Bacterial Spore Antigens: a Review

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SUMMARY

Agglutinogens and precipitinogens have been detected in the spores of many species of aerobic and anaerobic spore-formers and these antigens are distinct from those of the vegetative growth phases of the corresponding organisms. Spore antigens are heat resistant and antibody response is elicited by the injection of autoclaved spores into rabbits. Injection of living spores leads to the development of antibodies reacting with vegetative cell antigens in addition to spore antibodies.

Agglutinogens tend to show subspecies distribution in some aerobic species but spore precipitinogens appear to be mainly species specific in aerobes. Less information is available about anaerobes where a similar pattern of specificity is suggested. Data about the antigenic structure of the spore and the antigenic changes accompanying sporulation and germination are scant; it is in these aspects of the subject that the most significant progress is to be anticipated.

INTRODUCTION

Do bacterial spores possess antigens distinct from those of the vegetative organism? If so, what is their taxonomic value? Do spores carry any of the antigens found in the vegetative forms? These questions have been the theme of much of the work on spore antigens and part of the present task will be to consider how far they have been answered—in short, to summarize our knowledge about the distribution of antigens between spore and vegetative form and of particular spore antigens among different spore-forming bacteria. Today interest is being focused on the detailed antigenic structure of the bacteria. Techniques for separating cells into their component parts and for studying antigens both in situ in the cells and in separated cell components are developing rapidly and are opening new approaches to the problems of cell analysis. By their very nature, spores would seem to be well suited to this type of study. We know very little indeed of the 'antigenic architecture' of spores or of the antigenic changes which accompany the formation and germination of spores. The appearance, in the last few years, of papers touching on these aspects of the subject suggests that the time is ripe for a thorough investigation and I hope that this review, by presenting the information available and suggesting possible lines of extension, may act as a stimulus for further work.

Technical problems; the pre-treatment of spore antigen

Observations on spore antigens date almost from the beginning of the century; some thirty papers have dealt directly with the subject. There are technical difficulties in work of this kind which stem from the nature of the spore itself and from
the mode of its production. The success of the different studies and the significance of the results obtained are largely reflections of the extent to which these difficulties have been overcome. The earliest paper dealing with spore antigens, that of Defalle (1902), which forms an admirable starting point for investigation of the subject, clearly shows that the author was aware of most of the technical problems which have since caused trouble.

The use of living spores as antigen. The use of viable spores for injection and for agglutination tests is open to criticism on several different accounts. Defalle (1902) suggested that living spores may germinate within the body of the animal after injection. This was subsequently shown to happen by several workers, notably Teale & Bach (1919) who found that spores of *Bacillus anthracis*, *B. mycoides* and *B. subtilis* germinated in large numbers when injected into animals. The resulting vegetative forms did not normally multiply or produce toxaemia but the possibility that these may stimulate the production of vegetative cell antibodies must be taken into account when the results of immunological experiments are being evaluated.

The agglutination test, in its usual form, involves a fairly long period of contact between antigen and antiserum at an elevated temperature and under these conditions living spores may well germinate—a change which is clearly undesirable when spore antigens are to be studied. Noble (1919) described a rapid method of performing the agglutination test in which shaking the reactants together at high concentration was followed by dilution. The results with a number of species compared well with those obtained by using standard techniques; Lamanna (1940a, 1940b), Lamanna & Eisler (1960), Lamanna & Jones (1961) and Norris & Wolf (1961) used a modification of Noble’s method to study the agglutination of viable spores. The method decreases the agglutination time to 30 min. and diminishes considerably the possibility of germination interfering with the result. Moussa (1956), working with anaerobic spore-formers, had little success with this technique.

Auto-agglutination of spores. The surface of the bacterial spore appears to be strongly hydrophobic (Lamanna & Eisler, 1960) and several workers have noted that spores of certain strains tend to agglutinate spontaneously when suspended in saline. Noble (1927) showed that when a suspension of vegetative *Bacillus anthracis* was shaken vigorously for 12 hr. and then allowed to stand overnight, coarse aggregates of bacteria settled out, leaving a homogeneous suspension which could be used for agglutination. Norris & Wolf (1961) used a similar method for preparing spore suspensions for agglutination. Nevertheless, some spore suspensions, even when treated by Noble’s method, show almost immediate clumping in the controls and it has been impossible to study a certain small proportion of these organisms by agglutination techniques. The addition of 5 mg. bovine serum albumin/ml. to the diluent to decrease spontaneous agglutination and the tendency for spores to be trapped in a meniscus was advocated by Lamanna & Jones (1961).

Contamination of spores with vegetative cell material

The most important factor which has caused confusion in the study of the antigenic structure of the spore has been the use of impure material for injection and agglutination. Growth on normal laboratory media rarely results in anything like 100% sporulation with the aerobic spore-formers; with the anaerobes the situation is even less satisfactory. Suspensions of old cultures must inevitably contain con-
Spore antigens

Siderable amounts of material derived from vegetative cells. Several workers have injected such preparations and have obtained antisera containing a mixture of spore and vegetative cell antibodies. There appear to be two approaches to this problem: the production of cultures consisting solely of spores or the active removal of vegetative cell debris.

