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STUDIES ON A CORTEXLESS MUTANT OF
BACILLUS CEREUS var. *ALESTI*

by

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and
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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
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ABSTRACT

A stage 4 sporulation mutant of a strain of *Bacillus cereus* var. *alesti* fails to synthesise a cortex although all other structural components appear normal. With terminal lysis the spore core as well as the sporangium is lysed. Both the uptake of ^{45}Ca and the synthesis of dipicolinic acid (DPA) are similar to these activities in the parent strain, but these components (DPA and Ca) are lost to the medium with the drastic lysis. The first stage of diaminopimelic acid incorporation, that into germ cell wall mucopeptide, is intact in the mutant; the second stage, that into cortical mucopeptide, is absent. Soluble mucopeptide precursors are accumulated in the mutant at the time of normal cortex formation. These biochemical studies, as well as phospholipid metabolism and freeze etch analysis demonstrate that the lesion lies in the outer forespore membrane. The precise biochemical block in the mutant was not determined, although there is some indication that the mutant cannot form amounts of lipid intermediates comparable to those in the parent.

In vitro preparations of the parent strain show a difference in sensitivity to methicillin, but not to vancomycin or bacitracin, of the cortex synthesising

system compared to that of cell wall mucopeptide. The mucopeptide product of the *in vitro* cortical system is sensitive to lysozyme whereas the products of both cell wall and germ cell wall systems are more resistant.

Refractility as indicated by light microscopy, electron microscopy of thin sections, and freeze fracture etching was increased and maintained in this cortexless mutant, A(-)1, by the addition during sporulation stage 4 of actinomycin D, which prevents the terminal lysis of spore core associated with sporulation in this organism. ^{45}Calcium uptake levels and DPA content were similarly maintained. The location of these components appears to be in the spore protoplast. In the parent A(-), treated with actinomycin D during stage 4, spore particles with similar morphology to the mutant, that is without a cortex and with the characteristics of refractility, were obtained. A major difference in sensitivity to actinomycin D between the processes of ^{45}Ca uptake and DPA synthesis was observed. Some heat resistance in A(-) made cortexless by actinomycin D could be observed. These studies indicate that the role of the cortex is not to produce the dehydrated refractile spore state but to maintain it.

I. INTRODUCTION

Sporulation in bacteria has been intensely studied for many decades. The morphological sequence of events in the formation of the mature spore has been clarified; the chemical changes occurring during sporogenesis have been elucidated and the composition of major spore components determined. One of the predominant features of the spore is the wide, poorly staining spore cortex, composed mainly of mucopeptide which is basically similar to that of cell wall, yet has some unique modifications. Unlike synthesis of cell wall mucopeptide, biosynthesis of cortical mucopeptide has not been worked out. The cortex has been shown to be essential for the production of stable, resting spores. However, its precise role in sporogenesis is not clear, although it has been implicated, together with calcium or calcium dipicolinate, in the dehydration of the spore core, and consequently in the production of refractility and heat resistance.

The characterisation of a cortexless mutant undertaken in this study, should help in the understanding of the process of cortical synthesis and should lead to greater clarification of the role of the cortex in the bacterial spore.

II. HISTORICAL REVIEW

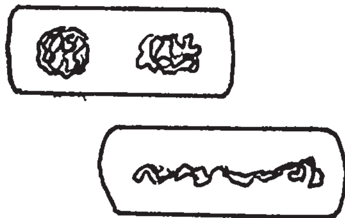
The Sporulation Process

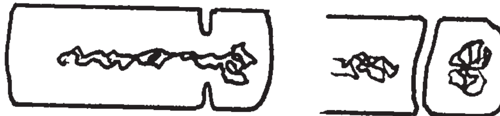
Studies on the morphology of bacterial spores and on the sporulation process to 1959 have been comprehensively reviewed by Robinow (1960). The sequential morphological changes occurring during sporulation in *Bacillus cereus* have been further elucidated, and correlated with some of the major physiological and chemical changes occurring at this time (Young and Fitz-James, 1959a,b; 1962). The description given by these authors is generally valid for a large number of species including *Bacillus subtilis* (Ryter, 1965; Ryter *et al.*, 1966), *Bacillus coagulans*(Ohye and Murrell, 1962) and *Clostridium histolyticum* (Bayen *et al.*; 1967). Although the changes during sporulation are continuous, for convenience of discussion, the sporulation sequence has been divided into seven stages (Fitz-James, 1965; Schaeffer *et al.*, 1965). These are shown in diagram 1.

Briefly, after vegetative growth ceases, there is an initial aggregation of nuclear material into an axial filament (Stage 1). Inward growth of the plasma membrane forms the forespore septum near one end of the cell (Stage 2). The forespore membrane continues to grow,

1. NUCLEAR CHANGES
AGGREGATION
AXIAL FILAMENT

2. FORESPORE SEPTUM
ENCLOSING SPORE NUCLEOID

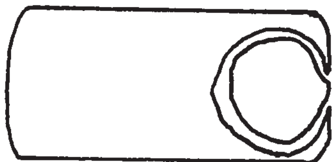


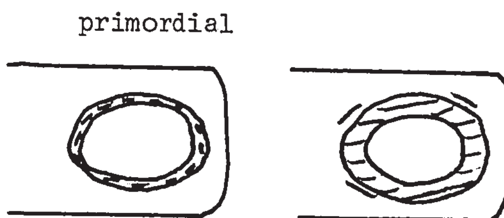


3. FORESPORE COMPLETION
(ENGULFMENT)

4. CORTEX FORMATION

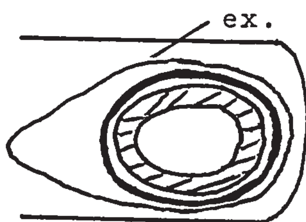
primordial





DAP uptake

5. COAT FORMATION



6. REFRACTILITY
PROCESS

7.LYTIC ENZYME

heat resistance

spore liberation

cysteine
uptake

Ca uptake and
DPA synthesis

Diagram 1

The stages of spore formation (Fitz-James, 1965)

eventually engulfing the forespore cytoplasm in a double membrane system within the sporangium (Stage 3). During Stage 4, mucopeptide is laid down between the double membrane, initially in a dense profile followed by a wider, less dense cortical zone. The forespore appears phase dark at this stage.

