was employed. Electronmicrographs of the cell wall preparation is seen in Plate 4.

Purity of the cell wall preparation was also checked by chromatographic analysis of hydrolysates. Samples were hydrolysed in sealed tubes with 1 ml. of 6 N HCl at 105°C for 18 hours. The hydrolysates were filtered and evaporated to dryness over a constant boiling water bath, and finally resuspended in 0.25 ml. of distilled water. Amino-acids were separated by 2-dimensional chromatography on Whatman No. 1 paper (46 cm. x 57 cm.) in a 20 inch "Two-way" sheet chromatank. The solvent system used was phenol + water descending for 36 hours followed by lutidine + water descending for 24 hours (Cummins & Harris, 1956). Chromatograms were dried and the spots located by dipping in ninhydrin in acetone (0.1%, w/v). The following substances were present:

DL diaminopimelic acid, alanine, glutamic acid, glycine, muramic acid, glucosamine and galactosamine. There were also traces of valine, leucine or isoleucine, and aspartic acid. These contaminants were almost entirely removed by further washing of the walls.

2.8.5. PROTOPLASTS

Weibull (1953) isolated protoplasts from B. megaterium

by controlled treatment with lysozyme. B. cereus is resistant to this enzyme and so an alternative method had to be found. Strange & Dark (1957) obtained lytic enzymes from extracts of spores and partial autolysates of B. cereus and showed that they dissolved isolated cell walls of the organism. When viable organisms were treated with the enzymes in the presence of sucrose and cobalt ions, good yields of stable protoplasts resulted. (Dark & Strange, 1957). The procedure described in this latter paper was followed using B. cereus M.8.

The buffer used in this experiment was McIlvaine's phosphate-citrate buffer. There were 2 stock solutions; A; 35.6g $Na_2HPO_4.2H_2O$ /litre; B; 21.01 g. citric acid /litre.

For buffer pH 5; 51.5 ml. A; 48.5 ml. B.

For buffer pH 7; 82.3 ml. A; 17.7 ml. B.

2.8.5.1. Preparation of Autolytic Enzymes

B. cereus M.8 was grown in sporulation medium in the usual way. Cells were harvested at an advanced stage of spore formation, washed twice in distilled water and resuspended in McIlvaine's buffer, pH 5. Cells from 1 litre of medium were suspended in 5 ml. of buffer, with the addition of 0.025 ml. of toluene /ml. of suspension. After 2 hours incubation at 30°C microscopic examination showed that many of the sporangia had lysed to release mature spores. The suspension was centrifuged at 4000 r.p.m. for 30 minutes to remove cells.

The autolysate was used without further treatment and constituted the enzyme solution for the preparation of protoplasts.

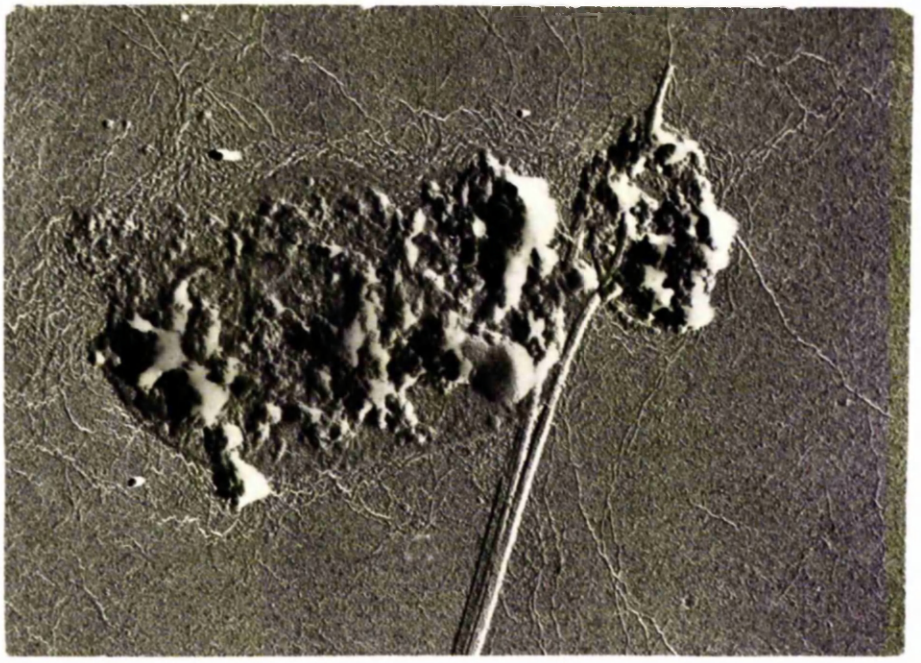
2.8.5.2. Preparation of Protoplasts

B. cereus M.S was grown in shaken culture in Lab-Lemco broth for 12-16 hours at 30°C. Cultures were harvested by centrifugation, the cells washed twice in McIlvaine's buffer pH 7 and resuspended in this buffer. Organisms from 1 litre of medium were taken up in 10 ml. of buffer. 2 ml. of bacterial suspension were mixed with 5 ml. of the enzyme solution (prepared as described in 2.8.5.1.) in the presence of 0.5 g. of sucrose and 1 mg. of $CoCl_2$. A control containing buffer instead of enzyme solution was set up and both tubes were incubated at 30°C.

Within 60 minutes, a few spherical forms were detected by phase-contrast microscopy and these increased in number until there was 60-70% conversion after 24 hours incubation. The suspension was centrifuged at 3000 r.p.m. for 20 minutes and the protoplast and cell mixture washed twice in 0.2M sucrose solution. Sedimented cells and protoplasts were then suspended in a minimal volume of distilled water. This resulted in complete lysis of the protoplasts and the residual rods and protoplast membranes were removed by centrifugation. The resulting supernatant was concentrated by vacuum ultrafiltration against distilled water to adjust the protein content to a level

PLATE 5

PROTOPLAST PREPARATION



ELECTRONM ICROGRAPH ×30,000

of 0.5 mg. of amino nitrogen/ml. This concentrated supernatant was used as protoplast preparation for further studies.

2.9. PHOTOGRAPHY

2.9.1. Photomicrographs

All photographs were taken on the phase-contrast microscope manufactured by W. Watson and Sons, Ltd., using the 35 mm. eyepiece camera produced by the same firm. For phase-contrast photomicrographs, a drop of the material to be examined was spread over a slide and allowed to dry in air prior to mounting in a drop of molten agar (1% Ionagar No. 2, in distilled water). This precaution was taken to avoid the movement of cells in a wet preparation. Ordinary light microscopy of stained organisms was used only for flagellar preparations.

Ilford Pan F 35 mm. film was employed.

2.9.2. Electronmicrographs.

All preparations were examined in the Seimens Elmiskop I electron microscope, using the single condenser system, with a 200 u condenser aperture, a 50 u objective aperture and an accelerating voltage of 60 kV. Suitable fields were photographed at a magnification of 6,000-10,000 diameters on Ilford N 50 thin film half-tone plates.

2.9.3. Gels

All gels were photographed by transmitted light. An

Exa 35 mm. camera with a close-up lens was used and the film was Microneg Pan 35 mm. (Ilford).

Agar and immuno-acrylamide gels were photographed against a black background; starch gels and stained acrylamide gels were taken against a white background.

2.9.4. Colonial Morphology.

Colonies on agar plates were photographed by reflected light with the Exa 35 mm. camera using Pan F 35 mm. film (Ilford).

All films were developed in Ilford ID 20 developer.

SECTION 3

RESULTS

3.1. SPORULATION OF BACILLUS CEREUS M.8

3.1.1. Growth in Fluid Sporulation Medium

Before selecting stages during growth and sporulation for detailed study, it was necessary to know the precise course followed during growth in fluid sporulation medium. Flasks containing 330 ml. of sporulation medium were inoculated with 1 ml. of an overnight Lab-Lemco broth culture of B. cereus M.8. The flasks were incubated at 30°C with constant agitation. The first sample was withdrawn immediately after inoculation, the second after 7 hours incubation, samples being taken hourly thereafter. Several tests were carried out on all samples.

(i) Viable counts

(ii) Heat resistance tests

(iii) Turbidity measurements

(iv) pH measurements

Methods used in performance of these tests are detailed in 2.4. In addition, each sample was examined by phase-contrast microscopy and typical cells photographed. Results are tabulated and represented graphically (Table 2, Figure 4). Cytological changes are shown in Plate 6. Young vegetative cells growing in this medium occurred singly or in pairs. At an age of 8-9 hours the cells aggregated to form clumps which increased in size and persisted through the entire course of spore formation, only dispersing at 20-24 hours, at which time the majority of cells contained mature spores and lysis of

TABLE 2

GROWTH IN SPORULATION MEDIUM

Sample (hours)	pH	% Trans-mission	Viable Count		Heat Resistant Count	
			cells/ml.	log. no.	cells/ml.	log.no.
0	5.8	100	$24^{x}10^{2}$	3.38	0	0
7	5.8	67	$28^{x}10^{6}$	7.45	0	0
8	5.9	47.5	$48^{x}10^{6}$	7.68	0	0
9	6.0	33.4	$10^{x}10^{12}$	13.0	0	0
10	6.1	25.4	$5.6^{x}10^{12}$	12.75	0	0
11	6.2	18.8	$3.2^{x}10^{12}$	12.51	0	0
12	6.2	16.6	$16^{x}10^{10}$	11.20	0	0
13	6.2	14.6	$24^{x}10^{10}$	10.38	0	0
14	6.2	15.7	$16^{x}10^{8}$	9.20	0	0
15	6.3	16.3	$28^{x}10^{6}$	7.45	0	0
16	6.4	17.5	$44^{x}10^{6}$	7.64	0	0
17	6.5	17.1	$40^{x}10^{6}$	7.60	0	0
18	6.5	16	$52^{x}10^{6}$	7.72	0	0
19	6.5	14.9	$6.8^{x}10^{8}$	8.83	0	0
20	6.5	13.6	$2.4^{x}10^{8}$	8.38	$7.2^{x}10^{4}$	4.86
21	6.5	12.7	$3.2^{x}10^{8}$	8.51	$1.6^{x}10^{6}$	6.20
22	6.4	11.7	$4.8^{x}10^{8}$	8.68	$28^{x}10^{6}$	7.45
23	6.4	11.8	$6.0^{x}10^{8}$	8.78	$4.8^{x}10^{8}$	8.68
24	6.6	10.8	$28^{x}10^{8}$	9.45	$5.6^{x}10^{8}$	8.75
30	6.6	10.7	$7.2^{x}10^{14}$	14.86	$4^{x}10^{14}$	14.60
36	6.6	13	$8^{x}10^{14}$	14.90	$6^{x}10^{14}$	14.78
40	6.6	12	$10.4^{x}10^{14}$	15.02	$9.6^{x}10^{14}$	14.98
48	6.6	10.2	$13.6^{x}10^{14}$	15.13	$12.4^{x}10^{14}$	15.09

the sporangia was commencing. For this reason, viable counts were inaccurate during the period of spore formation. The salient fact that emerges from viable counts and heat resistance tests is that the development of heat resistance was sudden and rapid, and did not occur until mature spores were detectable by phase-contrast microscopy. It is evident from the graph (Figure 4) that turbidity measurements offer a more reliable indication of the numbers of cells present in the samples. The rise in turbidity which occurred at 18-24 hours coincided with the maturation of the spores and was probably due to the difference in optical refractility between spore and vegetative cell. The pH of the culture was initially 5.8 and rose gradually to reach a level of 6.6 at 24 hours.

Cytological changes which occurred in the culture are shown in Plate 6. 7-9 hour cells appeared evenly dense under phase contrast, but from 12-14 hours, increasing granularity of the cytoplasm was noted. From 15 - 18 hours, forespores could be seen, and in older cultures mature spores or forespores could be seen in the majority of the cells. By 21 hours, all spores had become phase-bright and the release of spores into the medium had commenced. This was a gradual process which was completed by 30-36 hours. In 48 hour cultures only a few vegetative forms could be detected among the free spores.

FIGURE 4

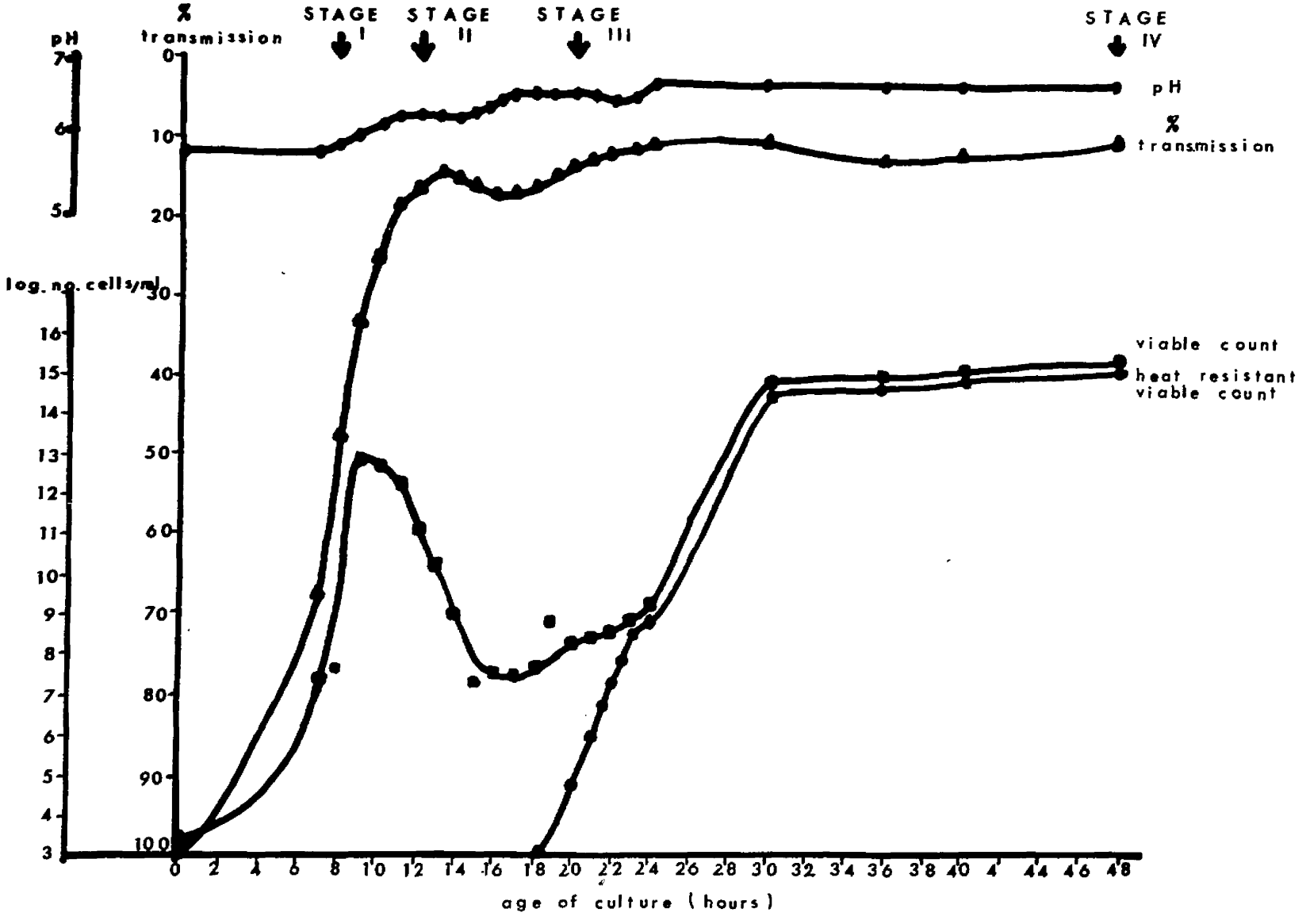
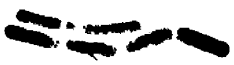

% transmission
STAGE I
STAGE II
STAGE III
STAGE IV
pH
log. no. cells/ml
pH
% transmission
viable count
heat resistant viable count
age of culture (hours)