Complete sporulation. Howie & Cruickshank (1940) described an asparagine agar medium which gave complete sporulation of an organism resembling Bacillus cereus and of B. mesentericus. Injection of a suspension of such a culture resulted in the production of antibodies specific for the spore stage and the antisera did not agglutinate vegetative cells. Occasionally complete sporulation has been claimed with more conventional media (Davies, 1951).

Active removal of vegetative debris. Mellon & Anderson (1919) used 'antiformin' to effect the lysis of vegetative cells and KOH was used by Krauskopf & McCoy (1937), Schweinsberg (1951) and Schlossberger (1951) for the same purpose. Antiformin consists of equal volumes of liq. sod. chlorinat. (B.P.; an aqueous solution of sodium hypochlorite containing 2-5-3-0 % available chlorine) and a solution of caustic soda (150 g./l.). The use of such vigorous agents is open to criticism, since they will almost certainly cause changes in the serological properties of the spores, paralleling the drastic chemical effects which are readily observed. This has led several workers to seek gentler methods of removing vegetative debris while leaving the spores intact and viable.

Delpy & Chamsy (1949) used autolysis in the presence of thiomersalate to remove vegetative material from old cultures of Bacillus anthracis and found that the spores remained viable and antigenically unchanged. Norris & Wolf (1961) used this method with considerable success for the purification of spores of several Bacillus species; it was not successful with all species. Norris (1957) described a bacteriolytic principle derived from B. cereus which effectively lysed the vegetative cells of all species of aerobic spore-forming bacteria against which it was tested. Spores of these organisms were not affected by the lytic principle and antigenically 'clean' spores were readily obtained. Walker (1959) successfully used lysozyme to remove vegetative debris from spore suspensions of thermophile aerobic spore-formers. Pancreatic extract was used by Moussa (1956, 1959) with some success to free spores of Clostridium septicum and C. botulinum from vegetative cell remnants. Meisel & Rymkiewicz (1957, 1958a, b, 1959) used purified preparations of lysozyme, deoxyribonuclease and trypsin to remove vegetative material from spores of C. tetani and other Clostridium species but, although this treatment prevented spore suspensions from being agglutinated by antisera produced against vegetative cells, vegetative cell remnants could still be seen under the microscope and the injection of enzymically purified spores yielded antisera containing both spore and bacillary antibodies. B. megaterium is highly sensitive to lysis by lysozyme and Tomesik & Baumann-Grace (1959) experienced no difficulty in using this reagent to free B. megaterium spores from vegetative cell remnants.

The nature of the spore antigen and its relationship to the antigens of the vegetative cell

The first observations on the antigenicity of bacterial endospores appear to be those of Defalle (1902) who injected old agar cultures of Bacillus mycoides, 'B. mesentericus vulgatus', B. subtilis, B. alvei and two attenuated strains of the anthrax...
bacillus into rabbits and tested the resulting antisera by slide agglutination. The cultures which he injected contained few, if any, vegetative forms. Antiserum produced against _B. mycoides_ spores agglutinated viable and autoclaved spores as did a serum prepared by injecting autoclaved spores. Vegetative cells gave rise to no spore agglutinins on injection although antibodies which agglutinated the bacilli were readily produced. Spore antisera showed some ability to agglutinate vegetative cells, a fact which the author attributed to the presence of vegetative debris in the suspensions used for injection.

Chimera (1916) found no evidence for the production of specific agglutinins within ten days of the inoculation of spores of _Bacillus anthracis_ and _B. subtilis_ into rabbits. Agglutinins active against bacilli had no action on the spores.

Mellon & Anderson (1919) injected two types of antigen in an investigation of the serological relationships between spores and vegetative cells of _Bacillus subtilis_. Their spore antigen was a formolized suspension of an old culture on nutrient agar; a formolized suspension of vegetative cells constituted their bacillary antigen. The resulting antisera showed complete cross-reaction, each agglutinating both types of antigen. Antiformin was then used to dissolve vegetative material from the spore suspension. After exposure for 18 hr. to this agent the vegetative material had lysed and many of the spores had lost the ability to retain the spore stain. The resulting preparation, after washing, was agglutinated by the spore antiserum but not by the bacillary antiserum, indicating that the cross-reactions had been due to the presence of bacillary remnants in the spore suspensions rather than to the sharing of common antigens by the two growth phases; this was confirmed by cross-absorption experiments.