In Stage 5, coat deposition and exosporium formation, and completion of cortex synthesis occur. Calcium uptake and dipicolinic acid (DPA) synthesis begin. The forespore becomes phase white .

Concurrent with the continuing uptake of calcium and DPA synthesis in Stage 6, the refractility reaction occurs; the spores become phase bright; in electron micrographs, the spore core is seen to be featureless and difficult to stain. Heat resistance is acquired. Finally, a lytic enzyme releases the mature spore from the sporangium (Stage 7).

A detailed review of the morphology of sporulation with discussion of parasporal inclusions, asporogenous mutants, and the effects of inhibitors on the sporulation process has recently appeared (Fitz-James and Young, 1969).

In the present study, attention was focussed on the synthesis and role of the cortex.

Chemical Composition of the Cortex

The cortex, the wide poorly staining zone first described in resting spores by Robinow (1953), consists largely of an insoluble mucopeptide polymer similar but not identical to that of vegetative cell walls. Recent reviews on the chemistry of bacterial spores include

detailed sections on the composition of the cortex (Murrell, 1967; 1969). Basically, the cortical mucopeptide has the structure shown below:

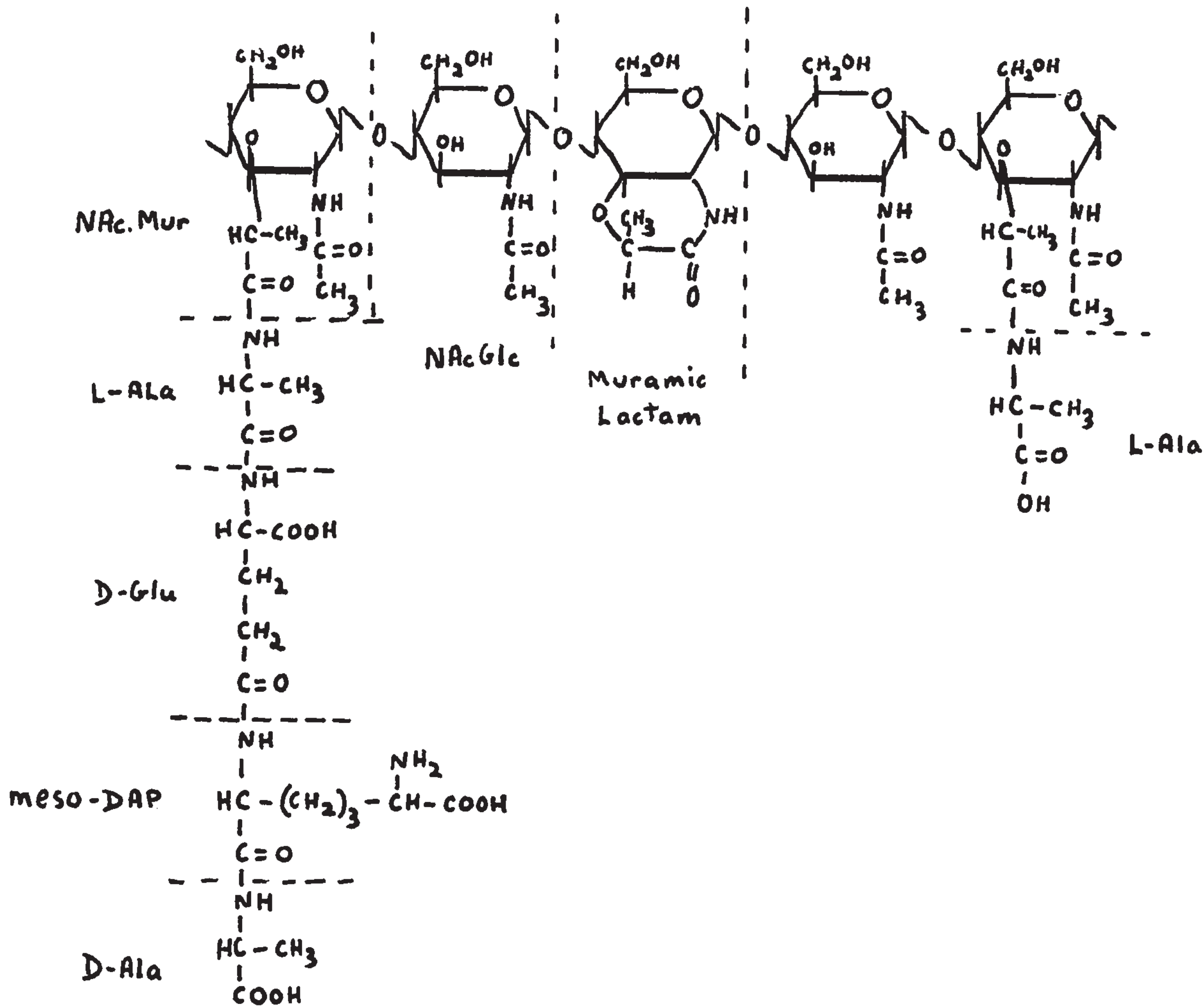


Diagram 2

Hypothetical average unit of cortical mucopeptide.
(After Murrell, 1969).