PLATE 6

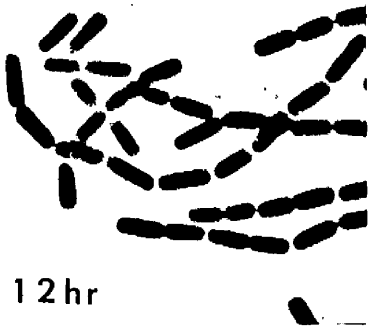
8hr

9hr

10hr

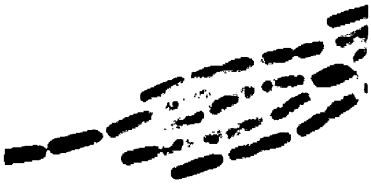


11 hr



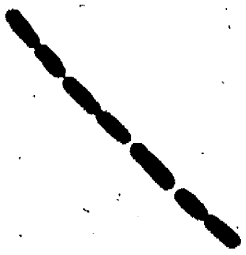
12hr

13hr

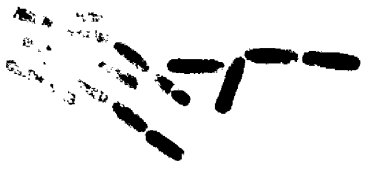


14hr

15hr



16hr

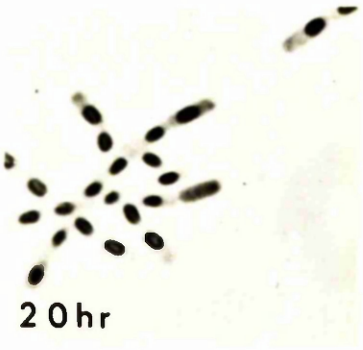


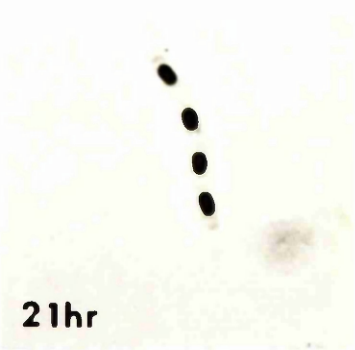
17hr

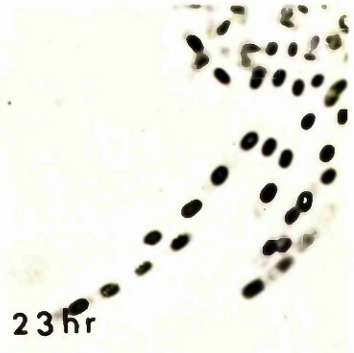
18hr

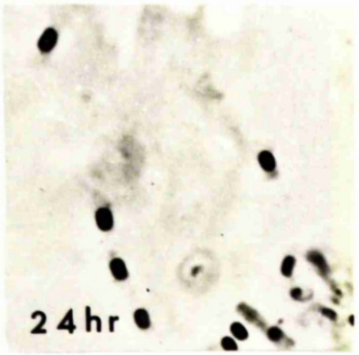
19hr

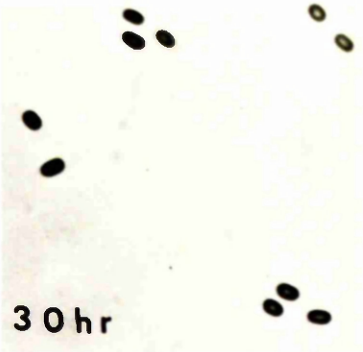
PLATE 6 contd

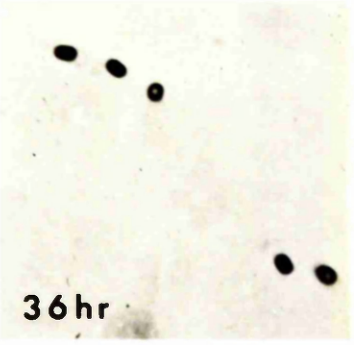


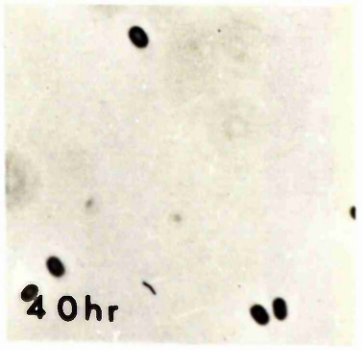


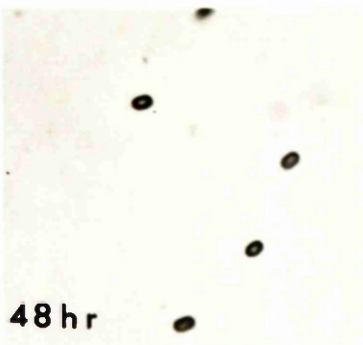












Phase contrast photomicrographs

B. cereus ×1200

3.1.2. Selection of 4 Growth Stages for Detailed Study of Enzymes and Antigens

From the information obtained from the experiment described in 3.1.1., it can be seen that the sporulation process in fluid sporulation medium falls into 4 main phases - vegetative, initial and final sporulating phases, terminating with the release of the mature spore. Accordingly, cells representative of these 4 phases were chosen for detailed study.

STAGE I CELLS: Vegetative cells from an 8 hour culture, appearing uniformly dense under phase contrast.

STAGE II CELLS: Vegetative cells from a 12 hour culture, showing distinct granulation, but no mature spores or forespores.

STAGE III CELLS: Sporulating cells from a 20 hour culture, the majority of cells containing mature spores or forespores.

STAGE IV CELLS: Mature spores released by the dissolution of the sporangia and washed free from vegetative debris.

B. cereus M.8 was grown in bulk in sporulation medium and harvested at one or other of these 4 stages, as judged by microscopic appearance. Extracts were prepared as described in 2.5. for the antigen and enzyme studies detailed in the following pages.

3.2. ENZYME CHANGES DURING SPORULATION

3.2.1. Esterases and Catalases

Extracts of cells at the 4 stages of sporulation were

analysed by starch gel electrophoresis (see 2.7.3.). On completion of electrophoresis, the 2 middle slices of the gel were stained to detect the presence of catalase and esterase. These 2 enzymes appear to exist as multimolecular systems in B. cereus M.8 and the patterns change during sporulation.

Stage I cells contained only one esterase (a esterase) and one catalase (a catalase). Stage II cells possessed an additional esterase (b) and an additional (b) catalase. These 2 new molecular forms were less mobile under electrophoresis than the a forms. Stage III cells showed the same pattern as Stage II cells. Stage IV cells contained no detectable esterase and only the b catalase.

3.2.2. Heat Resistance of the Enzymes

Heat sensitivities of the enzymes in cell disintegrates was investigated using the method detailed in 2.5.6.

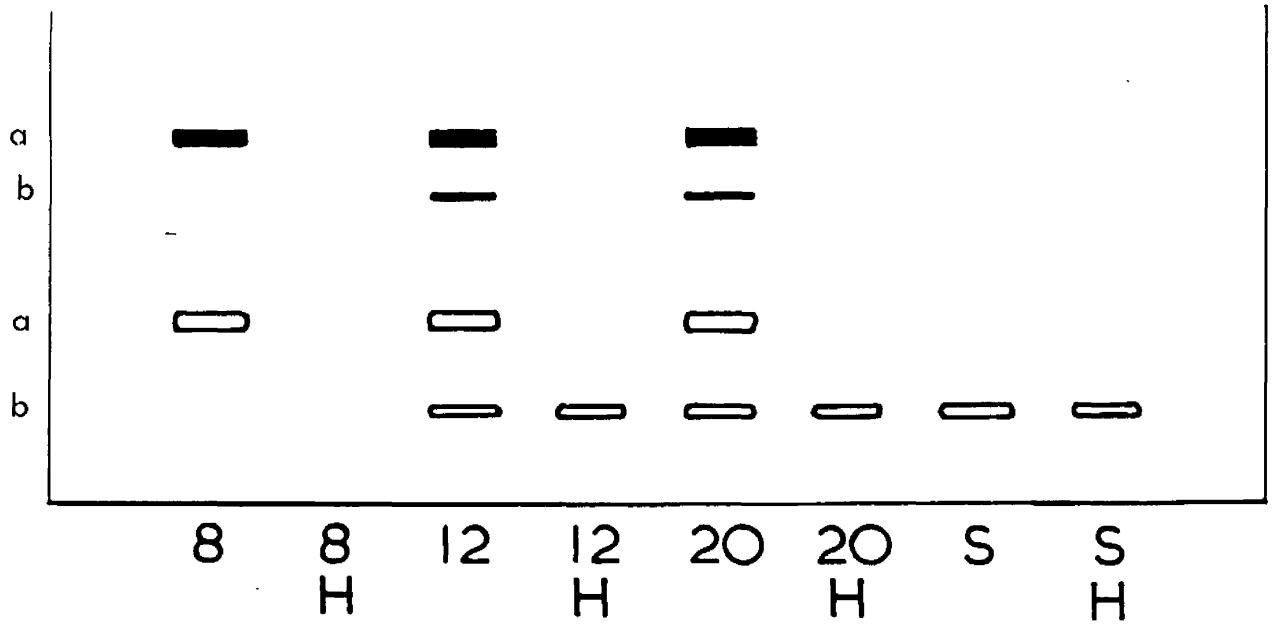
Esterases: Both a and b esterases were sensitive to moderate heating. The a esterase was inactivated by heating at 50°C for 5 minutes; the b esterase resisted this degree of heating, but was inactivated at 60°C for 5 minutes.

Catalases: The a catalase was inactivated at 60°C in 5 minutes, but the b catalase showed a remarkable degree of heat resistance, being unaffected by 30 minutes at 80°C and resisting 5 minutes at 90°C; it was inactivated completely by exposure to 100°C for 5 minutes.

Figure 5 summarises, in diagrammatic form, the effects of

FIGURE 5

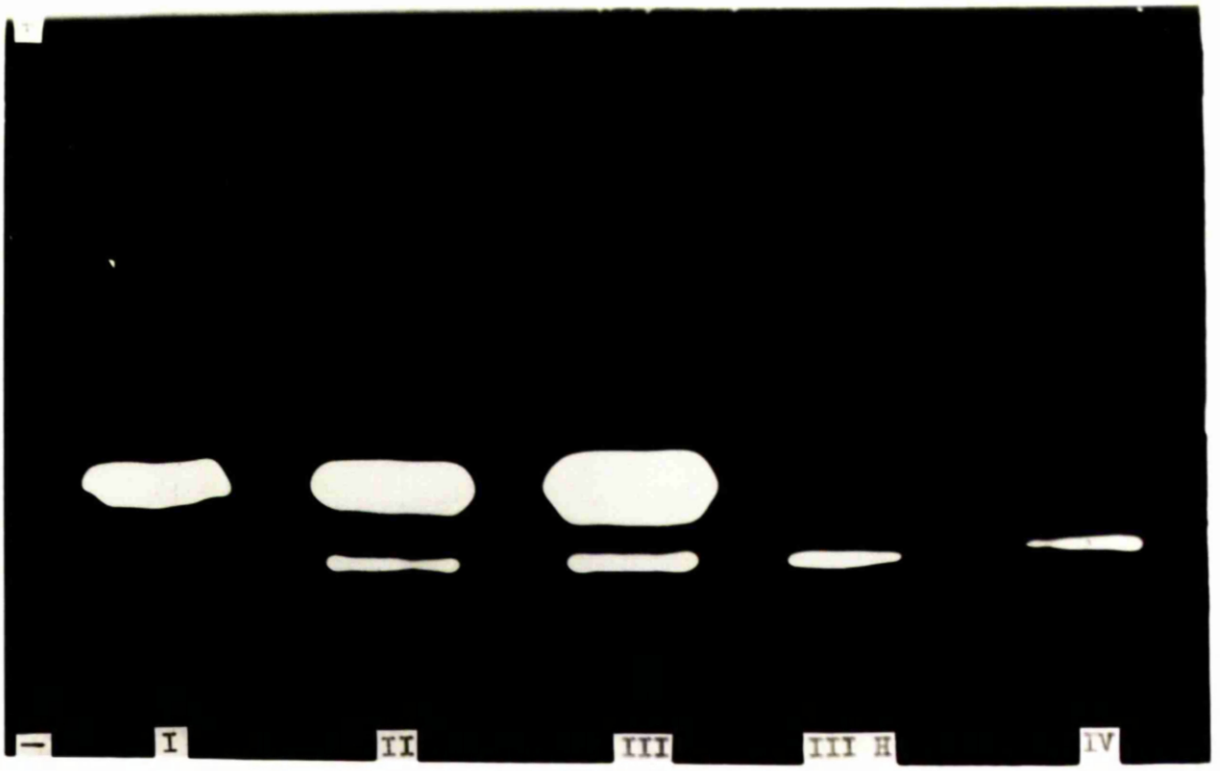
EFFECT OF HEATING ON CATALASES AND ESTERASES OF BACILLUS CEREUS



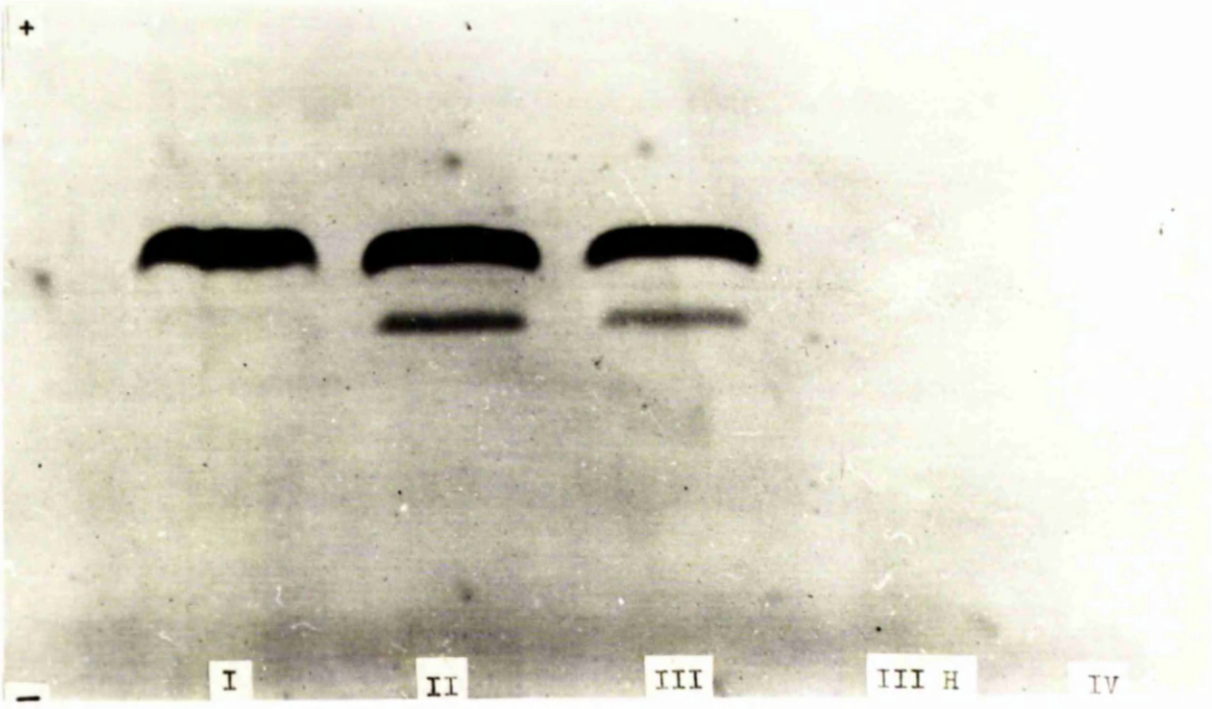
H = HEATED AT 80°C × 15 MINUTES

CATALASE ESTERASE

PLATE 7



Catalase



Esterase

exposure to 80°C for 10 minutes on the different enzyme components of the extracts.

Plate 7 shows catalase and esterase stains of starch gel slices.

3.3. ANTIGEN CHANGES DURING SPORULATION

3.3.1. Antigens Present in the 4 Growth Stages

Extracts of the cells at the 4 stages of sporulation were examined by immunoelectrophoresis in agar gels (see 2.7.2.). 4 antisera were used in the study:-

ANTISERUM 1: against uncentrifuged disintegrates of Stage I cells.

ANTISERUM 2: against uncentrifuged disintegrates of Stage II cells.

ANTISERUM 3: against uncentrifuged disintegrates of Stage III cells.

ANTISERUM 4: against uncentrifuged disintegrates of Stage IV cells.

Stage I cells revealed at least 15 antigens when tested with Serum 1, 10 with Serum 2, 15 with Serum 3, and 3-5 with Serum 4.

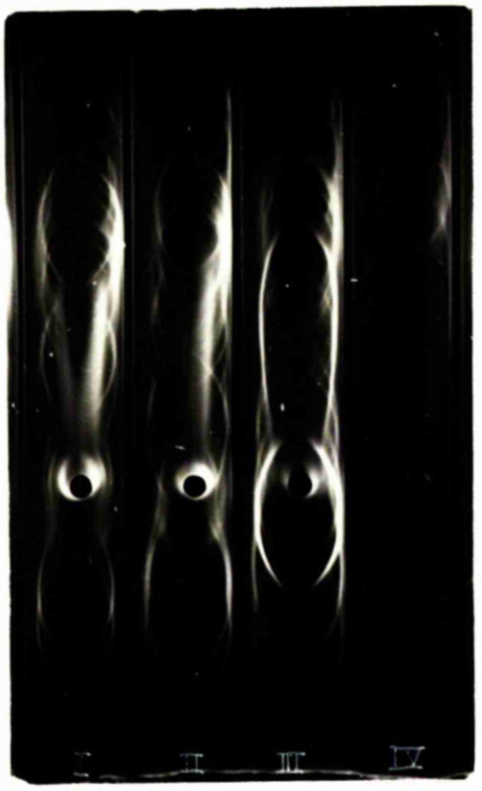
Stage II cells appeared to have an antigenic composition very similar to that of Stage I cells.

Stage III cells showed at least 15 antigens with Serum 1, 12 with Serum 2, 15 with Serum 3, and 12 with Serum 4.