Krauskopf & McCoy (1937) attempted to demonstrate the presence of bacillary H antigen in spores of _Bacillus niger_ (presumably _B. subtilis_ var. _niger_). Two types of antigen were injected: whole vegetative cells; spore suspensions treated with KOH to dissolve any vegetative material which may have been present. Both of the resulting antisera agglutinated bacillary O antigen, whole vegetative cells, KOH-treated spores and untreated spores (the latter suspension almost certainly containing much vegetative cell material). Absorption experiments gave complex results which are difficult to analyse. The main facts to emerge were: (a) KOH-treated spores absorbed antibodies specific for themselves from both antisera: (b) whole vegetative cells apparently absorbed all of the antibodies from the treated spore antiserum. The main conclusion—that the spore contained no specific antigen—would seem to be unwarranted in view of the fact that no antiserum to normal untreated spores was examined. Ferry & Fisher (1924) showed that organisms dissolved in antiformin or NaOH still retained their antigenicity and Professor J. W. Howie (personal communication) found that KOH did not remove vegetative antigen from spore suspensions of Krauskopf & McCoy's organism which normally contained large amounts of vegetative cell material. It is not clear from Krauskopf & McCoy's paper how thoroughly their injection preparations were washed; inadequate removal of dissolved vegetative material might have been responsible for some of their results.

Howie & Cruickshank (1940) reported an investigation of the antigenic properties of spores of aerobic and anaerobic bacteria. Antisera prepared against old cultures of _Clostridium sporogenes_ contained both spore and bacillary agglutinins. H and O
bacillary antibodies could be absorbed by the corresponding antigens leaving intact specific spore agglutinins. An organism resembling *Bacillus cereus* spored completely on asparagine agar and gave rise, on injection, to a specific spore antiserum. *B. mesentericus* (probably *B. pumilus* or *B. subtilis*) behaved similarly and with this organism it was shown that autoclaving did not destroy the spore agglutinogens.

Lamanna (1940a, b) injected old agar cultures of aerobic spore-formers into rabbits and demonstrated agglutinins specific for the spore stage in the resulting antisera by differentially absorbing out bacillary antibodies. Later Lamanna (1942) used an acid extraction method to obtain precipitating antigens from spores of *Bacillus subtilis* and *B. vulgatus* and used them to clarify the relationship between these two organisms.

Bekker (1944) prepared antisera to living and autoclaved spores of *Bacillus anthracis, B. mesentericus* and *B. ubiquitarius* and to H and O vegetative antigens of the same organisms. The spore antisera in general showed high agglutinin titres for the homologous antigens and low anti-bacillary titres. Living spores of *B. mesentericus*, however, gave rise, on injection, to considerable amounts of vegetative H antibody which the author attributed either to vegetative material in the spore suspension or to germination occurring after injection. Autoclaved *B. mesentericus* spores gave no such H antibody response. In no case was the antigenicity of the spores affected by autoclaving.

In an extensive investigation involving *Bacillus cereus, B. subtilis, B. vulgatus, B. agri, B. brevis* and *B. sphaericus* Doak & Lamanna (1948) showed that treatment of spores with KOH brought about changes in their serological properties. In addition to a high titre of spore agglutinins, antisera to viable spores of *B. cereus* also showed a small amount of bacillary antibody which was attributed to the presence of vegetative debris in the spore suspension. After treatment with KOH a similar spore preparation on injection gave bacillary antibodies in amounts comparable with those of the spore agglutinins. The authors concluded that the spore had antigenic components in common with the vegetative cell but that vigorous treatment was needed to expose them. Prolonged injection of living vegetative cells gave rise to the production of small amounts of spore agglutinins but the authors’ conclusion, that this indicated the presence of spore antigens in the vegetative cells, should perhaps be regarded with caution; the possibility of spores being present in small numbers in the injection material needs to be taken into account. Absorption experiments indicated that the antigenic structure of the spore was complex; one strain of *B. cereus* had at least three surface antigens. Both spores and vegetative cells induced the formation of precipitins and these were absorbed simultaneously with the agglutinins. In two cases agglutinins were absorbed by precipitinogens.

Schlossberger (1951) injected KOH-treated spores of *Bacillus sphaericus*, following Doak & Lamanna’s (1948) method of purification, but found vegetative cell antibodies as well as spore antibodies in the resulting antisera. A bacillary O antiserum agglutinated KOH-treated spores and treated spores absorbed O agglutinins from antisera prepared against vegetative cell O antigen. O and H agglutinins were absorbed from spore antisera and antibody specific for the spore stage could then be demonstrated.

Schweinsberg (1951) used KOH to remove vegetative cell material from spore suspensions of *Bacillus sphaericus*. Spore suspensions were incubated at 37°
for 6 hr. with m-KOH and then washed three times. Spore antiserum agglutinated KOH-treated spores and also bacillary H and O antigens and the author concluded that spores contained H and O antigens identical with those of the bacilli while vegetative cells contained only minimal amounts of spore antigen. Schweinsberg’s own results, however, show that KOH treatment did not inactivate bacillary antigens (especially not the O antigen); the period of washing may well have left residual antigenic material adsorbed to the spore surface. Indeed in this case it is unnecessary to postulate adsorption; B. sphaericus spores carry a legacy from the vegetative growth phase in the form of a tenacious sporangium wall remnant which envelops the spore and may well carry antigens derived from the vegetative cell.