Initial evidence for the composition of the cortical mucopeptide came from studies on the muropeptides (repeating units of the mucopeptide polymer) found in "germination exudates" (Powell and Strange, 1953; Strange and Powell, 1954). These germination muropeptides were similar to those found in disrupted spores, as is expected since spore lytic enzyme breaks up the cortex during germination. They were mainly non-dialysable and had a molecular weight of approximately 15,300 (Record and Grinstead, 1954). The fact that in some preparations, the peptides were partially dialysable and had lowered viscosity was suggested by Strange and Powell (1954) to be due to differences in chain length caused by greater depolymerisation.

Analysis of non-dialysable fractions of many Bacillus species showed that the major constituents, acetylhexosamine, alanine, α, ε-diaminopimelic acid (DAP), and glutamic acid occurred in the mole ratio of 6-8: 3: 1:1, (Warth *et al.*, 1963b; Murrell and Warth, 1965). Warth (1965) determined in detail the composition of *B. coagulans* non-diffusible muropeptide, and found glucosamine, muramic acid, L-alanine, D-alanine, D-glutamate and meso-DAP in the mole ratio of 2.8:3:1.6: 1.0:1.0:1.0.

Cross-linking of muropeptides to form the three dimensional mucopeptide polymer occurs by the joining of the free amino group of DAP to a carboxyl group in a

neighbouring chain, usually that of the penultimate alanine. As about 80% of the amino groups of DAP and 3.8 carboxyl groups per polymer unit are free in *B. coagulans*, the cortical polymer must be loosely cross-linked and have a considerable excess of carboxyl groups (Warth, 1965).

As well as a lower amount of cross-linking, further differences between the spore and cell wall polymers have been documented (Warth and Strominger, 1969). The major ones are: (i) the occurrence of a unique spore component, the lactam ring (see diagram 2) on a large number of muramic residues (55% in *B. coagulans*), thus limiting the extent of cross-linking possible in spore mucopeptide, and producing a more plastic structure. All spores examined have this lactam component. (ii) Many muramic residues are substituted by L-alanine only, while in cell wall mucopeptide all residues are substituted by peptides. (iii) All spore mucopeptides yet studied have the same undifferentiated tetrapeptide originally isolated from *Edcherichia coli* (Primosigh *et al.*, 1961), whereas many modifications of this structure occur in cell wall mucopeptide especially in gram-positive bacteria.

Significant amounts of the other major cell wall polymer, teichoic acid, have not been found associated with the cortex, as demonstrated by the low

phosphorus content of insoluble cortical material (Warth, 1965; Strange and Powell, 1954).

Studies on muropeptides either from germination exudates, smashed spores or lysozyme-digested preparations of cortical integuments do not give any information on substances possibly occurring *in vivo* within the cortex, but which are released on its disruption (Warth *et al.*, 1963a,b).

Studies on the inner germ cell wall layer found attached to isolated cortical integuments indicate that it also has a mucopeptide composition. Digestion of integuments from *B. subtilis, B. coagulans,* and *B. stearothermophilus* by lysozyme removed this layer as well as the cortex indicating a composition similar to cortex (Warth *et al.,* 1963a,b). In *B. cereus* strains the germ cell wall was resistant to lysozyme. However, the vegetative cell wall of this organism is likewise resistant. Ferritin-labelled antibodies to cell wall react with this germ cell wall layer (Walker *et al.*, 1966). Vinter (1965) has demonstrated that during the first period of uptake of labelled DAP into *B. cereus* NCIB 8122, the DAP is incorporated into mucopeptide which remains intact during germination, that is, into germ cell wall mucopeptide rather than the cortex.

No work has yet appeared on the precise mode of biosynthesis of cortical mucopeptide. Tipper and

Pratt (1970) have demonstrated the synthesis of a new spore enzyme responsible for addition of DAP to the growing cortical mucopeptide precursor in *B. sphaericus*. This organism is unusual in that its cortex contains the typical DAP residue in its peptide side chains, whereas the vegetative cell wall contains lysine. Thus, a special enzyme for cortex synthesis is a necessity in the *B. sphaericus* spore system.

The chemistry of the intermediates of cortex formation and the enzymes involved is yet to be worked out. However, the system for cell wall mucopeptide synthesis has been extensively investigated. Presumably cortical synthesis is a basically similar process since in both systems, precursors must pass through a membrane barrier before polymerisation. Study of this system may indicate ways of approaching the problem of cortical synthesis.

Cell Wall Mucopeptide Biosynthesis

This process has been studied most extensively, mainly by Strominger and co-workers, in *Staphylococcus aureus* and *Micrococcus lysodeikticus*, but it is essentially identical in many other species except for species-specific modifications to the peptide side chain.

The basic pathway is summarised in diagram 3 below.