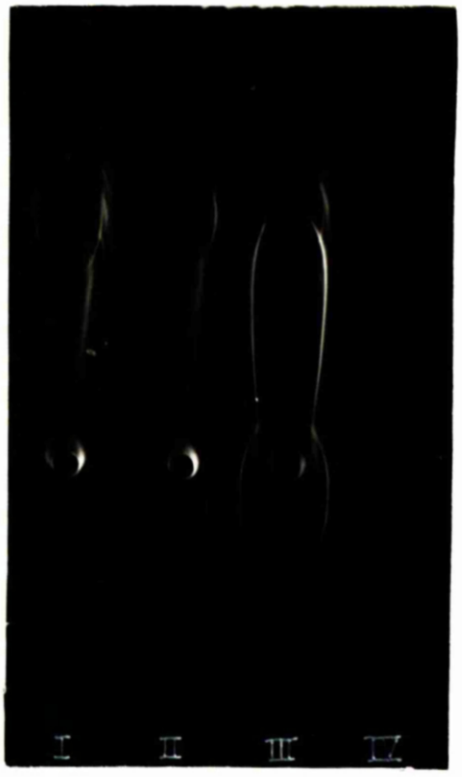
Stage IV cells possessed 9 antigens which reacted with Serum 1, 6 with Serum 2, 7 with Serum 3, and 12 with

PLATE 8

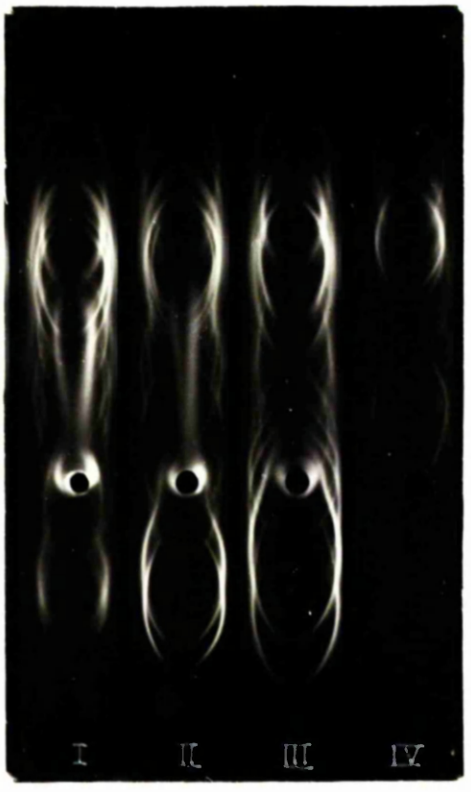
ANTIGENS OF B.CEREUS M.8



SERUM 1



SERUM 2



SERUM 3

SERUM 4

Serum 4.

The appearance of precipitin lines and the overall pattern of changes from stage to stage is shown in Plate 8.

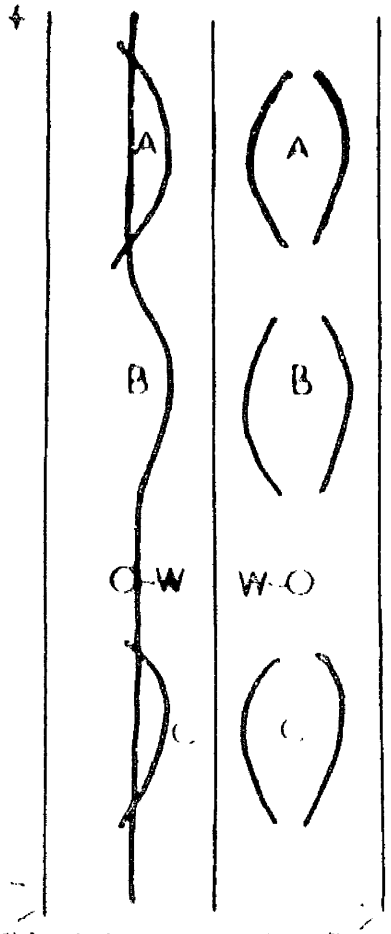
3.3.2. Correlation of Precipitin Lines

When comparing, side by side, extracts of cells harvested at different growth phases, it was noted that particular antigens showed slightly different electrophoretic mobilities in different preparations. This meant that it was sometimes difficult to identify individual precipitin lines when comparing the 4 extracts. The technique of Osserman (1960) using terminal antigen-containing trenches was used to facilitate the identification of the various antigens.

A diagrammatic representation of the theory behind this method is shown in Figure 6. An extract containing antigens A, B, and C was submitted to electrophoresis through an agar gel in the usual manner and serum troughs cut parallel to the run. 2 trenches were filled with antiserum to A, B, and C, the last one being charged with an extract containing only the antigen B. Antiserum diffusing towards the electrophoretically separated antigens formed precipitin arcs at A, B, and C. Antigen B diffusing towards the antiserum from a parallel trench formed a straight band of precipitation. Arcs due to the precipitation of antigens A and C crossed this band in reactions of non-identity, but the precipitin arc of antigen B united with the band in a reaction of identity.

FIGURE 6

CORRELATION OF PRECIPITIN LINES



ANTIGEN B ANTISERUM

W = ANTIGEN WELL

PLATE 9

CORRELATION OF PRECIPITIN LINES

CW P P

SERUM 1

CW P P

SERUM 3

P - PROTOPLASTS CW - CELL WALLS

Using this technique, it was possible to compare directly antigenic constituents of all electrophoretic mobilities in the antigenic extracts of B. cereus. Where the number of precipitin lines was large, it became difficult to compare individual lines. For this reason, only isolated cellular components and those antigens which withstood heating at 80°C for 10 minutes were studied in this manner.

A typical example of end-trench analysis can be seen in Plate 9.

3.3.3. Heat Resistant Antigens

Thermostability of the various antigens was tested by heating extracts at 60, 80, and 100°C for periods of 10 minutes immediately prior to analysis (see 2.5.6.). A typical heat resistance test is shown in Plate 10.

Results presented in this thesis were obtained with extracts heated at 80°C for 10 minutes. This time/temperature combination was chosen for special study since it kills vegetative cells of B. cereus but has no lethal effect on mature spores. Figures 7-10 show diagrammatic summaries of the effect of heating at 80°C for 10 minutes on the antigens present in the 4 extracts.

STAGE I CELLS: When tested against homologous serum, 7 antigens a, b, c, d, e, f, and g were found to resist heating at 80°C for 10 minutes. 5 of these (a, d, e, f, and g) were detected with Serum 2, 6 (b, c, d, e, f, and g) with Serum 3 and 3 (c, d, and e) with Serum 4.

STAGE II CELLS: An additional antigen, h, was observed

when tested with Serum 1, the other reactions being identical with those of Stage I cells.

STAGE III CELLS: A marked increase in the proportion of heat resistant antigens was noted at this stage. With Serum 1, 8 resistant antigens were seen (a, b, c, d, e, f, g, and k), 9 (a, d, e, f, g, k, o, n, and q) with Serum 2, 10 (c, d, e, f, g, k, m, n, o, and q) with Serum 3, and 8 (c, d, e, g, k, m, n, and o) with Serum 4.

STAGE IV CELLS: 5 heat stable antigens (c, d, e, f, and l) were detected with Serum 1, 6 (d, e, f, l, o, and n) with Serum 2, 7 (c, d, e, f, l, n, and o) with Serum 3, and 8 (c, d, e, l, m, n, o, and p) with Serum 4.

The antigens d and e were detected at all phases in the sporulation process and with all antisera. These antigens showed moderately high electrophoretic mobility towards the anode, and the close relationship in their behaviour indicates that they are probably similar molecules. The slower running components showed greatest variation from stage to stage.

Spore extracts (Stage IV cells) contained 4 of the heat resistant antigens of young vegetative cells (Stage I) c, d, e, and f, in addition to 5 (l, m, n, o, and p) which were found only in Stage III cells or spores. 2 antigens, k and q were unique to Stage III cells. Heat resistant antigens in the 4 extracts are summarised in Table 3.

TABLE 3

EXTRACT	ANTIGEN															
	a	b	c	d	e	f	g	h	k	l	m	n	o	p	q	
STAGE I	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	
STAGE II	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	
STAGE III	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+	
STAGE IV	-	-	+	+	+	+	-	-	-	+	+	+	+	+	-	

3.3.4. Nature of the Heat Resistant Antigens

Immunoelectrophoretic analyses of the 4 extracts heated at 80°C for 10 minutes were developed with each of the 4 antisera. After 48 hours incubation, the gels were washed, dried, and stained with a modification of the periodic acid-Schiff reaction (2.7.2.3) in order to determine whether any of the thermostable antigens were polysaccharide in nature.

Antigens c, d, e, f, g, k, l, m, n, and o gave a positive reaction with this stain.

It would appear, therefore, that the majority of the antigens of B. cereus which withstand heating at 80°C for 10 minutes possess polysaccharide components. Only the antigens a, b, h, q, and p gave a negative result.

PLATE 10

HEAT RESISTANCE TEST

B. CEREUS M.8 EXTRACT I

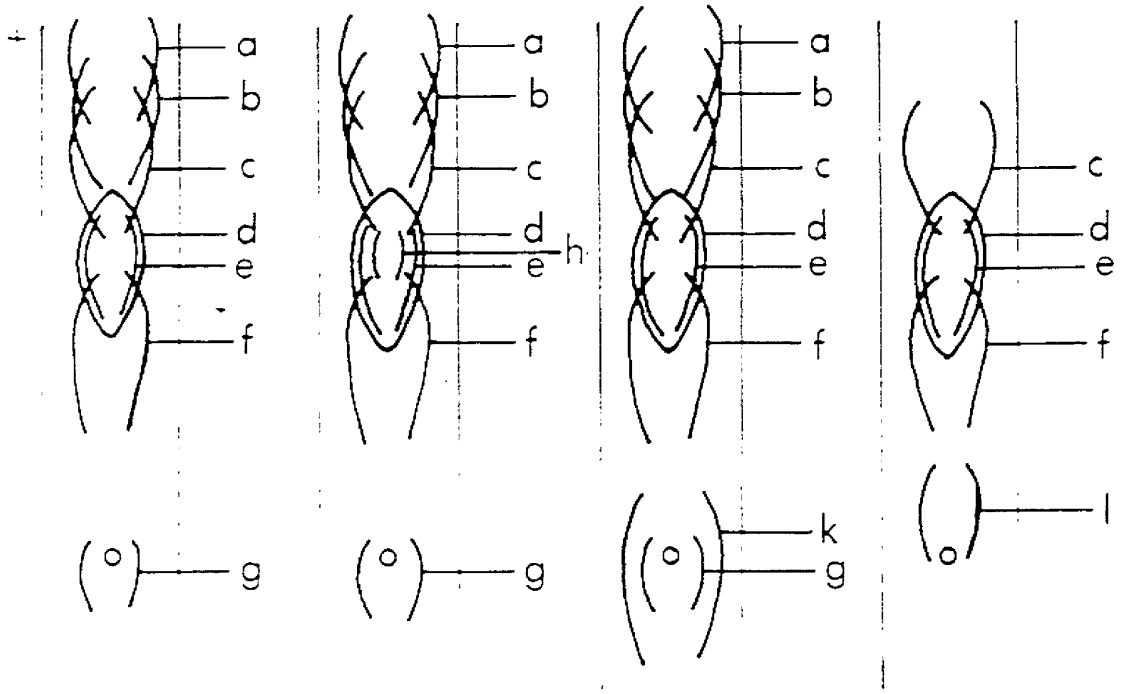
UN. 60°C 80°C 100°C UN.