Davies (1951) claimed that old nutrient agar cultures of Bacillus polymyxa consisted entirely of spores and used such material for the production of antisera. Injection of living spores resulted in the formation of high titre H agglutinins in addition to spore antibodies. This was not the case when heat-killed spores were injected and heated material was used throughout for the production of spore antisera. Bacillary O and H antigens and spore antigens were quite distinct showing no cross agglutination.

Norris & Wolf (1961) showed heat-resistant agglutinogens and precipitinogens in the spores of each of twelve Bacillus species and found them to be completely independent of the vegetative cell antigens. With four organisms (Bacillus cereus, B. subtilis, B. licheniformis, B. alvei) an examination of the relationship between spore and vegetative cell antigens was made. In the first three of these species the spore contained no vegetative antigens as surface components, but B. alvei spore suspensions did contain H antigen, the presence of which might have been associated with the persistence of the wall of the sporangium as an exterior membrane around the spore in this species. Injection of autoclaved spores did not result in the formation of vegetative cell antibodies with any of the twelve species studied. The effect of injecting viable spores was investigated with B. cereus and B. subtilis and in both cases vegetative H antibodies appeared along with spore antibodies in the resulting antisera. Spore suspensions of these two organisms were not agglutinated by specific H antisera prepared against their vegetative cells and the spores were incapable of absorbing H agglutinins from vegetative cell antisera. The spore antigens in general were not destroyed by autoclaving at 120° for 20 min. and autoclaved spore suspensions were used in the preparation of antisera for taxonomic purposes. Formamide extracts of the spores showed the presence of precipitinogens which reacted strongly with the specific antisera prepared against autoclaved spores. In many instances with both agglutinating and precipitating antigens evidence was obtained of the multiplicity of spore antigens.

Norris (unpublished work) has used gel diffusion techniques to study spore antigens of Bacillus cereus, and of crystal-producing B. cereus variants such as B. cereus var. alesti and has found one precipitinogen common to spores of all of these organisms. Several strains show more than one precipitinogen. A detailed study of B. cereus var. alesti, although showing several different antigens both in the spores and the vegetative cells, showed no sharing of antigens at all between the two growth phases.

Studies of anaerobic organisms have so far been fragmentary as compared with the work on aerobic species. Starin & Dack (1923) injected viable spores of Clostri-
&'pore antigens into rabbits and obtained a serum which showed no ability to agglutinate vegetative cells of this organism. The serum was not apparently tested against a spore suspension. Moussa (1956, 1959) studied the spore and vegetative cell agglutinogens of *C. septicum* and *C. chauvoei*. No attempt was made to remove bacillary material from the spores before injection but spores for agglutination were incubated for 6 hr. with pancreatic extract—a process which removed most of the vegetative debris but did not impair the antigenicity of the spores. The spores possessed agglutinogens distinct from those of the vegetative cells and the two species shared a common spore antigen. Injection of viable spores resulted in the formation of antibodies reacting with vegetative H and O antigens; this was largely prevented by autoclaving the spores before injection. No attempt was made to study precipitinogens with these organisms.

Sussman (1959) worked with *Clostridium histolyticum* and *C. sporogenes* and showed these organisms to share a spore agglutinogen. Antisera prepared against vegetative cells did not agglutinate spores and antisera prepared by injecting autoclaved spores contained, in addition to spore agglutinins, vegetative cell O agglutinins but no vegetative cell H antibody. Precipitinogens were not studied. Mandia (1955) studied the antigenic structure of *C. tetani*, *C. sporogenes*, *C. parabotulinum* and *C. histolyticum* and noted a heat resistant antigen shared by these species. Although Mandia did not suggest that this antigen is associated with the spore, the antigen preparations he used certainly contained spores and Sussman (1959) suggested that Mandia’s heat-resistant agglutinogen may have been a spore antigen.