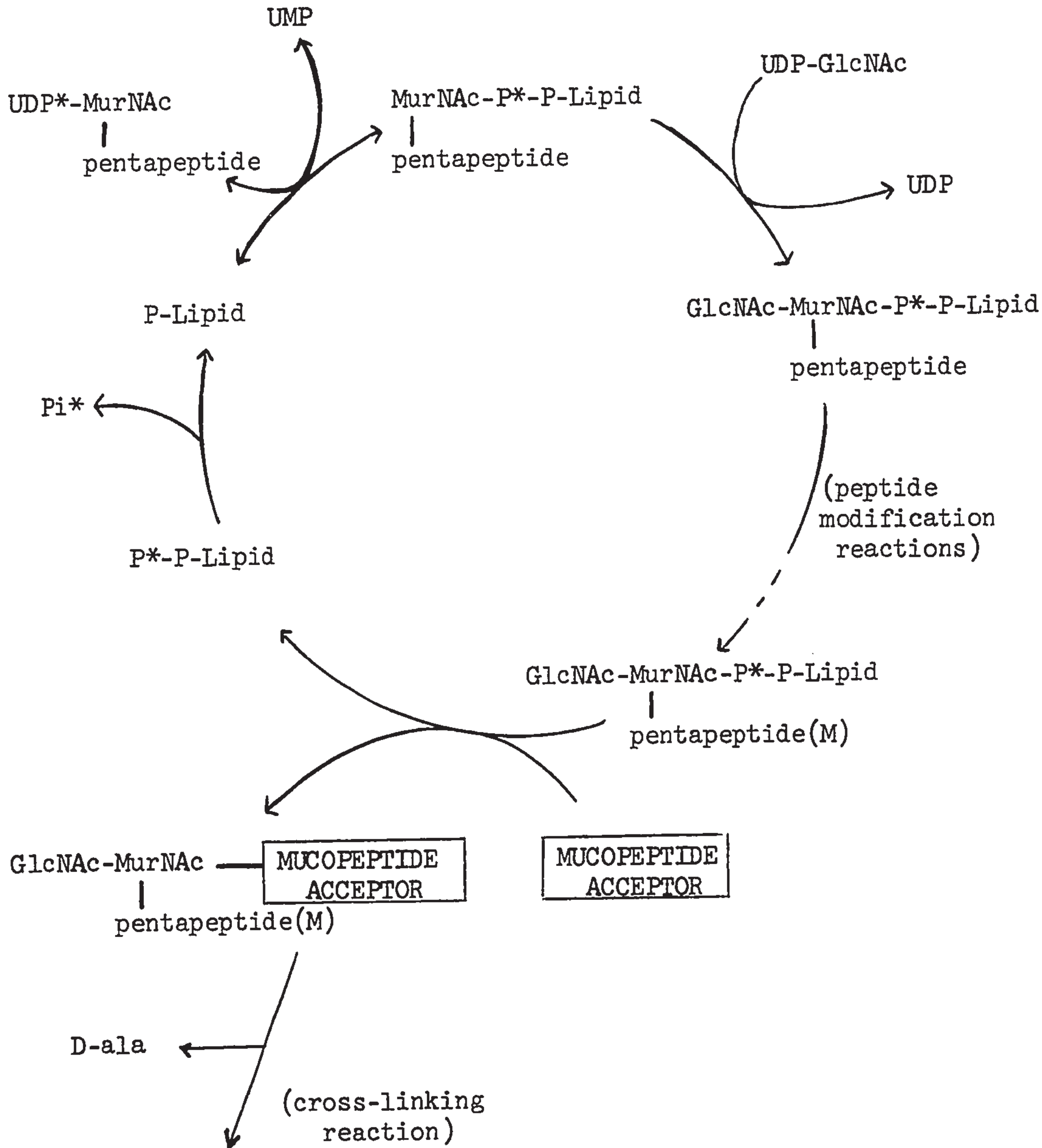


Diagram 3

Sequence of reactions leading to synthesis of peptidoglycan. Pentapeptide (M) represents the pentapeptide after the various modification reactions. P* represents the phosphate group that originates from UDP-Mur-NAc-pentapeptide. (Modified from Rothfield and Romeo, 1971).

In 1964, the first direct evidence that uridine diphospho-acetyl muramic pentapeptide (UDP-MurNAc-pentapeptide) and uridine diphosphoacetyl glucosamine (UDP-GlcNAc) are precursors of cell wall mucopeptide, was obtained by Chatterjee and Park (1964) and Meadow *et al.*, (1964), using fragments of cell envelope of *S. aureus*. Struve and Neuhaus (1965) produced evidence that MurNAc-pentapeptide was first transferred from its UDP derivative to an intermediate acceptor with release of UMP, and that addition of GlcNAc occurred at a later stage.

Direct evidence for this intermediate acceptor, its lipid-soluble nature, and the sequential formation of lipid-phospho-acetyl muramylpentapeptide and lipid-phospho-disaccharide pentapeptide was presented by Anderson *et al.*, (1965, 1967). The carrier lipid in peptidoglycan synthesis has been identified as a derivative of a C_{55} polyisoprenoid alcohol, and linkage of the glycosyl group of the intermediates to the lipid through a pyrophosphate bridge was established, (Higashi *et al.*, 1967).

Modifications of the peptide side chain occur while the disaccharide-pentapeptide is still linked to the carrier lipid. In *S. aureus*, this involves addition of a pentaglycine chain which later becomes the bridge cross-linking peptide chains in the fully formed mucopeptide polymer (Matsuhashi *et al.*, 1965). L-serine

and glycine are incorporated in *S. epidermidis* (Petit *et al.*, 1968); L-threonine in *Micrococcus roseus* (Roberts *et al.*, 1968). Modifications of the glutamyl residue may also occur at this time.