SERUM I

FIGURE 7

HEAT RESISTANT ANTIGENS B CEREUS M B

SERUM I



I II III IV

FIGURE 8

HEAT RESISTANT ANTIGENS B. CEREUS M.8

SERUM 2

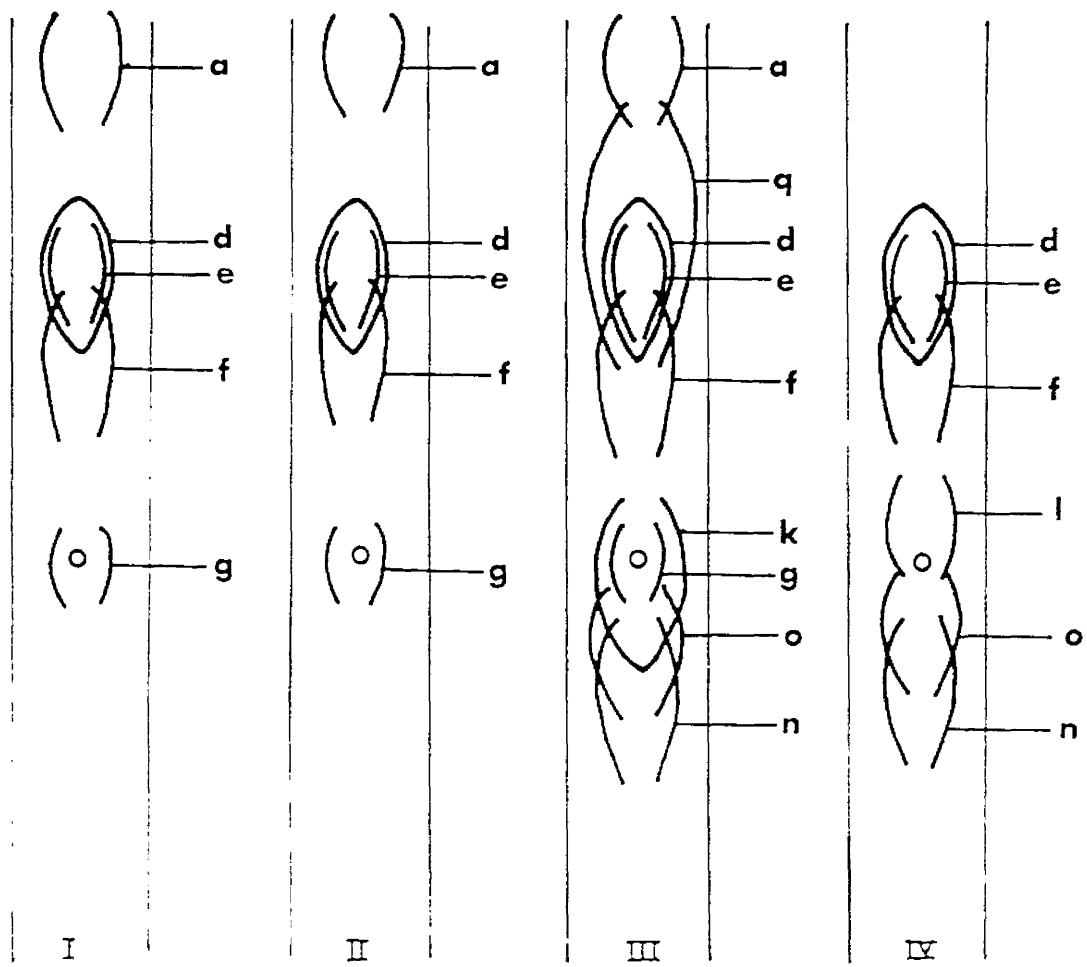


FIGURE 9

HEAT RESISTANT ANTIGENS B CEREUS M 8

SERUM 3

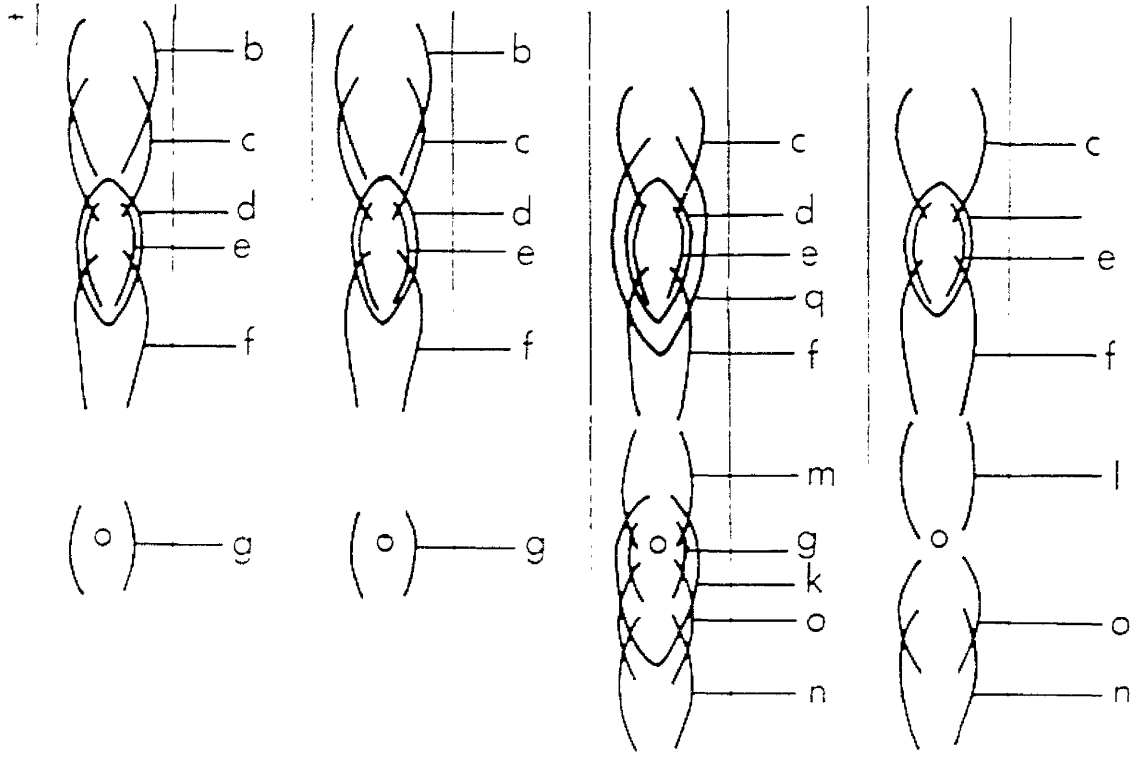
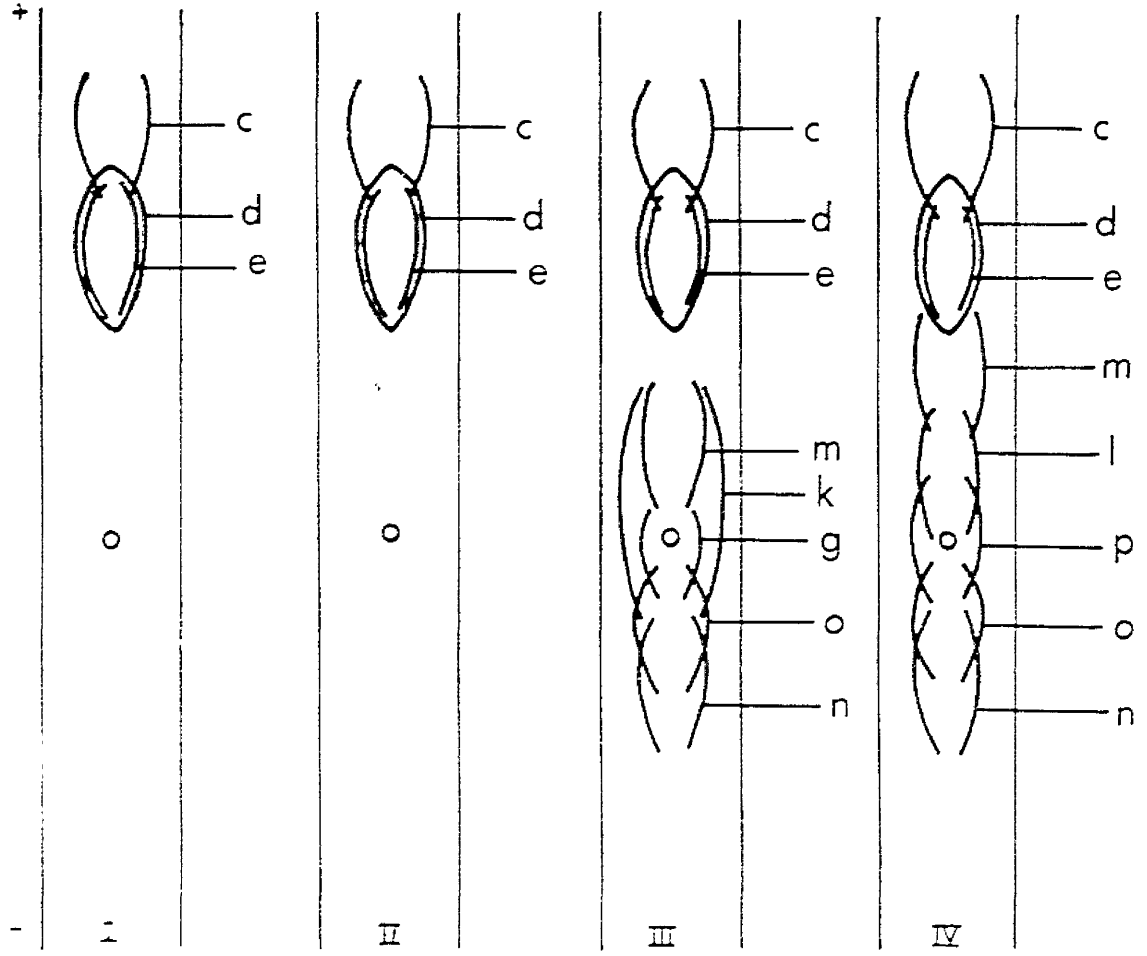


FIGURE 10

HEAT RESISTANT ANTIGENS B. CEREUS M.8

SERUM 4



3.4. IMMUNOLOGICAL SPECIFICITIES OF ENZYMES

3.4. IMMUNOLOGICAL SPECIFICITIES OF ENZYMESSalase were demonstrated in B. cereus M.8 by means of starch gel electrophoresis (3.2). In an attempt to discover the relationship between a and b catalase, and a and b esterase, the immunological specificities of the enzymes were investigated.

Extract of Stage III cells was submitted to immunoelectrophoresis in agar and the gels developed with Serum 3 for 48 hours. At the end of this period, the gels were washed and dried as described in 2.7.2.3 prior to the location of enzymes.

3.4.1. Spore and Vegetative Cell Catalases

2 areas of catalase-anticatalase activity were detected on treating the gel with H_2O_2 (2.7.2.3). The bands crossed, indicating a lack of antigenic relationship between the vegetative cell and spore catalase. (Figure 11).

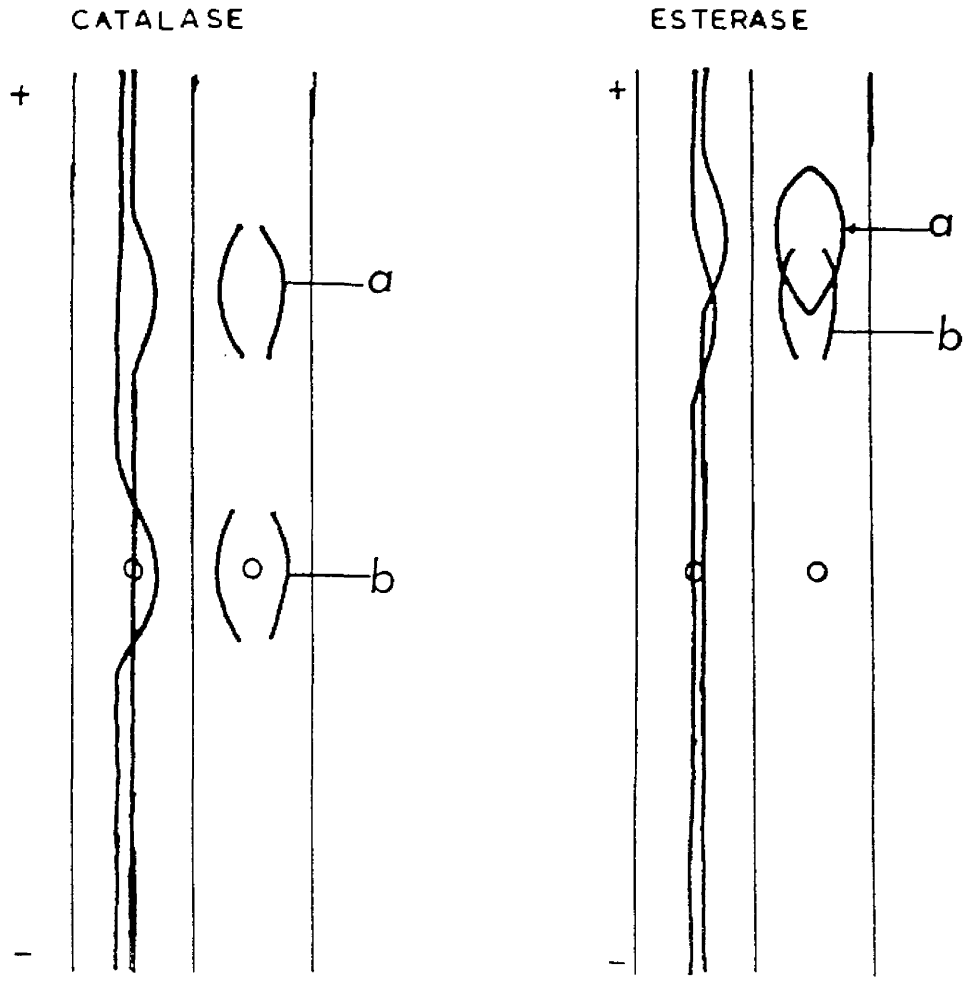
3.4.2. a and b Esterases

A duplicate gel was stained to locate zones of esterase activity as detailed in 2.7.3.3. 2 distinct bands were noted, and these crossed in a reaction of non-identity, demonstrating the antigenic dissimilarity of a and b esterase (Figure 11).

Since 2 areas of catalase activity and 2 areas of esterase activity were noted after the reaction of the enzymes with antibody, it would appear that the antigen-antibody combination did not interfere with the catalytic site of

FIGURE 11

IMMUNOLOGICAL SPECIFICITIES OF ENZYMES



the enzymes.

3.5. ANTIGENS OF CELL FRACTIONS

3 cell fractions were prepared; cell walls, protoplasts, and flagella. Only small yields of the last 2 were obtained and it was not possible to carry out a detailed analysis of them. Purity of the cell fractions was checked as described in 2.8 but the experiments were of a preliminary nature and caution must be exercised when interpreting the results.

Cell walls, protoplasts, and flagella were analysed by immunoelectrophoresis in agar gels, which were developed with each of the 4 antisera. Resistance of the antigens to heating at 80°C for 10 minutes was also investigated.

3.5.1. Cell Walls

9 antigens were detected in cell walls (Plate 11). All resisted heating at 80°C for 10 minutes and corresponded to antigens b, c, d, e, and g. Polysaccharide was present in all but b.

3.5.2. Protoplasts

Cytologically, the cells used for the preparation of protoplasts appeared to be of Stage II. Starch gel electrophoresis of the protoplast preparation revealed the presence of a and b catalase, and also of a and b esterase. When tested against Serum 1, 11 antigens were detected as compared with the 15 found in disintegrates of untreated

cells of Stage II.

7 antigens were present when tested with Serum 2 (10 in untreated cells), 9 with Serum 3 (15 in untreated cells), and 3 with Serum 4 (3-5 in untreated cells).

Thermoresistant antigens c, d, e, f, and g were present.

3.5.3. Flagella

No antigens were detected in the flagellar preparation with any of the 4 antisera.

Heat resistant antigens present in the cell fractions are summarised in Table 4.

TABLE 4

	ANTIGEN															
EXTRACT	a	b	c	d	e	f	g	h	k	l	m	n	o	p	q	
STAGE II	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	
CELL WALLS	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	
PROTOPLASTS	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	
FLAGELLA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

The antigenic patterns of cell fractions are shown in Plate 11.

3.6. ENZYMIC CONSTITUTION OF BACILLUS CEREUS M.8.P

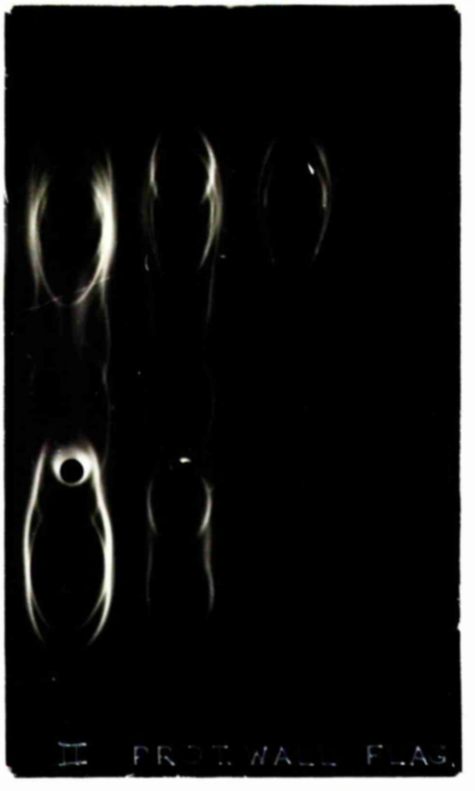
3.6.1. Choice of 5 Growth Stages for Study

PLATE II

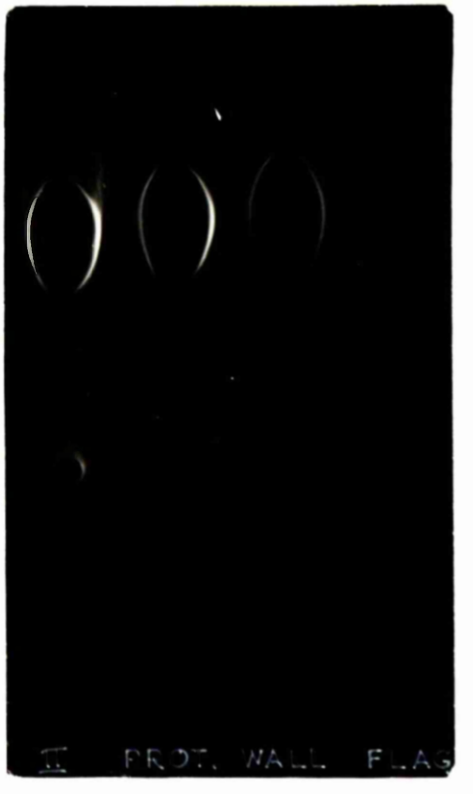
ANTIGENS OF CELL FRACTIONS

SERUM 1

SERUM 2



SERUM 3



SERUM 4

P_{24A} CELLS: A 24 hour culture on Lab-Lemco agar, with cells showing granulation, but few detectable pleomorphic forms.

P_{48A} CELLS: A 48 hour Lab-Lemco agar culture, with many swollen and distorted cells, some of which were undergoing lysis to leave cell walls.

P_{72A} CELLS: A 72 hour Lab-Lemco agar culture, the majority of cells of which had lysed to give large numbers of empty shells and a few pleomorphic rods.

P_{24B} CELLS: Phase-dark rods from a 24 hour Lab-Lemco broth culture. The cells were very similar in appearance to those of B. cereus M. 8 Stage I.

P_{48B} CELLS: Cells from a 48 hour Lab-Lemco broth culture. The rods showed distinct granulation when viewed under phase-contrast. No pleomorphic forms were detected.

B. cereus M.8.P was grown in bulk, and the cells harvested at these 5 phases. Extracts were prepared as described in 2.5.

Cells from broth cultures P_{48B} and P_{24B} appeared to be physiologically younger than the corresponding agar cultures. P_{24A} cells were more granular than P_{48B} cells. This fact was taken into account when interpreting the results of enzyme and antigen studies. At no point did the variant progress beyond the cytological appearance of Stage II of the parent strain.

3.6.2. Esterases and Catalases

Extracts of the 5 growth phases of B. cereus M.8.P were

analysed by electrophoresis in starch gels (2.7.3). After electrophoresis, slices of the gel were stained to detect the presence of (a) esterase and (b) catalase.

(a) Esterase: P_{24B} and P_{48B} cells contained both esterases. Only the b form was detected in the other 3 extracts.

(b) Catalase: All extracts appeared to have only a catalase activity. The heat resistant b catalase was not present at any time.

3.7. ANTIGENIC STRUCTURE OF BACILLUS CEREUS M.S.P

3.7.1. Antigens Present in the 5 Growth Stages

Extracts of the 5 growth stages of the phantom variant were studied antigenically by immunoelectrophoresis in agar gels (2.7.2).

P_{24A} cells showed 14 antigens when tested with Serum 1, 6 with Serum 2, 12 with Serum 3, and 4 with Serum 4.

P_{48A} cells had an antigenic constitution essentially similar to P_{24A} cells.

P_{72A} cells possessed 10 antigens detectable with Serum 1, 8 with Serum 2, 14 with Serum 3, and 4 with Serum 4.

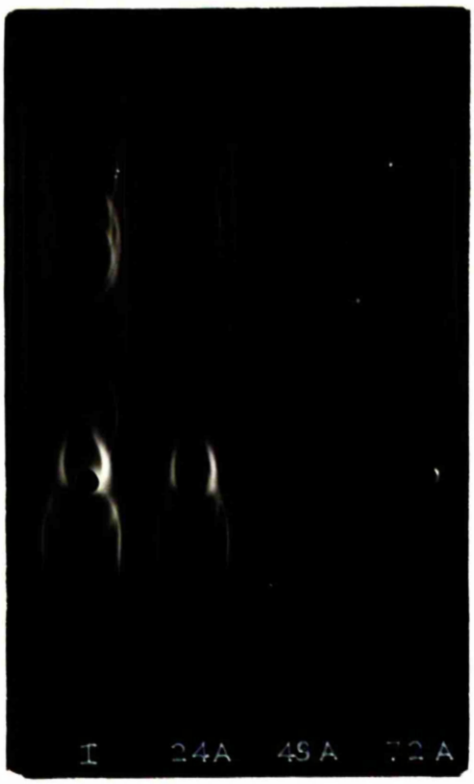
P_{24B} cells gave 11 precipitin lines when tested with Serum 1, 8 with Serum 2, 12 with Serum 3, and 4 with Serum 4.

P_{48B} cells closely resembled P_{24B} cells in antigenic constitution.