In a series of publications since 1957, Meisel & Rymkiewicz have extended our knowledge of the spore antigens of anaerobes and have applied their findings to taxonomic problems. These authors (Meisel & Rymkiewicz, 1957) demonstrated spore agglutinogens in *Clostridium tetani*, but found that antisera produced by injecting spore suspensions contained agglutinins active against H and O antigens of the vegetative phase, even when the spores were heated ‘to the limit of thermo-resistance’ before injection. Trypsin, lysozyme and deoxyribonuclease were used to free spores from the antigens of vegetative cells; but the majority of the spores were seen microscopically still to be retained within vegetative cell remnants. Antisera prepared against such enzyme-treated spores still agglutinated vegetative cell antigens as well as heated and enzyme-treated spores. Anti-H and anti-O sera, however, did not agglutinate enzyme-treated spores although they would agglutinate heated spores. The authors concluded on a basis of these findings that vegetative cell H and O antigens are present within spores of *C. tetani*. Similar results were obtained with *C. sporogenes* (Meisel & Rymkiewicz, 1958a) and it was shown that enzyme-treated spores gave rise to H and O antibodies even when these spores were autoclaved before injection, so that the result could not be explained by the germination of spores within the animal body. The same pattern of behaviour was later demonstrated in *C. histolyticum* and *C. botulinum*. The authors summarize their findings as follows ‘...while anti-H and anti-O sera no longer agglutinated enzyme-treated spore-containing suspensions, immunization with such enzyme-treated spores was followed by the formation of two types of antibodies, those reacting with the spore antigens and those reacting with the vegetative cell H and O antigens’ (Meisel & Rymkiewicz, 1958b). While studying the kappa toxin of *C.
welchii, Meisel, Alelrycht & Rymkiewicz (1959) obtained the collagenase—which was neutralized by anti-welchii serum but not by anti-histolyticum serum or normal horse serum—in extracts of enzymically ‘purified’ spores.

The value of spore antigens in taxonomy

Taxonomy of the aerobic spore-formers. The classical work of Smith, Gordon & Clark (1952), while bringing order into the classification of the genus Bacillus, has led to the suppression of many species names. The interpretation of some of the earlier work on spore antigens is difficult since incorrectly labelled or inadequately described species were frequently used. Nevertheless, one cannot help but be impressed by the degree of consistency between the findings of different workers when these are analysed in the light of changing nomenclature and present-day taxonomy.

Defalle (1902) reported cross agglutination between spores of Bacillus mycoides and the anthrax bacillus. A B. mycoides serum gave high titre agglutination with spores of B. mycoides, B. subtilis and the anthrax bacillus, and low titre agglutination with those of ‘B. mesentericus vulgatus’ and B. alvei. By using the complement-fixation technique cross-reactions were demonstrated between spores of B. mycoides, ‘B. mesentericus vulgatus’ and B. subtilis. B. mycoides and the anthrax bacillus are now known to be closely related to one another and the finding that they possess a spore antigen in common is not surprising. Neither is the cross-reaction between B. mycoides and B. subtilis when it is realized that the strain of B. subtilis concerned was almost certainly one of the ‘large-celled’ (Michigan) strains, later shown by Conn (1930) to be B. cereus. ‘B. mesentericus vulgatus’ would appear to have been B. licheniformis, B. pumilus or a true B. subtilis and the B. alvei used was probably correctly named. Thus the picture emerging from Defalle’s work is not at variance with more recent ideas on classification.

The first extensive taxonomic work involving the use of spore antigens was that of Lamanna (1940a, b) who used a shaking method based on that of Noble (1919) to study spore agglutinogens. With the ‘small celled’ species Lamanna was able to demonstrate three clear-cut serological groups: a Bacillus subtilis group, a B. mesentericus group and a B. agri group. Included in the B. mesentericus group was B. vulgatus Flugge. Lamanna’s B. subtilis was the Ford strain now known as B. licheniformis (Gibson, 1937, 1944). His B. vulgatus was the true (Marburg) strain of B. subtilis and his B. agri is now recognized as B. brevis. B. mesentericus appears to have been a B. pumilus and the cross-reaction between B. pumilus and B. subtilis serves to emphasize the close relationship which exists between these two species as judged from their morphological and physiological characteristics and is in agreement with the later findings of Norris & Wolf (1961). Less success attended the investigation of ‘large celled’ species. Bacillus cereus could be separated from other large-celled organisms and some strains of B. mycoides (which is now considered to be a variety of B. cereus) were shown to cross-react with this species. Later Lamanna prepared acid extracts from spores of the Marburg and Ford strains of Bacillus subtilis and from B. vulgatus and showed them to give precipitin reactions with the homologous spore antiserum. Extracts from the Marburg strain and from B. vulgatus cross-reacted with one another, thus supporting the conclusion, arrived at by a study of their biochemical behaviour, that these two organisms were identical.
and that the Ford strain was a distinct species (Lamanna, 1942). The Marburg strain is now recognized as the true *B. subtilis* and the Ford strain is re-named *B. licheniformis* (Gibson, 1937, 1944). Bekker (1944) studied only a few strains of *B. anthracis*, *B. mesentericus* and *B. ubiquitarius* but found spore agglutinogens to be species specific in his limited series of organisms. *B. mesentericus* in this case was probably *P. pumilus*. *B. ubiquitarius* cannot be identified.

Shattock (1955) quoted a personal communication from Davies & Proom to the effect that spore antigens are species specific in several species of aerobic spore-formers; Chu (1951) used spore agglutination reactions with some success to distinguish between *B. cereus* and the anthrax bacillus. These species had antigens in common but cross-absorption enabled useful diagnostic antisera to be produced.

Doak & Lamanna (1948) found cross agglutination between *B. cereus*, *B. brevis* and *B. sphaericus* spores and between those of *B. brevis* and *B. agri*, the latter organisms probably being identical.