After modification, the disaccharide -peptide unit is released from the carrier lipid and added to the growing cell wall acceptor to form linear mucopeptide strands. The carrier lipid is released as the lipid-pyrophosphate and must be dephosphorylated before it can be reutilised (Siewert and Strominger, 1967). The final reaction is the cross-linking between peptide chains. In *E. coli*, this has been shown to involve transpeptidation with the release of the terminal D-alanine residue (Izaki *et al.*, 1966).

A number of antibiotics have as their major site of action, specific reactions in the pathway of mucopeptide synthesis. Bacitracin has been shown to specifically prevent dephosphorylation of the lipid pyrophosphate carrier (Siewert and Strominger, 1967). Penicillin interferes with the transpeptidation reaction (Izaki *et al.*, 1966) presumably due to its structural analogy to the D-alanyl-D-alanine end of the penta-peptide (Tipper and Strominger, 1965).

Vancomycin, shown by Anderson *et al.*, (1967) to prevent utilisation of lipid intermediates, appears from the studies of Perkins (1969) to combine specifical-ly with peptide chains ending in D-ala-D-ala. Thus,

in vivo, it would bind strongly to any unlinked peptide chains in the cell wall and to lipid intermediates. *In vitro*, it would also bind to soluble precursors as permeability barriers are removed.

The Roles of the Cortex, Dipicolinic Acid and Calcium in the Formation of Heat Resistant Spores.

(a) The requirement for an intact cortex.

A fully-formed cortex is an essential layer for the formation and maintenance of the stable, refractile spore state. Fitz-James (1965) has shown that addition of penicillin during cortex formation causes the production of a wide, spongey cortical layer, and that, with the beginnings of terminal lysis, the spore itself is lysed leaving only resistant coats and exosporium. Calcium and DPA are lost with this lysis. Addition of cycloserine also interferes with cortex formation and produces spores which leak calcium and DPA (Murrell, 1969). On germination, one of the first reactions is probably a breakdown of the cortical mucopeptide by spore lytic enzyme (Dring and Gould, 1971). Lysozyme treatment of sensitised spores again leads to breakdown of the cortical mucopeptide and a germination-like change in the state of the spore protoplast (Gould and Hitchins, 1963). Even mechanical damage can cause dissolution of the cortex by activation of lytic enzymes, and a subsequent rapid

loss in refractility (Lewis *et al.*, 1960; Lewis, 1969). Thus, interference with formation of the cortex prevents production of a normal, refractile spore, and any alteration, enzymatic or mechanical, in its structure leads to breakdown of the dormant spore state.

(b) The contractile cortex hypothesis.

The mechanism by which the cortex can make such a large contribution to dormancy, heat resistance and refractility, has been the subject of much discussion (Murrell, 1967; Vinter, 1969; Lewis, 1969). The most predominant hypothesis is that it acts as a contractile layer exerting pressure on the spore core and thereby causing a lowered water content in the core. This is the "contractile cortex" hypothesis of Lewis *et al.*, (1960).

That the cortex does exist in a contracted state in the spore is supported by several lines of evidence. Cytological studies on the cortex from disrupted spores have shown it to be more swollen and thicker in the disrupted rather than the intact spore, and that the inner margin (germ cell wall) of the isolated cortical integuments is in a highly folded state suggesting that on disruption, the cortex assumes an uncontracted state (Murrell, 1967; Warth *et al.*, 1963b).

Warth *et al.*, (1963b) proposed that the contraction of the spore mucopeptide from an initially swollen

state could be caused by the introduction into the cortex during sporogenesis of a high concentration of calcium or calcium dipicolinate (CaDPA). Further support for this came from studies of Hitchins and Gould (1964) who released phase bright "cores" still retaining mucopeptide, from dormant spores by smashing in acid media (pH 4.2). These spores responded to pH and calcium by changes in refractility and volume; at higher pH (> 6.5) they lost refractility and became swollen but this effect could be overcome by the presence of calcium. These results could be explained by contraction of the mucopeptide when its negative carboxyl groups were neutralised either by hydrogen ion at low pH or by calcium at higher pH values.

Vinter and Stasna (1967) partially restored phase brightness and reduced cell volume in germinating spores by addition of basic peptides, presumably causing contraction of remaining mucopeptide.

(c) <u>The location of calcium and DPA</u>.

This contractile cortex hypothesis assumes a cortical location of calcium or CaDPA. The precise spore location of calcium and DPA is not clear. Micro-incineration studies of spores (Knaysi, 1965; Thomas, 1964) showed a considerable amount of mineral ash, probably mainly calcium, in the core region. Thomas does point out that there may be contraction of ash

from the cortex into the central region.

Indirect evidence that calcium and DPA are located in the spore protoplast, comes from studies of Donellan and Setlow, (1965), in which unidentified photoproducts of hydrolysed DNA from ultra-violet irradiated spores were similar to those of irradiated DNA dried previously in the presence of CaDPA.

However, the uptake of calcium and synthesis of DPA at the time of continued cortex synthesis, the rapid release of these components during germination when the cortex is breaking down, the electrostatic binding of calcium to mucopeptide, and the requirements of the "contractile cortex" hypothesis, argue for a cortical location, although there is no direct evidence of this.

The facts that DPA, first isolated by Powell (1953) and making up to 15% of the spore dry weight, is found in approximately a 1:1 molar ratio with calcium, and that on germination (or a variety of chemical or mechanical treatments), it is released as the calcium chelate (Powell and Strange, 1966; Murrell, 1969), have lead many workers to conclude that these two components are located together in the spore. The dependence of the final DPA content on the level of calcium in the sporulation medium, and on the time of addition during sporulation, of calcium to calcium-deficient medium (Young and Fitz-James, 1962; Black *et al.*, 1960), sug-

gests some relationship between the two.