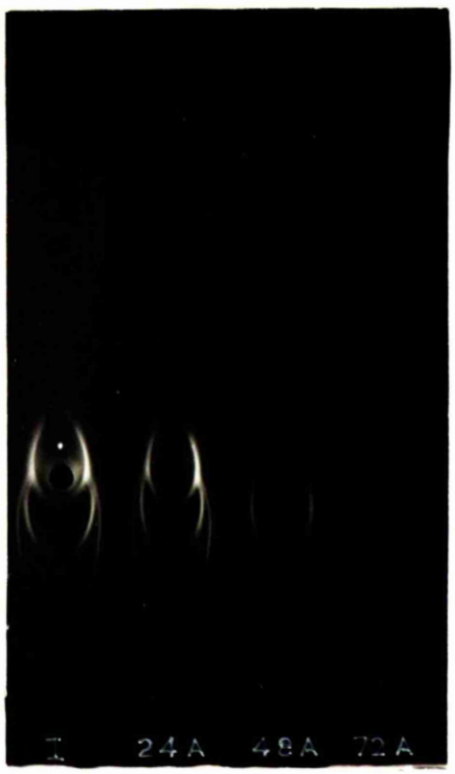
The general pattern of antigenic changes between the 5 stages of B. cereus M.S.P and a comparison with the parent

PLATE 12

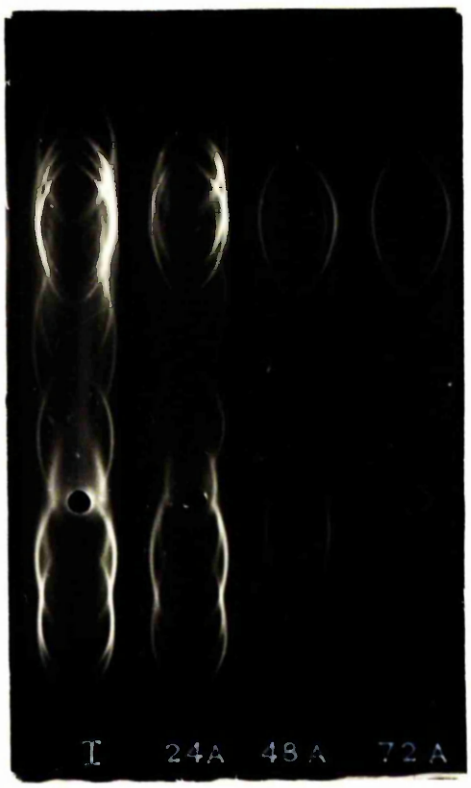
ANTIGENS OF B. CEREUS M.8.P



SERUM 1



SERUM 2



SERUM 3

SERUM 4

PLATE 13

ANTIGENS OF B. CEREUS M.8.P

SERUM 1

SERUM 2

SERUM 3

SERUM 4

strain is seen in Plates 12 and 13.

3.7.2. Heat Resistant Antigens

As with the thermostable antigens of B. cereus M.8, correlation of precipitin lines in the different extracts was carried out by the technique of end-trench analysis (3.3.2). Antigens present in the phantom variant were compared with those of the parent strain by use of this method.

Extracts of the 5 growth stages were heated at 80°C for 10 minutes (2.5.6) immediately prior to electrophoresis. Figures 12-15 summarise diagrammatically the effect of this degree of heating on the antigens present in the extracts.

P_{24A} CELLS: When tested with Serum 1, antigens b, c, d, e, f, and g were detected, with Serum 2, b, d, e, and g, with Serum 3 c, d, e, f, and g, and with Serum 4 c, d, and e.

P_{48A} CELLS: These had an antigenic constitution identical with P_{24A} cells. Antigen e formed an elongated precipitin arc.

P_{72A} CELLS: Antigens c, d, e, f, and g were seen when tested against Serum 1, d, e, and g with Serum 2, c, d, e, f, and g with Serum 3 and c, d, and e with Serum 4. Alteration in the shape of the arc of antigen e was again noted.

P_{24B} and P_{48B} CELLS: Both these growth stages had an

antigenic constitution identical with P_{24A} cells.

3.7.3. Nature of the Heat Resistant Antigens

Extracts of the 5 growth stages of the phantom variant were heated for 10 minutes at 80°C before running immunoelectrophoretic analyses. The gels were developed with each of the 4 antisera for 48 hours and then washed, dried and stained with the modified periodic acid-Schiff reaction described in 2.7.2.3.

Antigens c, d, e, f, and g all gave a positive result, indicating the presence of a polysaccharide component. Heat resistant antigens of the phantom variant are summarised in Table 5.

TABLE 5

EXTRACT	ANTIGENS															
	a	b	c	d	e	f	g	h	k	l	m	n	o	p	q	
STAGE I	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	
P_{24A}	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	
P_{48A}	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	
P_{72A}	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	
P_{24B}	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	
P_{48B}	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	
STAGE IV	-	-	+	+	+	+	-	-	-	+	+	+	+	+	-	

FIGURE 12

HEAT RESISTANT ANTIGENS B. CEREUS M.8.P

SERUM I

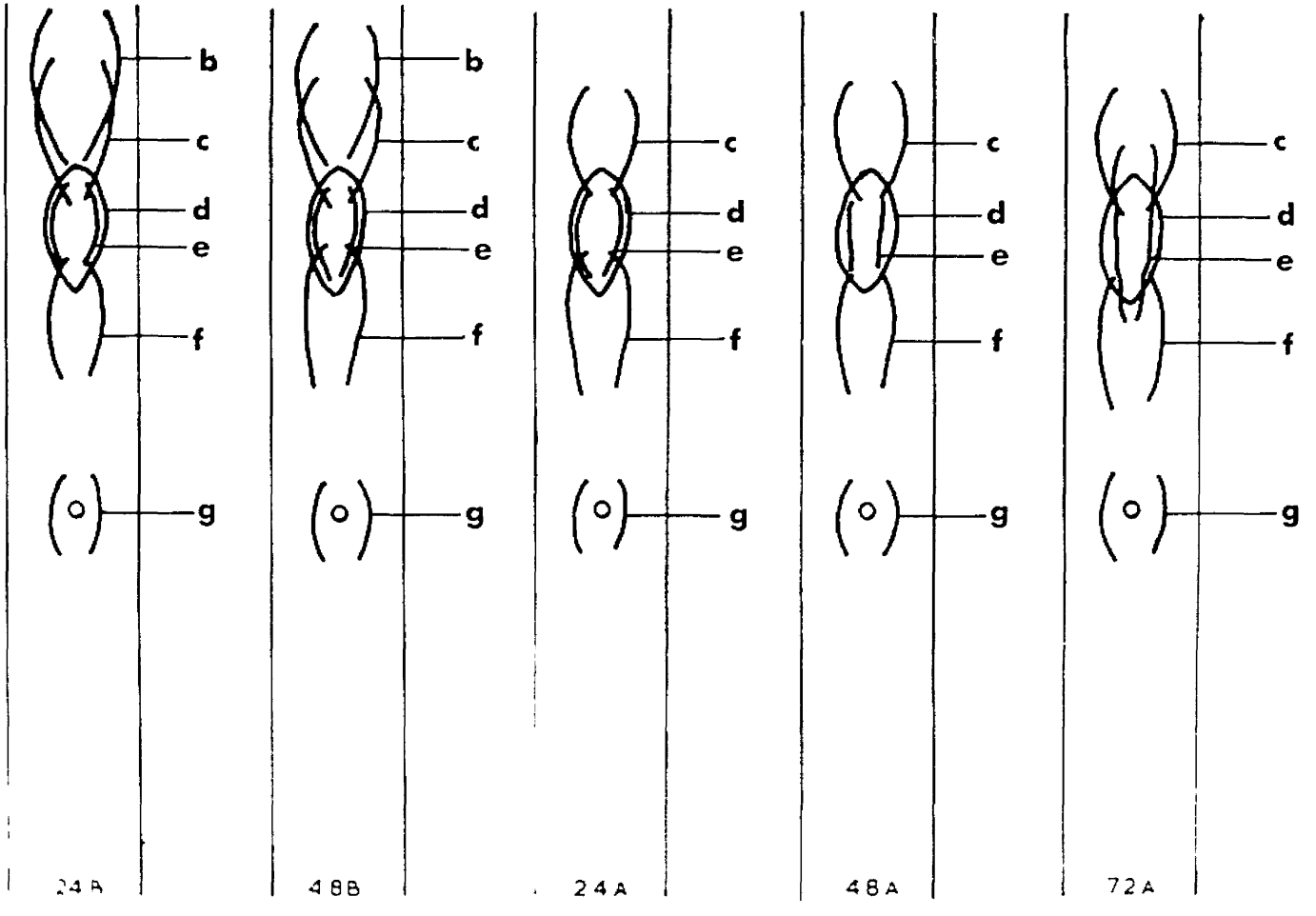
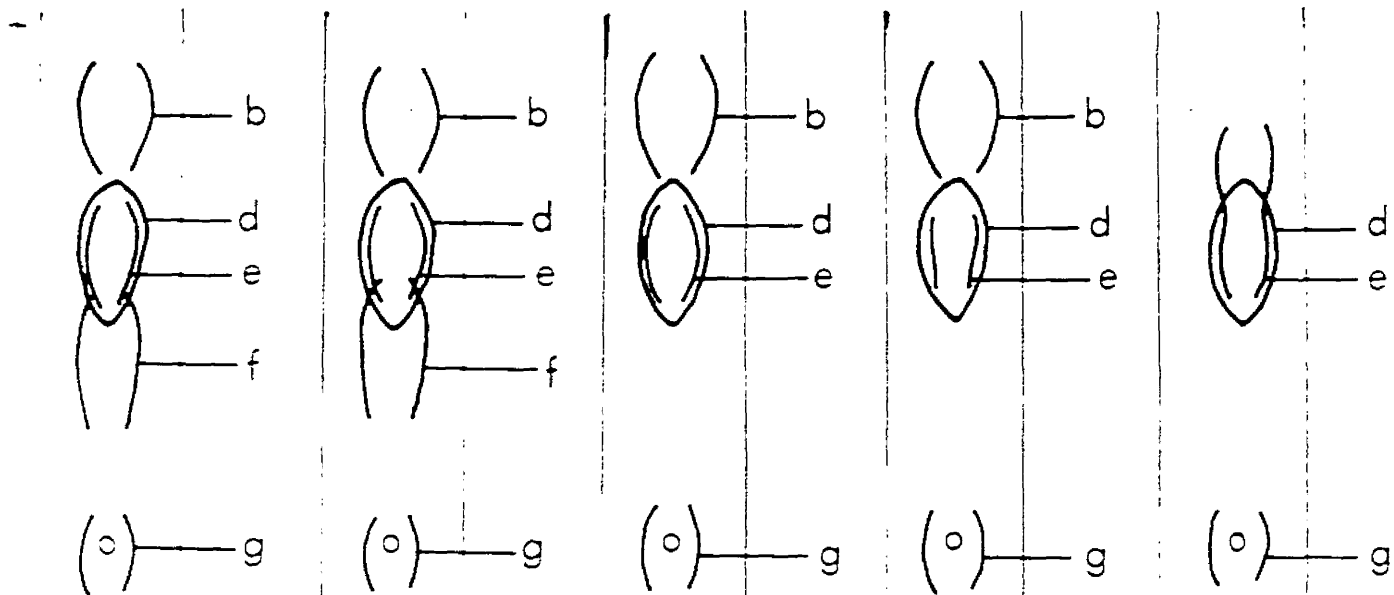


FIGURE 13

HEAT RESISTANT ANTIGENS *B. CEREUS* M.B.P.

SERUM 2



24B 48B 24A 48A 72A

FIGURE 14

HEAT RESISTANT ANTIGENS B. CEREUS M 8.P

SERUM 3

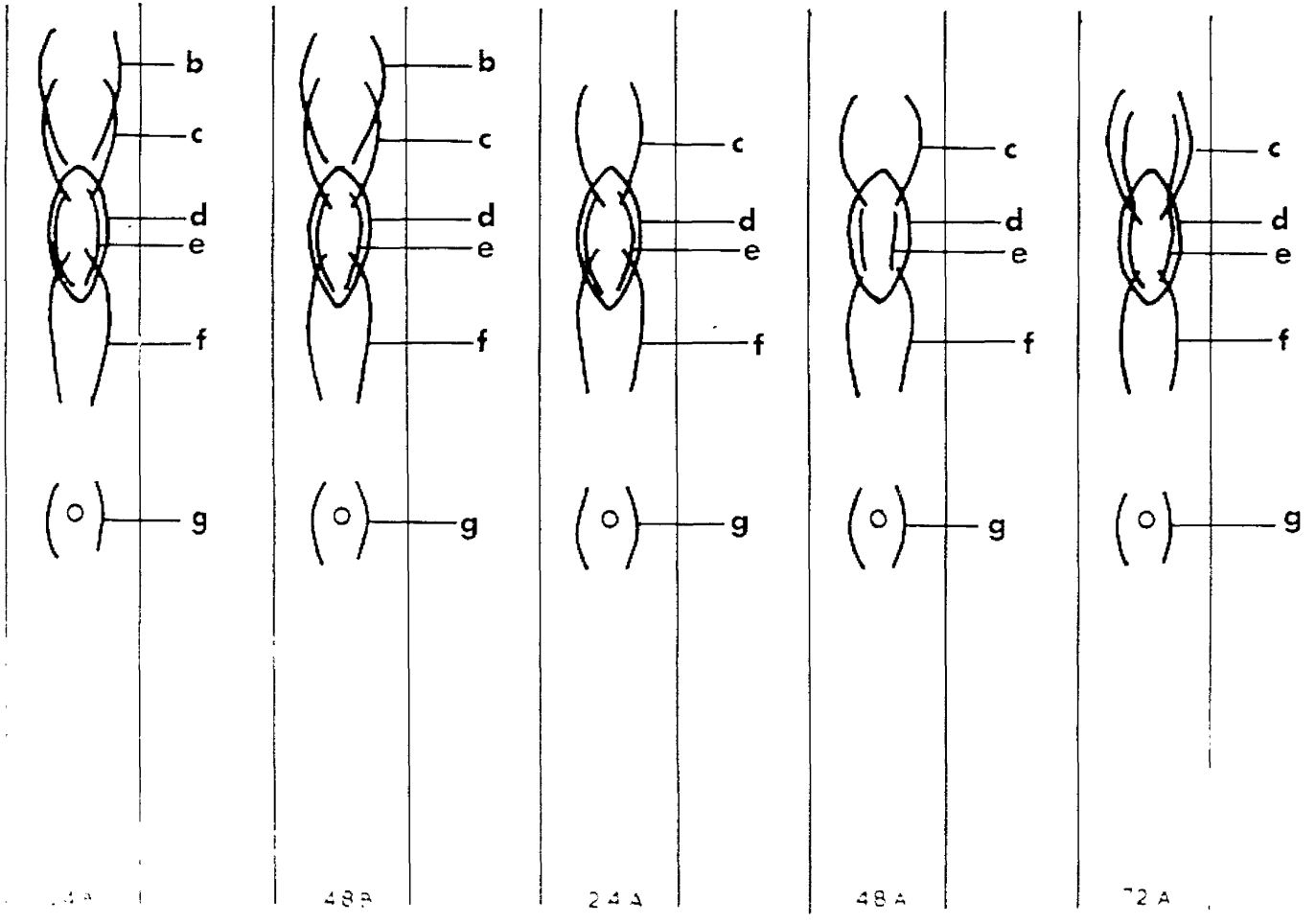
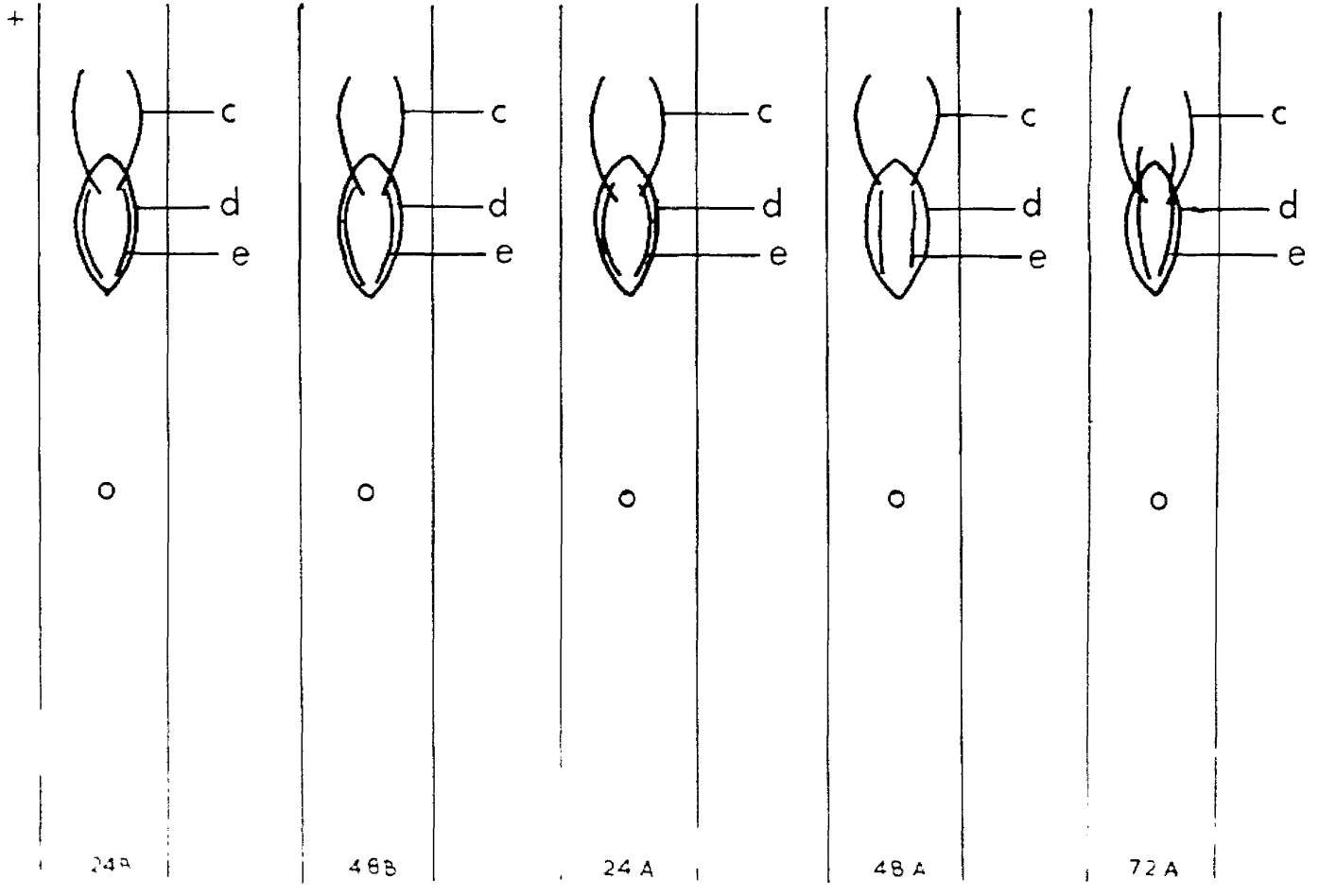