The work of Davies with *Bacillus polymyxa* (1951) did much to focus attention on the taxonomic possibilities of spore antigens. Spores of all of the thirty-nine strains of *B. polymyxa* investigated were agglutinated by the seven spore antisera prepared and were capable of absorbing all of the homologous agglutinins. No cross-reactions occurred with the spores of fifteen other *Bacillus* species comprising eighty-one different organisms. Precipitinogens were not studied in this investigation.

Norris & Wolf (1961) studied twelve different *Bacillus* species in an attempt to extend the work of Davies with *B. polymyxa* and to assess the taxonomic value of spore and vegetative cell antigens in aerobic spore-formers. With some organisms, for example, *B. subtilis* and *B. licheniformis*, the spore agglutinogens proved to be species specific, being represented in each of a considerable number of strains of the species. But this was not the case with other species. For instance, with *B. cereus* an antiserum to autoclaved spores of one strain agglutinated spores of only seven of twenty-seven different strains. Spore precipitinogens on the other hand proved generally to be species specific. *B. cereus*, *B. megaterium*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. coagulans*, *B. polymyxa*, *B. circulans*, *B. alvei*, *B. brevis*, *B. laterosporus* and *B. sphaericus* each possessed a spore precipitinogen characteristic of each and every strain of the species studied. Spores of *B. pumilus* contained at least two agglutinogens one of which was shared with *B. subtilis*, thus tending to confirm the very close relationship between these two organisms suggested by their physiological properties. Faint cross-reactions involving precipitinogens were observed between spores of *B. cereus* and *B. megaterium*, *B. subtilis* and *B. pumilus*, *B. pumilus* and *B. coagulans*, and between *B. alvei* and *B. sphaericus*, but in each case these reactions were easily distinguished from the strong specific reactions. Spore extracts of *B. circulans* and antisera prepared against autoclaved spores of *B. circulans* showed a wide tendency to cross-precipitation with spore extracts and antisera of representatives of the other species; but, again, these reactions were readily distinguished from the strong reaction seen with strains of the homologous species.

Walker (1959) studied thermophilic *Bacillus* species and recognized three groups based on physiological characteristics. His group 1 could be subdivided into two clearly related but not identical biochemical types. He was able to show spore agglutinogens in groups 1a, 1b, and 2 but injection of spore material of representa-
tives of his group 3 led to the death of experimental animals and spores of these organisms could not be studied antigenically. The spore antigens of group 1a proved to be group specific, that is to say they were represented in all the strains studied; but this was not so with the corresponding antigens of group 1b, where evidence of at least two spore agglutinogens was obtained. A small proportion of group 1b strains was agglutinated by group 1a antisera. Spore agglutinogens of group 2 proved to be group specific and were not represented in members of the other groups. In spite of several attempts by different methods Walker was unable to extract precipitinogens from the spores of any of these thermophilic aerobic bacteria. He was also unable to demonstrate any cross-reaction between thermophilic and mesophilic members of the genus Bacillus.

Lamanna & Eisler (1960) used spore agglutinogens to attempt to distinguish between Bacillus anthracis and B. cereus but were unable to do so. Antisera prepared against washed spores and absorbed with the homologous vegetative cells showed widespread cross-reactions when tested for ability to agglutinate spores of several strains of the two organisms. Spore precipitinogens received only a cursory examination, but here also spore extracts from the two organisms reacted with antisera to both. Agglutination and agglutinin absorption tests were used by Lamanna & Jones (1961) to study the relationship between B. cereus, B. cereus-like insect pathogens and B. anthracis. Extensive cross-agglutination occurred and these organisms could not be distinguished from each other serologically. My own unpublished observations agree with these findings.

Dowdle & Hansen (1961) studied a phage-fluorescent antiphage staining system for identifying the anthrax bacillus and included some observations with fluorescent spore antibodies. Fluorescent staining of spores was obtained when a Bacillus anthracis spore antiserum was tested against all of twenty-nine strains of B. anthracis, thirty-three of thirty-six B. cereus including ten mycoides types, three of three B. thuringiensis, one of six B. licheniforms, one of seven B. megaterium, and two of thirteen B. subtilis. These results accord well with those of other workers but suggest a somewhat wider distribution of anthrax spore surface antigen among the various species.

**Taxonomy of the anaerobic spore-formers.** The classification of the anaerobic spore-formers, especially of the non-pathogenic species, still presents problems and the study of clostridial spore antigens is complicated by the technical difficulty of obtaining satisfactory spore yields. Studies with such antigens are as yet few, but the findings strongly suggest that, as with the aerobes, they may be of great value to the taxonomist. Moussa (1956, 1959) demonstrated a common spore agglutinin in Clostridium septicum and C. chauvei and Sussman (1959) made a similar observation with C. histolyticum and C. sporogenes. Meisel & Rymkiewicz (1959) investigated the applicability of spore agglutination reactions to the classification of various Clostridium species. With thirty strains of C. sporogenes, C. histolyticum, C. botulinum types A and B, C. sordelli, C. bifermentans and C. tetani, they showed species specific agglutinogens in each case and also detected minor antigens. The only instance of inter-species cross-agglutination occurred between C. sporogenes and C. histolyticum, a finding which agrees with that of Sussman (1959). The only studies involving clostridial spore precipitinogens appear to be the preliminary observations of Dr P. D. Walker (personal communication) who has compared the agglutinating
and precipitin reactions of several spore antisera. He has found that precipitinogens can readily be extracted from spores of anaerobes and that they show a pattern of species specificity in their distribution.