The idea that calcium is chelated with DPA presents some problems in that the ability of calcium to neutralise electrostatic repulsions in the mucopeptide is effectively annulled (Hitchins and Gould, 1964). Recently, Murrell *et al.*, (1969) have suggested that CaDPA chelates occupy intracellular spaces within the mucopeptide network, and that they may remove water (by formation of the sesquihydrate) from the protoplast as the cortex contracts. In this hypothesis, other cations are assumed to cause this contraction.

An alternative solution is that calcium and DPA have separate locations within the spore (calcium mainly bound to the inner membrane and DPA in the core), and that it is only on germination that the two components combine (Fitz-James, 1971).

(d) Water content of spores.

Lewis *et al.* (1960) proposed the contractile cortex hypothesis to account for the concept of a low water content of the spore protoplast, reintroduced by Powell and Hunter, (1956), and thus, for the spore properties of dormancy, heat resistance and refractility. The equilibration of most or all of the water in a spore pellet with external heavy water (Murrell, 1961; Black and Gerhardt, 1962; Marshall and Murrell, 1970) indicates that the spore is freely

permeable to water, although permeability barriers to larger molecules do exist (Lewis, 1969). However, the amount of water present in the spore core is a matter of controversy. Earlier estimates that spores contain about 70% water (Murrell, 1961; Black and Gerhardt, 1962) have been reduced to as low as 40 to 50% for *B. subtilis*, and *B. coagulans* (Marshall and Murrell, 1970). Such studies cannot give direct information on the distribution of the water within the spore. Murrell and Scott (1966) have shown that heat resistance of spores of widely varying resistance at a_w of 1, at a relative humidity of about 30% (a_w of 0.3) is at a maximum and varies very little between species. Thus, the spores respond as if the most sensitive part is at an a_w near 0.3. To account for this the spore core must be under pressure, as is proposed in the contractile cortex hypothesis (Lewis *et al.*, 1960; Lewis, 1969).

Calculations based on a low protoplast volume (20% of spore volume) containing 1/3 of the spore protein and all the CaDPA, give a protoplast with about 10% of water (dry weight) (Marshall and Murrell, 1970). Thus, these authors conclude that a very dry protoplast is unlikely, except in species such as *B. coagulans*, which have a low protoplast volume. However, Fitz-James (1971) has reported that smashing of ethanol-washed viable spores in ethanol, releases

only 5% of the dry weight as water, the lowest limit of the technique. Similarly, by drying lyophilised but viable spores at 100C, Hyatt and Levinson (1968) found the water content of spores to be about 5% of the dry weight.

Another theory to account for lowered water content in the core is that the core exists as an insoluble and heat stable gel, with cross-linking between macromolecules producing a high polymer matrix with some entrapped free water (Black and Gerhardt, 1962). There is no direct evidence to support this. A similar idea has been proposed by Marshall and Murrell (1970); the protoplast is thought to gel and contract under the action of metal ions and other "gelation co-molecules". During this gelation, water would be extruded, helped by the development of a contractile cortex while the protoplast is in a minimum volume state.

(e) Heat resistance.

Heat resistance is one of the most striking properties of the mature spore. Although different species appear to form spores similar in structure and DPA content, these spores differ by a factor of 10^5 in heat resistance in a dilute aqueous system (Murrell and Scott, 1966). There is no simple explanation for this great difference in resistance of spores, nor for the large increase in heat resistance of spores

over vegetative cells. It would seem that many factors, both structural and chemical, are important in producing the mature spore state.

The requirement for an intact cortex for maintenance of the spore state has been discussed previously (Section a), and the lowered water content of the spore core has been discussed above. Murrell and Warth, (1965) from an extensive analysis of many species with a 700 fold range in heat resistance, found a significant correlation between DAP content (and consequently degree of cross-linking of the mucopeptide) and heat resistance. The magnesium-calcium ratio of spores also was directly related to heat resistance-the higher the calcium compared to magnesium, the greater the resistance. The role of DPA was also investigated. Although the actual content of DPA varies comparatively little in species exhibiting a large range of heat resistance, suggesting that a direct relationship between DPA content and heat resistance does not exist, (Murrell and Warth, 1965), nevertheless, DPA appears essential for the production of the heat resistant state (Church and Halvorson, 1959; Black *et al.*; 1960). The isolation of DPA-deficient mutants, low in heat resistance unless supplemented with exogenous DPA, appears to confirm the role of DPA in heat resistance (Halvorson and Swanson, 1969). However, whether DPA contributes to heat resistance by participation in contraction of the cortex, by capture of water from the spore

protoplast as CaDPA sesquihydrates in the cortex, or by stabilisation of spore biopolymers by the formation of coordination complexes with amino acids or peptides and ions such as Ca and Mg, (Tang *et al.* 1968) is not known. Better evidence for the exact location of DPA within the spore would help distinguish between these possibilities. For further discussion of the possible roles of DPA see Murrell, (1969) and Lewis (1969).

In conclusion of this section, it can be said that the location and role of calcium and DPA in spores, the extent to which the cortex is involved in production of the mature spore, the precise state of water in the core, and the mechanism of heat resistance, are still not completely understood.