FIGURE 15

HEAT RESISTANT ANTIGENS B. CEREUS M.B.P

SERUM 4



3.8. PROTEIN CHANGES ACCOMPANYING SPORULATION

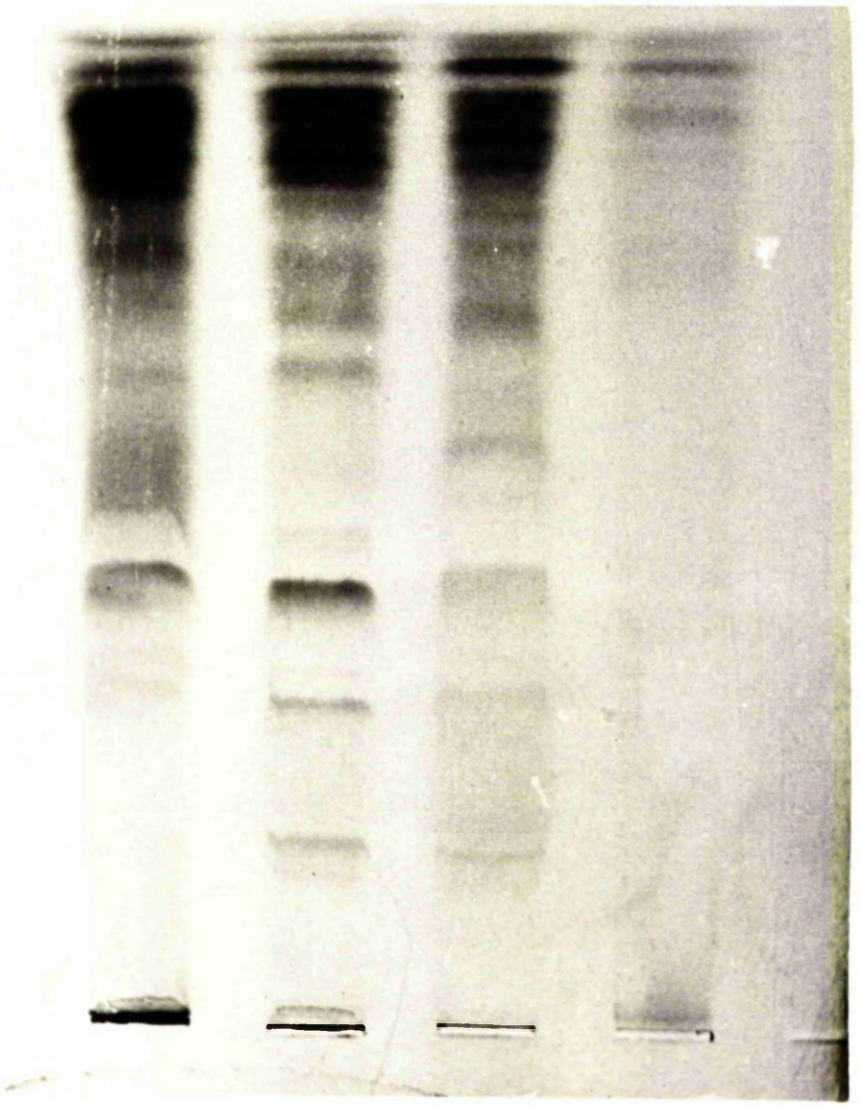
Electrophoresis in polyacrylamide gels (2.7.4) of extracts of the 4 growth stages of B. cereus M.8 was used to follow alterations in the protein constituents of cells as spore formation progressed. After electrophoretic separation, the gel was sliced and stained with naphthalene black to locate protein bands. Before attempting to correlate bands from extract to extract, standardisation of electrophoretic separation was necessary. All gels were run until the front artifact reached a distance of 10 c.m. from the sample inserts. After staining, it was noted that gel slices increased slightly in size due to the uptake of water. To overcome this, the stained preparations were photographed and the magnification of the print adjusted to give an electrophoretic path length of 10 cm.. Adopting the technique of Fowler et. al., (1963) protein bands were given migration numbers, the front artifact representing a migration number of 100. By using this method, it was possible to compare protein bands from gel to gel.

3.8.1. Protein Patterns of the 4 Growth Stages

Vegetative and sporulating cells appeared to have fairly similar patterns of protein constituents. 17 bands were detected in young vegetative cells, 18 in Stage II cells, and 15 in Stage III cells. Mature spores possessed only 5 protein components, which had a high electrophoretic mobility, all the slower running proteins of vegetative and sporulating cells having disappeared.

PLATE 14

PROTEINS OF B. CEREUS M.8



I II III IV

The appearance of protein bands in the 4 extracts is shown in Plate 14.

3.8.2. Heat Resistance of Proteins

8 of the proteins of Stages I and II, 7 of Stage III, and 4 of Stage IV withstood heating at 80°C for 10 minutes. A diagrammatic summary of heat stable and heat labile proteins of the 4 growth stages is seen in the first part of Figure 16.

3.9. PROTEINS OF BACILLUS CEREUS M.S.P

Extracts of the 5 growth stages of the phantom variant were analysed by electrophoresis in polyacrylamide gels. Results were recorded by the method detailed in 3.8.

3.9.1. Protein Patterns of the 5 Growth Stages

Broth cultures of the organism possessed a larger number of protein components than did agar cultures. P_{24B} cells had 12 bands, and P_{48B} cells showed an additional 4. Both these extracts had a pattern essentially similar to that of Stage I cells of B. cereus. Extracts of P_{24A} and P_{48A} cells resembled those of P_{24B} cells and possessed 12 bands. Only 11 were seen with P_{72A} cells.

3.9.2. Heat Resistance of Proteins

Of the 12 protein bands of P_{24B} cells, 6 resisted heating at 80°C for 10 minutes. 8 of those of P_{48B} cells were thermostable. Extracts of agar grown cultures all had 7 heat stable antigens.

PLATE 15

PROTEINS OF B.CEREUS M.8.P

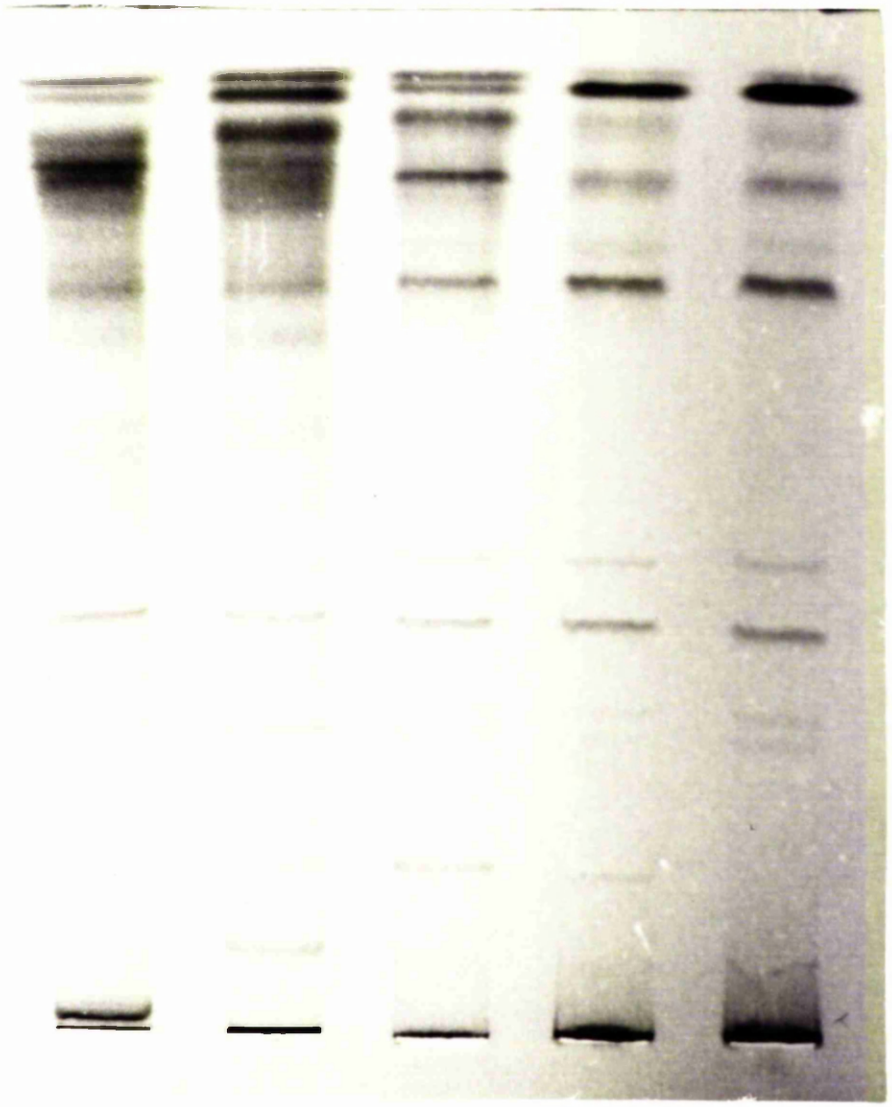
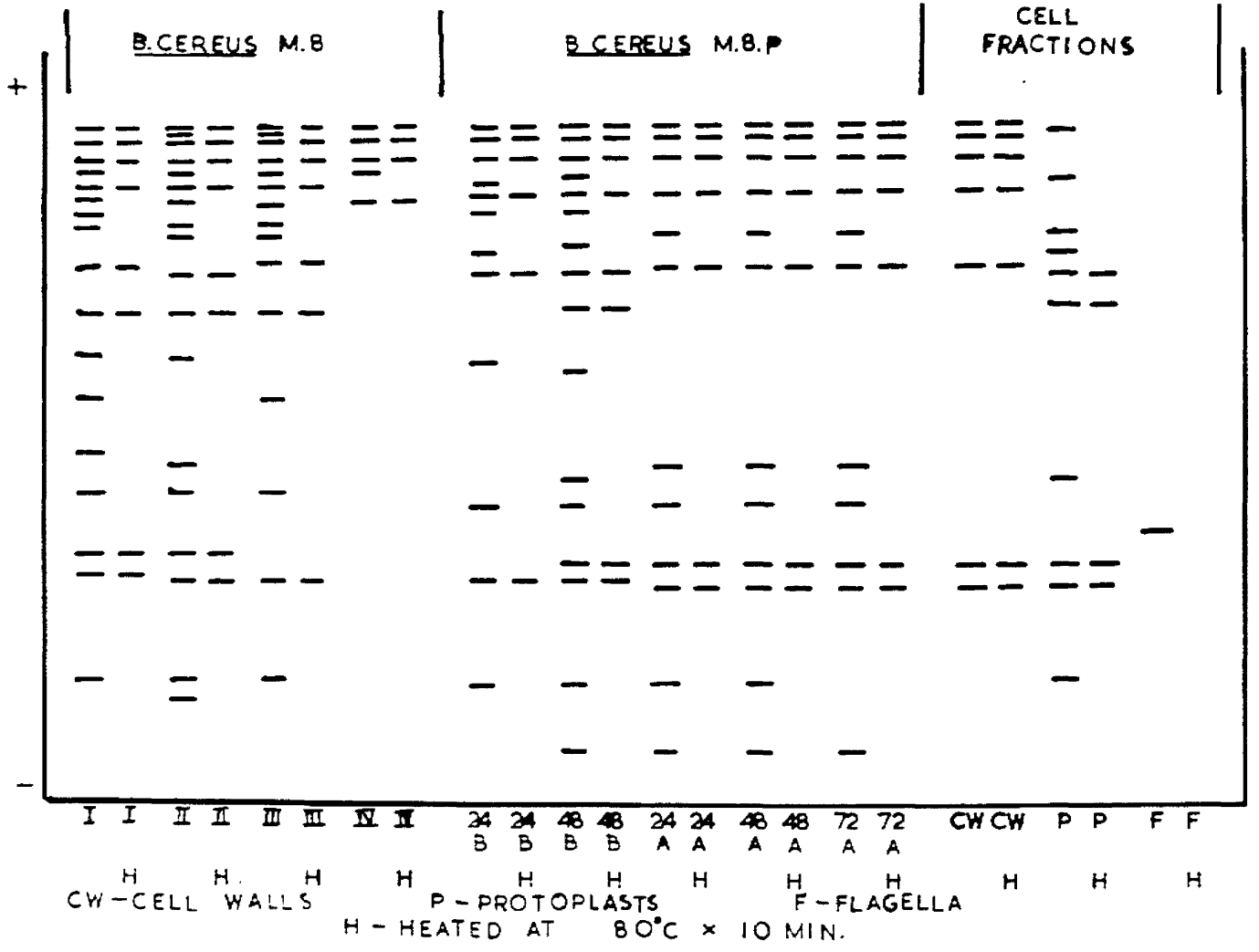


FIGURE 16

PROTEINS OF B.CEREUS



Protein patterns and heat resistance tests of the 5 growth stages of the phantom variant are summarised in the middle section of Figure 16.

3.10. PROTEINS OF CELL FRACTIONS

Preparations of cell walls, flagella, and protoplasts were investigated by electrophoresis in polyacrylamide gels in order to determine their protein components.

Results were again recorded by the method outlined in 3.8.

3.10.1. Cell Walls

Cell wall preparations contained 6 protein bands, all of which resisted heating at 80^{0}C for 10 minutes.

3.10.2. Protoplasts

10 protein bands were noted in protoplasts. Only 4 of these were thermostable.

3.10.3. Flagella

A single, heat labile protein band was detected in flagella.

Protein patterns of cell fractions are shown in the last part of Figure 16.

3.11. CORRELATION OF PROTEIN AND ANTIGEN PATTERNS

Resolution of constituents of cell extracts in 7% polyacrylamide gels was found to be much superior to that obtained in agar gels. For this reason, an attempt was made to correlate stained protein bands with lines of antigen-antibody precipitate. A technique combining electrophoretic separation in a 7% polyacrylamide gel

with double diffusion analysis in agar was evolved, and particulars of the method are given in 2.7.4.4. Although the results obtained by this procedure do not contribute substantially to the knowledge of antigens of B. cereus, a brief account of them is given here because it is felt that the technique is a potentially valuable one, and with further development it might yield interesting information. A typical example of immuno-analysis in polyacrylamide gel is shown in Plate 16.

3.11.1. Proteins and Antigens of Bacillus cereus M.8

Components of the 4 cell extracts of B. cereus M.8 were separated by electrophoresis in polyacrylamide gels. After slicing, one slice was stained to locate protein bands. A second slice was used for immuno-analysis (2.7.4.4.) Only 3 antisers, 1, 3, and 4 were employed.
In Stage I cells, at least 6 antigens were detected with Serum 1, 6 with Serum 3 and 4 with Serum 4.
Stage II cells possessed 11 antigens which reacted with Serum 1, 10 with Serum 3, and 5 with Serum 4.
10 antigens in Stage III cells formed precipitin lines with Serum 1, 12 with Serum 3, and 7 with Serum 4.
Extracts of mature spores showed 2 antigens when tested with Serum 1, 3 with Serum 3, and 5 with Serum 4.
A diagrammatic summary of proteins and antigens of B. cereus M.8 is shown in Figure 17.

3.11.2. Proteins and Antigens of Bacillus cereus M.8.P

Extracts of the 5 growth stages of B. cereus M.8.P were

submitted to electrophoresis in polyacrylamide gels prior to immunoanalysis as detailed in 2.7.4.4. Gels were developed with Sera 1, 3, and 4.

Extracts of agar grown cultures all contained 6 antigens detectable with Serum 3, and 4 with Serum 4. There was, however, a marked alteration from stage to stage in antigens reacting with Serum 1. P_{24A} cells showed 8 such antigens, but only 5 were present in P_{48A} cells, and 4 in P_{72A} cells.

P_{24B} cells possessed 8 antigens detected with Serum 1, 5 with Serum 3, and 4 with Serum 4.

P_{48B} cells showed 8 antigens with Serum 1, 7 with Serum 3, and 4 with Serum 4.

Proteins and antigens of B. cereus M.S.P are tabulated in Figure 18.