The antigenic architecture of the spore

The use of classical serological techniques of the kind discussed above provides the investigator with little information about the site and chemical nature of the antigens studied. Agglutinogens may be assumed to be surface components of the cell but the chemically-extractable precipitating antigens (almost certainly polysaccharide hapten in the extracted form) may be surface or deep seated components. Recently methods have been developed for studying in greater detail the distribution of antigens in different parts of the cell. A few attempts have been made to apply these techniques to the problems of antigen composition of spores. Tomesik (1956) observed bacteria suspended in specific antisera under the phase-contrast microscope and obtained a great deal of information about the distribution of antigens at the cell surface and in particular cell components. Fowler & Harrison (1953) first noted that when spores of *Bacillus subtilis* reacted with specific agglutinating antisera at dilutions such that pre-zoning occurred an 'exudate' could be seen around the periphery of the spores under the phase-contrast microscope. Tomesik & Baumann-Grace (1959) have taken this observation further with *B. megaterium*. By the use of lysozyme, pure spore suspensions were produced and spore antisera were prepared which agglutinated the homologous antigen. By phase-contrast specific antigen-antibody reactions could be seen around spores suspended in spore antiserum. Wet Indian ink preparations demonstrated a layer of viscous material surrounding spores of several strains and it was apparently antigens associated with this layer which were responsible for the observed reaction. Antisera prepared by injecting vegetative cell antigens failed to elicit this reaction. The authors referred to the slimy layer as the 'exosporium' and to the reaction as the 'specific exosporium reaction'. However, the term exosporium has been used loosely in the literature and should be reserved for the discrete membrane surrounding free-lying spores of certain species—particularly *B. cereus* and its varieties (Norris & Watson, 1960). Empty spore walls and incompletely germinated spores gave a specific 'spore coat' reaction analogous to the cell-wall reaction seen with vegetative cells in the presence of specific antisera. Agglutination tests revealed a complex cross-reaction pattern with spores of different strains of *B. megaterium*. There were no species specific spore agglutinogens and thirty-six strains could be divided into five antigenic types. Spore antisera did not react at all with vegetative cells.

Cherry & Freeman (1959) prepared antisera against encapsulated vegetative cells of the anthrax bacillus and found that these antisera gave a precipitin reaction with supernatant fluids from cultures of capsulated and non-capsulated strains. Spores resisted disintegration by freezing and thawing and by the use of a homogenizer. But they could be disintegrated by thorough shaking with glass beads, when precipitating antigen was released into the supernatant fluid and could be detected by antiserum prepared against capsulated organisms. It should be noted that the materials used for injection almost certainly contained small numbers of spores. The authors prepared fluorescent antibodies and used them primarily to detect bacillary antigen in cultures of the anthrax bacillus and in tissue sections.
from infected animals, and from human anthrax cases. The main reactions observed were associated with capsular antigens. Intracellular spores did not take up fluorescent antibody but in some cases slight staining of the periphery of free-lying spores was observed. In some instances it was clearly shown that the spores retained on their surface capsular material of bacillary origin. Capsular staining was not given by fluorescent spore antiserum.

Vennes & Gerhardt (1959) carried out detailed studies of the antigenic characteristics of isolated structures and surface components of *Bacillus megaterium* and proposed to extend this work to include the spores of this species. In a brief note (Vennes & Gerhardt, 1957) these authors reported that they were unable to demonstrate common antigens between spore coats and vegetative cells in *B. megaterium*. The use of separated spore coats excluded the possibility of spores germinating on injection in these experiments.

**Antigen changes at spore-formation and germination**

Only one published observation refers to the antigenic changes associated with the passage of the cell from vegetative to spore phase or vice versa. Howie & Cruickshank (1940) tested spores of an organism similar to *Bacillus cereus* at different stages in germination, against spore and vegetative cell antisera. Spore agglutinogens decreased sharply during germination and bacillary antigens appeared.

Gel diffusion analysis and immuno-electrophoresis have enabled J. R. Norris & R. Przyborowski (unpublished observations) to show antigenic changes during sporulation of *B. cereus* and *B. cereus* var. *alesti* which show a pattern of behaviour more complicated than might have been assumed from a study restricted to agglutinogens. Both stages are antigenically complex, with several antigens readily demonstrable in both the vegetative and spore phases. The disappearance of bacillary antigens and the simultaneous appearance of spore antigens are clearly seen when cells at different stages in spore-formation are tested against vegetative cell, sporulating cell and spore antisera. Spore antigen appears first in a readily extractable form but becomes more intractable as the spore assumes its mature state.