Sporulation Mutants

Asporogenous mutants have played an important part in mapping the position of sporulation markers on the genome, in determining sporulation-specific reactions and in elucidating the control mechanisms involved. Sporulation mutants have been classified as asporogenous (Sp^-) or oligosporogenous (Osp) (Schaeffer *et al.*, 1965). Asporogenous mutants form spores only by reversion to the wild type; oligosporogenous mutants will sporulate at low frequency under normal conditions of sporulation, and the spores so produced, when recultured, again sporulate

at the same low frequency. Thus, these Osp mutants possess all necessary information for sporulation, but cannot properly express it.

Sporulation mutants can be classified cytologically by examination with the electron microscope in the terminal stages of sporulation. They can be named by the sporulation stage at which the block occurs. Mutational blocks at all stages of the normal sporulation process have been found in *B. subtilis* (Ryter *et al.*, 1966; Schaeffer *et al.*, 1965), *B. cereus* (Fitz-James, 1965) and *Clostridium histolyticum* (Bayen *et al.*, 1967). Some of these mutants are difficult to classify as "late" structures may be formed, whereas earlier ones may not (Schaeffer, 1969).

As the total number of cistrons which affect the ability of the bacterium to form a mature spore is great(approximately 100 estimated for *B. subtilis*; Halvorson, 1965;), the number of different mutations possible, giving rise to the cytological classes, is also extremely large.

The asporogenous mutants probably of most interest are those affecting gene products that occur only during sporulation, and for which sporulation-specific functions are known. Such mutants would include those affecting synthesis of dipicolinic acid, of spore coats and of cortex. Characterisation of

these mutants should help in understanding the control of sporogenesis and the biochemical events involved in the synthesis of cortical mucopeptide and the production of the mature, heat resistant spore.

It was with this point of view that this study of a Stage 4 cortexless mutant of *B. cereus* var. *alesti* was undertaken. Previous brief descriptions of this mutant have been made (Fitz-James, 1965; Young, 1964).

III. METHODS

Organisms and cultivation. The parent organism used was a non-crystal-forming strain, A(-), of *Bacillus cereus* var. *alesti** described by Fitz-James and Young (1959). The cortexless mutant, A(-)l, was derived from A(-) by ultraviolet treatment by Dr. I.E. Young (York University, Toronto). It was first recognised by its failure to form the normal creamy-white sporulated colony of the parent. Because the spores formed were unstable and lysed, the mutant colony became pale after 18 to 20 hr of incubation. Both stocks were maintained on plates of nutrient agar (Difco), the mutant being subcultured every 7 days. Under these conditions of subculture, A(-)l appears to be a stable mutant, as no revertants have been found. For sporulation in liquid medium, the techniques and medium used were those of Young and Fitz-James (1959b). In experiments on ^{32}P incorporation, the salts part of the medium was reduced to one-tenth the usual phosphate

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*In keeping with previous published work from this laboratory, the name *B. cereus* var. *alesti* has been maintained, although the original crystal forming strain, (A+), should now be considered a variant of *Bacillus thuringiensis*, (see Bergey's Manual of Determinative Bacteriology).

concentration and was supplemented with tris(hydroxymethyl) aminomethane (Tris) at a final concentration of 0.01 M.

Microscopy. Sporulation was carefully followed by phase-contrast microscopy. In some experiments, the per cent of phase whitening in the culture was estimated by simple counting of the number of spore particles in any stage of phase whitening. This was a rather subjective procedure, but it did allow the degree of synchrony and stage of sporulation to be compared from one experiment to another.

Air-dried and air-mounted nigrosin smears prepared by a method devised by Dr. C. Robinow, as described by Fitz-James (1971) were examined by bright field microscopy and used for the detection of refractility.

Samples were prepared for electron microscopy by the method Kellenberger *et al.* (1958) with some modifications. Samples were mixed with 1/30 th volume of 1% OsO_4, and centrifuged immediately; the pellet was worked up in 0.3 to 0.5 ml of warmed (45 C) 2% agar and centrifuged. The cell layer was removed, cut into 1-mm cubes, and fixed in 0.5 ml of 1% OsO_4 with 1 or 2 drops of the tryptone medium for 2 to 3 hr. The original method was then followed. For freeze-etching, cells were harvested and rapidly frozen in Freon 22. Freeze-etching was carried out in a Balzers unit (AG, Fürstentum Lichtenstein). A Phillips 200 electron microscope was used for all

electron micrographs.

Heat resistance. Diluted samples of culture were heated at 60C for 30 min, a treatment sufficient to kill all vegetative cells, although well below the tolerance of spores of the parent strain. One ml samples were plated with nutrient agar using the pour plate technique. Unheated samples were also plated. Colonies were counted after 24 hr growth at 30C. As the cultures were often badly clumped, a 10 sec sonication in a M.S.E. sonicator running at 110 V was employed before dilution. This procedure broke up the clumps into single cells, some doublets and occasional chains of 3 or 4 cells. Thus heat resistance figures are approximate as only colony forming units were counted.

Chemical analysis. Dipicolinic acid (DPA) was estimated by the method of Janssen *et al.* (1958).

^{45}Calcium uptake was measured as described by Young and Fitz-James (1962) with slight modifications. A final concentration of 0.2 μCi ^{45}Ca/ml was used, and the membrane filters were placed in vials with 10 ml of scintillation fluid (see below).

Extraction of soluble pool materials was carried out by the technique of Gale (1947).

The method of Ernster *et al.* (1950) was used for total phosphate determinations on samples ashed with 60% perchloric acid.

Protein was estimated by the method of Lowry *et al.* (1951).

N-Acetylamino sugar esters were estimated by the method of Strominger (1957).