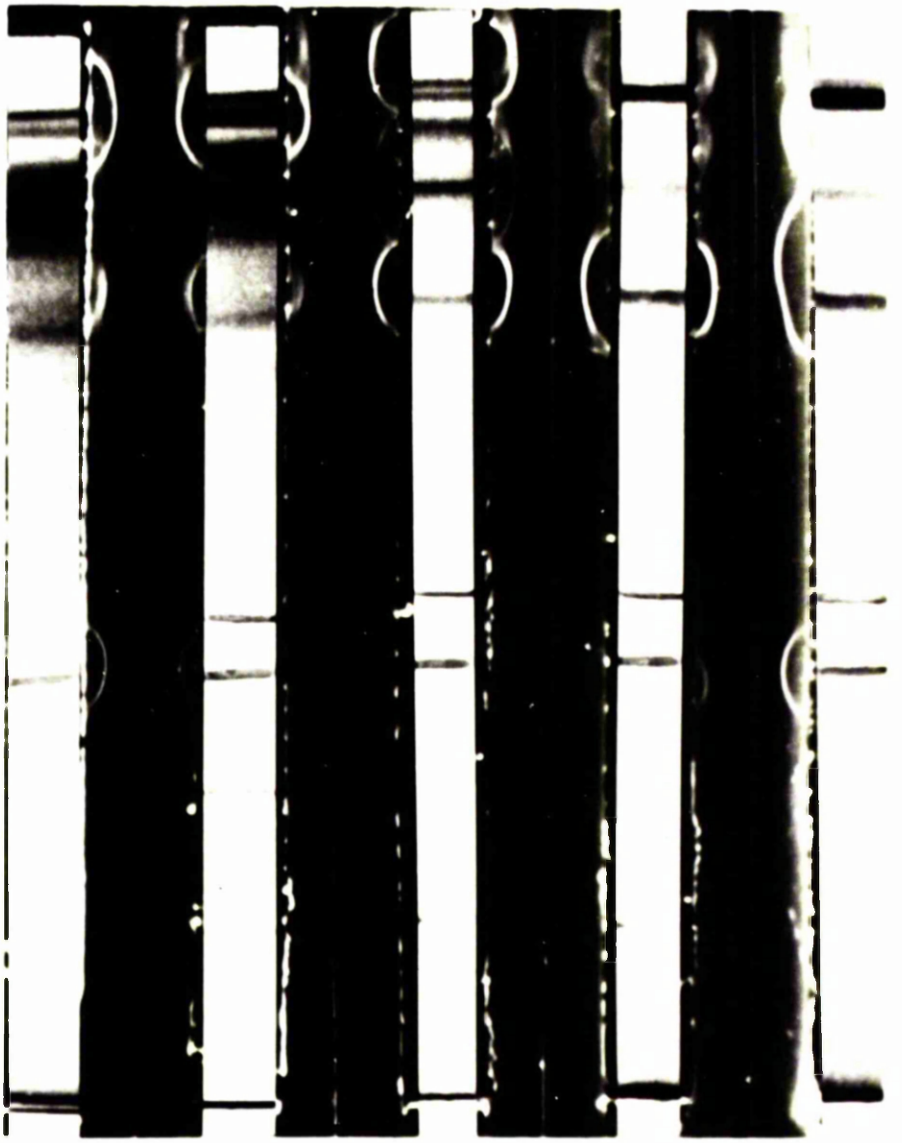
From the results outlined in the 2 foregoing paragraphs, it is evident that this method of immuno-analysis cannot be compared directly with the results obtained by immunoelectrophoresis in agar gels. Fewer antigen-antibody precipitin arcs were detected in polyacrylamide than in the corresponding preparations in agar gels. This cannot be explained by inadequate separation, since resolution in polyacrylamide gels is greater than in agar. Some antigens may not diffuse satisfactorily from the polyacrylamide into agar.

Note: It is possible to detect polysaccharide bands in polyacrylamide gels by the periodic acid-Schiff reaction

(2.7.4.3). 45 minutes exposure to sodium periodate is sufficient, but the gel slice has to be left in 1% pararosaniline hydrochloride for at least 16-24 hours before pink bands are visible. The exact timing of this staining reaction is still being investigated. For this reason, results so far obtained with this stain are not presented.

PLATE 16

IMMUNO-ANALYSIS IN POLYACRYLAMIDE



B. CEREUS M.B.P

SERUM 3

FIGURE 17

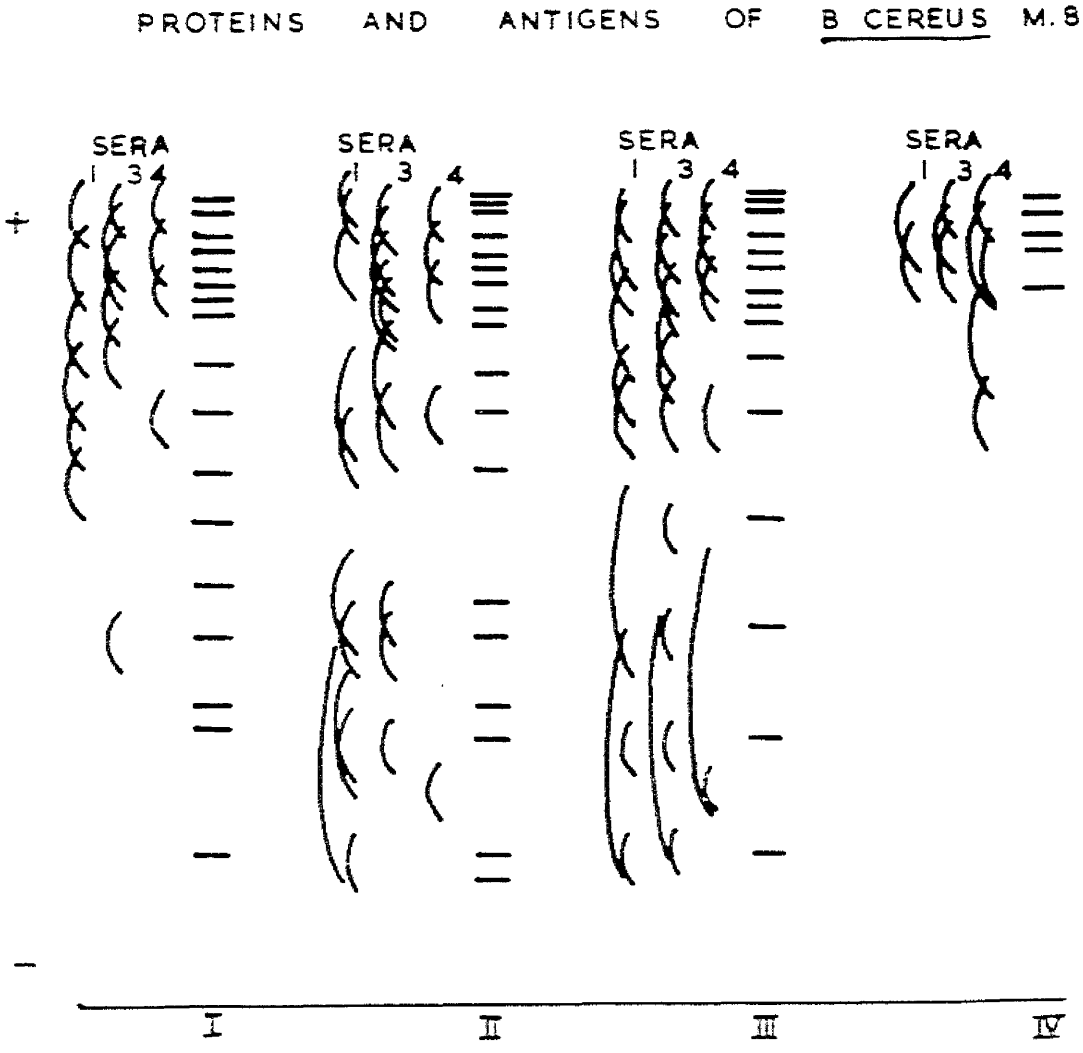
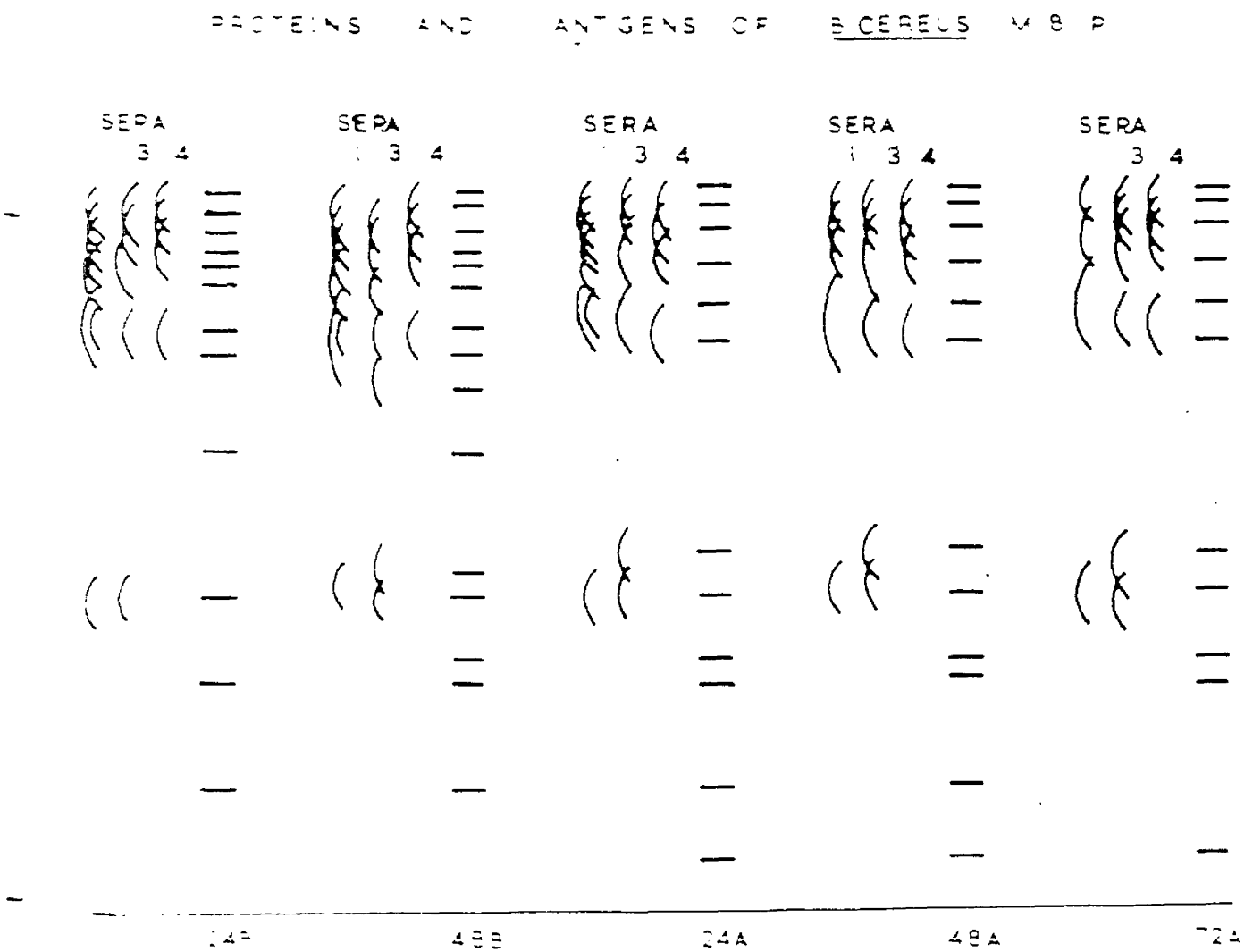

PROTEINS AND ANTIGENS OF B CEREUS M.8
SERA
1 3 4
SERA
1 3 4
SERA
1 3 4
SERA
1 3 4
+
−
I
II
III
IV

FIGURE 18


PROTEINS AND ANTIGENS OF B CEREUS M B P
SERA
3 4
SERA
3 4
SERA
3 4
SERA
3 4
SERA
3 4
24B
49B
24A
49A
72A

SECTION 4

DISCUSSION

4.1. INTRODUCTION

Bacterial endospore formation is a complex process which has been carefully studied by biochemists and cytologists. With its unusual stability to heat and disinfectants, the spore is an intriguing form of life, but the mechanism of its resistant properties remains largely unknown. Nor is the biological role of the spore understood. It can be of little value as a reproductive function as only one spore is normally produced per cell. Since spores are resistant cells, early bacteriologists decided that they were produced specifically to enable the organisms to survive unfavourable conditions such as depleted nutrients and toxic metabolic products. Although the properties of the spore must be a useful factor in survival, they are not essential. Many organisms, particularly those whose habitat is soil withstand unfavourable conditions without spores. The spore is however, well adapted for aerial dispersal.

In more recent times, evidence has been put forward to suggest that sporulation is controlled genetically, and involves the phenotypic manifestation of genetic determinants not otherwise expressed during growth. Such a process could involve an episome as the genetic determinant. Episomes are genetic elements which are not essential for the survival of the cell, but which can exist in 2 states - either as integrated units replicating with the bacterial chromosome, or autonomous units

replicating independently. Thus, due to some external stimulus such as a change in culture conditions, there might be a detachment of the episome giving rise to sporulation. Reintegration of the episome would take place on germination of the spore.

Whatever the stimulus for sporulation, the process by which vegetative cells produce spores is an exceedingly complex one, involving many drastic changes. One of the most notable physiological changes is the formation of dipicolinic acid, a compound absent from vegetative cells, but representing 10% of the dry weight of spores, (Halvorson, 1962). This substance appears to be closely associated with the heat resistance of the spore. The suggestion that a period of "de novo" protein synthesis is involved in the initial steps of sporulation is supported by the evidence that sporulation is paralleled by the utilisation of the free amino acid pool (Halvorson, 1962). The heat stability of the spore implies that many of its constituents, especially nucleic acids and enzymes, must have a higher resistance to thermal denaturation than is normal for such compounds. There must therefore be a higher degree of rigidity of molecular structure.

Differences in molecular components of vegetative cells and spores should be reflected by differences in antigenic make-up. By following the latter, it might be possible to throw some light on the molecular changes involved, and perhaps also on the origin of spore constituents.

These may be either vegetative cell constituents altered in some way so as to become heat resistant, or they may be synthesised "de novo" during the growth and maturation of the spore.

4.2. EXPERIMENTAL RESULTS

4.2.1. Methods of Study

Before considering the results obtained, some attention must be paid to methods of study. The antigenic distinction between spore and vegetative cell has been investigated almost exclusively by agglutination tests, or by precipitation reactions with crude chemical extracts of cells (see Review). Such techniques yield little information about the nature and site of the antigens involved. Agglutination concerns only antigens situated at or near the cell surface. Ultrasonic disintegrates of cells have a more complete antigen content than chemical extracts, and analysis of such disintegrates gives a better overall conception of the antigenic changes occurring within the sporulating cell. Such complex mixtures of cell components as ultrasonic disintegrates call for methods of study other than agglutination and precipitin ring tests. Double diffusion in agar gel does not give a high enough degree of resolution of the mixture. It was for this reason that the possibility of applying electrophoretic techniques to the problem was explored. Separation of the various antigens of an extract under

the influence of an electric field in agar gel prior to reaction with antibody made possible a more accurate enumeration of the antigens involved. Although agar is a suitable supporting medium for immunoelectrophoresis, it does not give as high a degree of resolution as do starch or polyacrylamide gels. These gels were employed for the electrophoretic separation of cell disintegrates in an effort to see whether the methods which have proved valuable in other fields could be turned to account in bacteriology.

4.2.2. Enzyme Changes

Two enzyme systems were followed by starch gel electrophoresis. Young vegetative cells possessed a single heat labile catalase. Stage II and Stage III cells showed the presence of an additional thermostable enzyme which persisted to become the sole catalase of the mature spore. Lawrence and Halvorson (1954) described a heat resistant catalase in intact spores of B. terminalis and Murrell (1955) found a similar enzyme in spore of B. subtilis. Both these enzymes lost their heat resistance on extraction from the spore by crushing. Sadoff (1961) reported the appearance of a thermostable catalase in cells of B. cereus during sporulation. The marked difference in heat resistance of these molecules could be explained in 2 ways. The 2 molecules may be entirely different, the spore catalase being a new molecular species with a higher intrinsic stability

synthesised during the process of spore formation, or else the vegetative cell catalase may become bound to other cell components during sporulation, thus acquiring a higher degree of structural rigidity. The fact that spore catalase retains its thermostability in aqueous extracts suggests that dehydration is not a major factor in heat stability.

In order to ascertain the relationship between the 2 molecular forms of catalase, their immunological specificities were investigated (3.4). This proved the 2 enzymes to be immunologically distinct. Sadoff (1961) partially purified vegetative cell and spore catalase of B. cereus and attempted to study the immunological specificity of the spore enzyme by immunodiffusion in cellulose acetate membrane, but failed to demonstrate catalase activity in the antigen-antibody complex. He did, however, find evidence from thermal inactivation and guanidine inhibition studies to suggest that there were definite differences between spore and vegetative cell catalases. The evidence, combined with a lack of antigenic relationship between the 2 catalases of B. cereus M.8 point to the existence of 2 structurally different forms of the enzyme in B. cereus. The spore catalase would thus appear to be quite distinct from the vegetative cell enzyme and to be elaborated within the cell during sporulation. The asporogenic variant B. cereus M.S.P possessed only the thermolabile catalase.

At no stage in growth was the spore catalase detected. This finding was not unexpected as the variant never progressed beyond the granular appearance of Stage II cells, before the cells became distorted and began to autolyse. Indeed the granular appearance may well be an initial stage in autolysis rather than the onset of spore formation. During spore formation, there are 2 distinct series of changes in the cell. One is connected with growth and sporulation, and the second with autolysis of the sporangium. Changes in esterase would appear to be of this second type, and seem to be unconnected with the formation of the spore in which esterases were not detected. Neither esterase of sporulating cells was notably thermoresistant (3.2). Broth cultures of the phantom variant contained both esterases, but agar cultures possessed only the b esterase. This might be explained by the fact that even 24 hour agar cultures appear physiologically older than 48 hour broth cultures. It is possible therefore, that in agar cultures, the esterase system is being destroyed by the autolytic process.