**Conclusions**

That the bacterial endospore possesses antigens which are not represented in the vegetative growth phase is now well established; heat-stable agglutinogens and precipitinogens have been demonstrated as specific spore antigens in a wide variety of aerobic and anaerobic species, and indeed, considering the striking changes in morphology and chemical constitution which accompany sporulation, it would be surprising if there were not corresponding changes in the antigenic composition of the cell. It is more difficult to determine whether or not spores also contain antigens characteristic of vegetative cells. Injection of spores, living or dead, usually results in the formation of antibodies which react with bacillary antigens as well as specific spore antibodies, but the former must often result from the presence of vegetative debris in the spore preparations. Observations based on the use of spore suspensions from which bacillary material has been removed by chemical or enzymic treatments must be interpreted with caution since processes which result in the visual removal of cell remnants often do not inactivate the associated antigens. When we consider
the anatomy of mature spores we find that they are of three types: (i) naked spores where the spore escapes completely from the disintegrating sporangium; (ii) spores which escape from the sporangium but are completely surrounded by a membranous exosporium which is synthesized within the sporangium at sporulation so that the outer surface of the mature spore is no longer part of the spore wall proper; (iii) spores which remain permanently encased within the wall of the sporangium. Organisms of the first type (such as *Bacillus subtilis* and *B. megaterium*) show a clear-cut morphological break between the spore and vegetative phases; this is also true of *B. cereus* where the exosporium is a true 'spore structure' absent from young vegetative cells. Organisms of the third type, however (such as *B. alvei*, *B. sphaericus* and *Clostridium tetani*), show no such sharp distinction between the two phases and such information as we possess suggests that there is a corresponding carry-over of surface bacillary antigens into the spore stage of these organisms.

It would be reasonable to expect that the spore protoplasm would contain antigens or antigen precursors associated with the vegetative cell but claims that this has been demonstrated appear, on careful scrutiny, to be open to question and no definite conclusions can safely be drawn. Unequivocal proof seems most likely to come from an exhaustive analysis of spore and bacillary components of the type planned by Vennes & Gerhardt (1959).

Knaysi (1948), reviewing the state of knowledge of the bacterial endospore, concluded that 'In view of the sad state of the taxonomy of the spore-formers it is hoped that further study of the spore antigens will prove helpful in developing a sound knowledge of this interesting and important group of bacteria'. Since then order has come to the genus *Bacillus*, largely as a result of the work of Smith *et al.* (1952) in the U.S.A. and of Knight & Proom (1950) and of Proom & Knight (1955) in Britain and there remain relatively few taxonomic problems connected with the aerobic spore-formers. A few organisms are of uncertain status and some strains appear to be intermediate in type between recognized species, for instance, between *B. cereus* and *B. megaterium* and between *B. alvei* and *B. circulans*. It has been suggested that non-pathogenic strains of *B. cereus* can, by serial passage under appropriate conditions, be converted into pathogenic strains of crystal-forming insect pathogens (LeCorroller, 1958; Toumanoff, 1956). Norris & Wolf (1961) studied the spore precipitinogens of a few intermediate *Bacillus* types and were able to allocate them to definite species and it seems likely that a study of spore antigens could help in the solution of problems of this kind.

From the point of view of taxonomy it is with the anaerobes that spore antigens seem most likely to make valuable contributions. Progress is likely to be governed by the development of suitable media and methods for spore production which is often poor with the clostridia. Nevertheless, interesting results have come from the few studies so far reported. The anaerobic spore-formers offer a promising field for research into the nature and taxonomic significance of spore antigens.

The resistance of bacterial spores to drying and to degrees of heat rapidly fatal to vegetative cells is of great practical importance. But the resistance of spores, their dormancy and their ability to germinate (sometimes in a matter of seconds) when exposed to suitable conditions are also characteristics of fundamental biological importance. Much attention is being directed today towards the elucidation of the details of spore structure and of the processes of spore formation and germination.
Naturally enough cytologists and the biochemists are the most active participants in this work and recent symposia (Halvorson, 1957; Jacobs & Clegg, 1957) consist almost entirely of their contributions and bear witness to the extensive advances which are being made. The peculiar properties of the spore, however, are likely to be reflected in the nature of the proteins and other antigenic materials of which it is composed and a more detailed knowledge of these antigens and of their synthesis is eminently desirable. Serological techniques are extremely sensitive and their application to the investigation of spore structure could well be rewarding from several points of view. I hope that this review, by collecting together the information already available and suggesting lines along which the subject might profitably be developed, may stimulate research and contribute towards a better understanding of spore-forming bacteria and of the spores which they produce.

REFERENCES


Spore antigens 407