The 2 molecular forms of esterase were also examined immunoelectrophoretically and found to be antigenically distinct.

4.2.3. Antigen Changes

Changes in the antigenic content of cells at 4 growth stages were followed by immunoelectrophoretic analysis.

Antisera were prepared against each of these 4 stages by inoculation of rabbits with uncentrifuged disintegrates of the appropriate cells. At least 4 rabbits were used for each preparation and great variation in immunological response from animal to animal was noted. This variation appeared to depend on the antigen injected rather than the animal itself. Antiserum to Stage I cells was obtained after a primary series followed by a booster series. Stage II cells never stimulated the production of a really satisfactory serum in spite of repeated booster doses, and the results obtained with this serum (Serum 2) serve to substantiate those of the other sera rather than to yield fresh information. Injection of disintegrates of Stage III and Stage IV cells led to the death of animals during the primary or first booster series, apparently due to a shock reaction. To avoid this, animals being immunised with these antigens were inoculated intramuscularly in the hind leg. This gave rise to a satisfactory antiserum after the primary and first booster series.

Immunoelectrophoretic observations reveal a complex system of antigens in B. cerus. Some of these antigens are only synthesised at certain stages in the growth cycle, but some appear to be present at all times.

Greatest variation occurs in the components moving towards the cathode. These antigens appear in young cells, increase in number and intensity in Stages II and III, but are only seen faintly in mature spores. A second set

of slow-moving antigens, detectable only with antiserum to disintegrated spores, are first noted in Stage III cells and are then incorporated into the mature spore. The majority of fast-moving components detected in Stages I-III with antisera 1 and 3 are not found in mature spores.

4.2.3.1. Thermostable Antigens

Correlation of all the antigens from extract to extract was not feasible due to the multiplicity of precipitin lines. Since a time-temperature exposure of 10 minutes at 80°C proves lethal to vegetative cells of B. cereus but permits the survival of spores, it seems probable that antigens which resist this degree of heating may be of some importance in the survival of the spore.

7 thermostable antigens were detected in Stage I cells, 4 of which persisted throughout growth and sporulation and were found in mature spores. These 4 antigens, c, d, e, and f, were found to contain polysaccharide. The other 3 thermoresistant antigens of Stage I, a, b, and g, were present in late sporulating cells (Stage III), but were absent from spores. Antigen g has a polysaccharide component and therefore its thermoresistance is not surprising. On the other hand, antigens a and b failed to stain by the periodic acid-Schiff reaction. Both antigens possess a fairly high electrophoretic mobility. Proteins owe much of their heat lability to tertiary structure. Polypeptides and peptides have a less intricate make-up and are thus less susceptible to heat

denaturation. It seems possible that a and b might be small polypeptides or peptides.

2 antigens k and q were found only in cells in the later phase of sporulation and 5 (l, m, n, o, and p) appeared only in Stages III or IV. In all, 9 heat resistant antigens (c, d, e, f, l, m, n, o, and p) were found in mature spores, antigen p being the only one without a polysaccharide component.

The most striking overall fact which emerges from these antigenic studies is the marked increase in heat resistance of cell constituents as sporulation progresses. Of the antigens detected in Stage I cells, less than 50% resist heating at 80°C for 10 minutes. With mature spores, however, few of the antigens are thermolabile. Fewer antigens were detected in spore extracts than in vegetative cells, but it is apparent that most of them can withstand adverse conditions of temperature, even in aqueous extracts.

These findings imply that during sporulation 2 processes are occurring: "de novo" synthesis of spore constituents and also incorporation of vegetative cell material into the spore. Physiological evidence supports this view (Halvorson, 1962).

Cavallo, Falcone and Imperato (1963) appear to be the only workers who have carried out a comparable study of the detailed antigenic structure of spore-forming bacteria. They detected 3 antigens in ultrasonic extracts of

B. subtilis spores and demonstrated the appearance of additional antigens during spore germination. They did not find any of the antigens of resting spores in vegetative cells. Lund and Norris (1963) followed germination of B. cereus M.8 and their results substantiated those obtained with sporulation.

4.2.4. Location of Antigens within the Cell

After following the broad pattern of antigenic changes from vegetative cell to spore, the question arises - "are these antigens located in any particular cell structure?" In an endeavour to answer this question, cell walls, protoplasts and flagella were prepared from B. cereus. The cell fractionation procedures used appeared to give reasonably pure cell constituents, but the preliminary nature of this work must be emphasised.

4.2.4.1. Cell Walls

Cell wall preparations were found to contain 5 antigens, all of which were thermostable. 4 of these antigens gave a positive reaction with the periodic acid-Schiff stain, indicating the presence of a polysaccharide moiety. 3 of these antigens (c, d, and e) were detected in spore extracts as well as in extracts of vegetative and sporulating cells. Walls of Gram-positive organisms appear to be composed mainly of a mucocomplex consisting of a peptide and amino sugars (Salton, 1960). Up to 30% amino sugarhas been found in the walls of B. cereus (Salton, 1958). The antigens of isolated cell walls of

B. cereus M.8 show that there are at least 5 different fractions, one of which is a molecule of fairly high electrophoretic mobility and which does not possess a polysaccharide moiety. Of the 4 polysaccharide containing antigens, 3 are of high electrophoretic mobility while the fourth is a slower moving component. This difference in electrophoretic mobility presumably reflects a difference in molecular size. Antigen g is present in all vegetative and sporulating cells, but is absent from mature spores. Strange and Dark (1956) showed that spore coats of *B. cereus* contain a variety of amino acids in addition to characteristic components of the mucocomplex of the cell wall, and it is suggested that the main constituent of the coats was a structural protein. The cell wall antigens, c, d, and e which are present in the mature spore may well be the mucocomplex components of the cell wall which Strange and Dark detected in the spore coat. Ultrasonic treatment of the cells could split the link between these components and the structural protein, leaving the carbohydrate moiety free to migrate in an electric field.

4.2.4.2. Protoplasts

Protoplasts contained fewer antigens than vegetative cells, but appeared to share some antigens with cell walls (antigens c, d, e, and g). There are several possible explanations for this observation. Perhaps the most obvious is the use of a protoplast preparation which is contaminated with cell wall remnants due to inadequate

washing. In the preparation of protoplasts by the action of autolytic enzymes on cells suspended in buffered sucrose solution, only 60-70% conversion from rods to spherical forms was observed, and the protoplast and cell mixture was only washed twice in sucrose prior to the lysis of the protoplasts in distilled water. Under these circumstances it is possible that some cell wall material might be present in the final extract. Another factor to be taken into consideration is the mode of action of the autolytic enzymes. These cell wall lytic enzymes, obtained by autolysis of sporulating cells of B. cereus, attack vegetative cells and cell wall preparations releasing hexosamine-containing peptides (Strange & Dark, 1957). There would appear to be 2 distinct lytic systems in such autolysates. The authors suggest that enzyme V with a pH optimum of 4.5 is concerned with the release of free spores from the sporangia, and enzyme S, of pH optimum 8.0, with the lytic processes accompanying spore germination. The exact linkages attacked by these enzymes is unknown, but typical cell wall amino sugar-peptide complexes are released from the walls. It is possible, therefore, that the cell wall antigens present in the protoplasts may be part of the wall which remains unaffected by these enzymes. A third explanation could be advanced. The antigens c, d, and e, were detected in vegetative and sporulating cells, mature spores, cell walls and protoplasts. Since these antigens

contain polysaccharide and are of fairly high electrophoretic mobility, they may be hapten groups which are found in various sites in the cell. On treatment with ultrasonic vibration, such groups could be separated from a larger molecule to which they were attached.

4.2.4.3. Flagella

The flagellar preparation did not appear to contain any antigen when tested against the 4 antisera. Since flagella are protein in nature and certainly antigenic, this was an unexpected finding. In the past, immunological studies on flagella have used mainly agglutination or complement fixation tests. Although subjected to ultrasonic vibration, it is possible that the molecules were too large to diffuse into an agar gel. Alternatively, treatment with ultrasound might have altered the antigenicity of the molecule in some way. On the other hand, a protein band was detected in flagellar preparations by polyacrylamide gel electrophoresis indicating that the protein component of the organs was able to migrate in an electric field. This being so, failure to detect flagellar antigens could be explained by inadequate antisera. B. cereus M.8 is not an actively motile organism, and for the purpose of obtaining detached flagella, motility was stimulated by passage through 0.3% agar.

0.3% agar.

Antigens for the immunisation of rabbits were prepared in bulk before cell fractionation was contemplated, hence

the organisms used for inoculation contained few, if any, flagella. To circumnavigate this difficulty, a rabbit was immunised with a purified flagellar preparation, but in spite of a primary series followed by a booster series of injections, a satisfactory antibody response has not yet been attained.

From these preliminary results of cell fractionation, it can be seen that such methods hold a promise for tracing the origin of spore constituents.

The only comparable work is that of Vennes and Gerhardt (1959). They carried out a detailed antigenic study of isolated cellular constituents of B. megaterium by quantitative complement fixation tests. The structures examined were capsules, cell walls, protoplasts, protoplast membranes, flagella, intracellular granules and nuclear cores. Sera were prepared against each of these fractions. (This was not done with B. cereus M.8, but would be desirable for detailed study). The authors found that the structures were antigenically distinct, apart from a possible polysaccharide antigen in capsules and cell walls. Low cross-reaction between flagella and protoplasts was attributed to the presence of flagella in the protoplast preparation. Unfortunately, spores were not studied. The lack of antigenic relationship between cell walls and protoplasts is not in agreement with the results described for B. cereus M.8. This may be accounted for by the different methods of preparation of the protoplasts.

B. megaterium is sensitive to lysozyme and this enzyme was employed for the removal of cell walls. Lysozyme action is believed to result in complete dissolution of the walls of B. megaterium as examined microscopically, chemically, and immunochemically (McQuillen, 1960). Tomcsik and Guex-Holzer (1954 b) also demonstrated the antigenic distinction between cell walls and protoplasts of B. megaterium.

TABLE 6

Antigens of cell structures of Bacillus megaterium (modified from Vennes & Gerhardt, 1959)

ANTIGEN	REACTION OF ANTISERA TO										
	a	b	c	d	e	f	g	h	i	j	k
CONTROL CELLS	+	+	+	-	-	+	-	-	-	-	-
FLAGELLATE CELLS	+	+	+	-	+	+	-	-	-	-	-
CAPSULATED CELLS	+	+	+	-	-	+	-	-	-	-	-
CAPSULAR POLYPEP	+	+	+	-	-	-	-	-	-	-	-
FLAGELLA	-	+	-	-	+	-	-	-	-	-	-
CELL WALLS	+	+	+	-	-	+	-	-	-	-	-
C. WALL POLYPEP	+	+	+	-	-	+	+	-	-	-	-
C. WALL POLYSAC	+	+	+	-	-	+	-	-	-	-	-
PROTOPLASTS	+	+	+	-	-	-	-	-	+	+	+
PROT. MEMBRANES	+	+	+	-	-	-	-	-	+	+	-
GRANULES	+	+	+	-	-	-	-	-	+	-	+
NUCLEAR CORES	+				-	-	-		+	-	-

a control cells
b flagellated cells
c encapsulated cells
d capsular polypeptide
e flagella
f cell walls
g cell wall polypeptide
h cell wall polysaccharide
i protoplasts
j protoplast membranes
k intracellular granules

TABLE 7

Antigens of Bacillus cereus

PREPARATION	ANTIGEN															
	a	b	c	d	e	f	g	h	k	l	m	n	o	p	q
STAGE I	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
STAGE II	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-
STAGE III	+	+	+	+	+	+	+	-	+	-	+	+	+	-	+
STAGE IV	-	-	+	+	+	+	-	-	-	+	+	+	+	+	-
P_{24A}	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P_{48A}	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P_{72A}	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-
P_{24B}	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
P_{48B}	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
CELL WALLS	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-
PROTOPLASTS	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-
FLAGELLA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

When comparing the antigens of cell fractions of B. cereus M.8 with those of B. megaterium described by Vennes and Gerhardt (1959), differences in immunological technique must be taken into account. Vennes and Gerhardt used quantitative complement fixation tests. While this is a very sensitive method, it should be remembered that not all antigens may fix complement in an immune reaction. Hence some common antigens may not be detected by this procedure. Tabular representations of the results of Vennes and Gerhardt (1959) and those obtained with B. cereus M.8 are presented for comparison (Tables 6 and 7).

4.2.5. Bacillus cereus M.8.P

The salient pointemerging from antigen and enzyme studies with B. cereus M.8.P is that constituents peculiar to cells of Stages IIIand IV of the parent strain were never detected. Although both esterases were present, only the heat labile catalase was produced. Similar behaviour was noted with the antigens. The antigen pattern of the phantom variant was practically identical with that of Stage I cells. Antigen a was never present, and in P_{72A} cells, antigen b had disappeared. Neither of these antigens contain a polysaccharide moiety and it was suggested (4.2.4.1) that they might be peptide molecules. If this is the case, such molecules could be degraded during the autolysis of the cells.

Such an asporogenic variant is potentially valuable, as a careful comparison with the parent strain might point

the way to some of the key steps in sporulation.

4.2.6. Protein Patterns of Cells

Electrophoresis in polyacrylamide gels has shown the protein patterns of various stages of growth of B. cereus and of the cell fractions. Perhaps the most important point arising from these observations is that little difference was noted between cells of stages I, II, and III, although a marked difference was apparent in Stage IV cells.

This serves to illustrate the sensitivity of antigen-antibody reactions for the detection of molecular changes. The technique of polyacrylamide gel electrophoresis gives good resolution of the gross protein constituents of an extract. Those present in smaller amounts can only be detected by means of specific enzyme activity, or by antigen-antibody reaction. It was for this reason that a method for immuno-analysis in polyacrylamide gels was devised. The results are presented for B. cereus to show the possible value and application of the method rather than for the information they yield.

4.3. GENERAL DISCUSSION

The antigenicity of the spore has been known to bacteriologists since the beginning of the century. In spite of this, very little attention has been paid to the serology of the spore. The various difficulties encountered in work of this kind and their circumnavigation

has been detailed in 1.2.1. Defalle (1902) demonstrated that injection of rabbits with various species of aerobic spore-formers would give rise to antiserum against the spores. Mellon and Anderson (1919) showed that spores and vegetative cells of B. subtilis were antigenically distinct, provided that steps were taken to free spore suspensions of vegetative debris.

Growth of B. mesentericus on asparaginate agar was followed by agglutination of the cells with antiserum to spores and to bacilli (Howie & Cruickshank, 1940). Cultures of increasing age showed an increasing titre with antiserum to spore and a decreasing titre with antibacillary serum. The authors concluded that the spores were antigenically distinct from vegetative cells.

In a series of papers (see Review) Iamanna and his co-workers investigated the serology of spores of several members of the genus Bacillus. Agglutinin-absorption experiments with spores and vegetative cells verified the antigenic distinction between spores and vegetative cells. An acid-extracted spore precipitinogen was used to study the relationships between various species. Agglutination studies on spores were used by Walker (1959) and Norris and Wolf (1961) in taxonomic studies. Similar observations have been made on the anaerobic sporeformers. Prior to the work described in this thesis, only agglutination reactions with intact cells and spores or precipitation tests with crude chemical extracts had been

used. Because of the limited information gained by such methods, the applicability of electrophoretic techniques to the problem has been explored.

Immunoelectrophoresis in agar has demonstrated the complexity and behaviour of the antigens of ultrasonically disintegrated cells of B. cereus. Appearance of spore antigens and disappearance of those of the vegetative cell during sporulation has been followed. The only comparable study is that of Cavallo, Falcone, and Imperato (1963) and appeared during the course of the work detailed here. These authors followed antigenic changes during germination of B. subtilis spores using immunoelectrophoresis of ultrasonic disintegrates of spores and cells. None of the antigens of resting spores was detected in vegetative rods. This was not the case with B. cereus M.8, where 4 of the antigens were common to all growth stages. Fewer antigens were detected in B. subtilis than in B. cereus and this may be a reflection of the efficacy of the antiserum.

Results with electrophoresis in starch and polyacrylamide gels indicate that such techniques can be applied in the field of bacteriology. Polyacrylamide gels have several advantages over starch, but gross protein stains are not as sensitive as immunological reactions.

Looking to the future, there are many possible continuations of the lines of research opened here. Only 2 enzyme systems have been studied, namely esterase and catalase.

Many other enzyme systems can be detected and studied after electrophoretic separation. Some preliminary experiments on proteinases have shown promise. An extension of enzyme changes is therefore suggested, along with a study of their immunological specificity.

Cell fractionation is another aspect which should yield much information. Isolation of other cell structures, in particular the exosporium, spore coat and forespore would give a much clearer idea of the antigenic structure of the spore and the origin of its constituents.

Antigenic changes on the surface of Cl. sporogenes during sporulation and germination have been followed using the fluorescent antibody technique (Walker & Batty, 1963). Spore antiserum was coupled to fluorescein isothiocyanate and vegetative cell antiserum to Lissamine Rhodamine B 200. Antigenic changes were accompanied by a change in fluorescence. Use of specific antisera labelled with fluorescent dyes would enable rapid and clear location of antigens either microscopically or in antigen-antibody precipitates.

In conclusion, the main object of the work described in this thesis has been to outline the antigenic changes accompanying spore formation in Bacillus cereus M.8 and to explore the possible application of new techniques in bacteriology. Having made a series of broad general observations, the way is now open for a more detailed study.

SECTION 5

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