RNA synthesis. RNA synthesis was followed by adding ^{14}C-uracil at a final concentration of 0.2 µCi/ml to cultures at the required stage of sporulation. Duplicate 0.1 ml samples were removed and added to 3 ml of ice cold 5% trichloroacetic acid (TCA) containing 100 µg of uracil/ml. After 1 hr in the cold, samples were filtered, washed thoroughly with 5% TCA containing 100 µg of uracil/ml and counted for radioactivity.

Incorporation of ^{3}H-DAP. In all experiments involving uptake of tritiated diaminopimelic acid (^{3}H-DAP), the culture was first trained for uptake by the addition of 5 to 20 µg DAP/ml and 100 µg lysine/ml, 10 to 20 min before addition of ^{3}H-DAP.

Studies on the incorporation of ^{3}H-DAP sequentially throughout the sporulation process were based on those methods of Vinter (1965) with modifications as described below. For each sample, a portion of the stock culture in the required stage of sporulation was placed in a warmed flask (30 C) containing DAP and lysine to a final concentration of 20 µg/ml and 100 µg/ml respectively. After 10 min aeration, 0.2 µCi of ^{3}H-DAP/ml was added, and the culture was shaken for a further 35 min. The sample was

precipitated with 5% TCA in the cold. Mucopeptide, extracted by the method of Park and Hancock (1960), was placed on membrane filters (Millipore Corp.) in duplicate, washed thoroughly, and counted for radioactivity.

Pulse-labelling of spores with ^{3}H-DAP. A sporulating culture of the parent strain, A(-), was divided in two. One half was labelled with ^{3}H-DAP (as described in the previous section) at the end of engulfment; the second half was labelled after phase whitening had taken place in 80% of the cells. At the end of each period of labelling, the culture was centrifuged and the pellet was suspended in the supernatant fraction obtained by centrifuging a culture in the same stage of growth. The cultures were allowed to complete sporulation, and after 36 hr the liberated spores were collected and washed thoroughly with saline by repeated centrifugation.

Germination. The labelled spore suspensions were heat-activated at 65 C for 20 min and then added to a germination medium consisting of 0.5 M Tris-hydrochloride buffer (pH 7.8) containing 10 mM L-alanine, 75µM adenosine, and 0.1 M glucose. The initial optical density at 650 nm was 0.25. In this system, over 80% of the spores became phase dark within 5 min and 99% within 10 min. Samples were removed with time, precipitated by 10% TCA containing 100 µg of DAP per ml, collected on membrane filters (Millipore Corp.), and counted for radioactivity.

In a parallel series of experiments, the labelled spores were made coatless by the technique of Aronson and Fitz-James (1968), and were subsequently digested with lysozyme (100 μg/ml). Samples were treated as above.

^{32}P and sodium acetate-1-^{14}C incorporation into lipids. ^{32}P (2 μCi/ml) or sodium acetate-1-^{14}C (0.2 μCi/ml) was added to the culture at the required stage of growth or sporulation; 10-ml samples were taken at several times, centrifuged, and washed, and the pellet was suspended in butanol: 6M pyridine acetate, pH 4.2 (2:1, v/v). For pulse-labelling experiments, the labelled culture was exchanged into "cold" medium obtained by centrifuging a culture in the same stage of growth; samples were subsequently taken as described.

Extraction of lipids. Total lipids were extracted from cells disrupted for 20 min in a Mickle disintegrator with butanol: 6M pyridine acetate, pH 4.2 (2:1, v/v), (Strominger *et al.* 1966). The butanol phase was washed twice with water, flash evaporated at 40C or less, dissolved in chloroform: methanol (3:1, v/v), and run through a Sephadex LH-20 column with the same solvent (Maxwell and Williams, 1967). Radioactivity was determined by evaporating samples of the column effluent in scintillation vials, dissolving in scintillation fluid, and counting.

Lipid fractionation. In some experiments, the method of Rouser *et al.*, (1964) or as modified by

Strominger *et al.* (1966) was used for fractionation of lipids into the major classes by diethylaminoethyl (DEAE) cellulose column chromatography. Fractions thought to contain lipid-mucopeptide intermediates were further fractionated on a silicic acid column equilibrated with chloroform: methanol (7:1, v/v), (Strominger *et al.*, 1966). The column was developed with stepwise increases in methanol concentration. The final fraction contained 80% methanol, 10% chloroform, 5% acetic acid and 5% water to elute more polar compounds.

Fatty acid analysis. Fatty acid analysis of lipids extracted by chloroform: methanol (2:1, v/v) from freeze-dried cells, was carried out on a 10 ft by 1/8 in polar column of 10% EGSS-X on Chromosorb P. A Beckman GC-45 analyser with a hydrogen flame detector was used. The temperature of the column was 200 C and the flow rate of carrier gas was 70 cc/min. Chromatogram peaks were identified by comparison with those of fatty acid methyl ester standards (NIH). Quantitative estimation of peak areas was made by the method of Carroll (1961).

Preparation of uridine diphosphate N-acetyl muramic pentapeptide labelled with ^{3}H-DAP. 500 ml of a culture in mid-log phase of growth (0.7-0.8 O.D. at 650 nm) to which 50 μg/ml of DAP and 100 μg/ml of lysine had been added 20 min previously, were centrifuged and suspended in warmed (30 C) Grelet salts medium with the following final concentrations of additives: 0.3M sucrose,

10^{-3} M $MgCl_2$, 100 μg/ml of lysine, 1 μg/ml of DAP, 5 μg/ml of vancomycin, and 0.5 μCi/ml of ^{3}H-DAP. After 60 min shaking, the cells were spun, washed with Grelet salts/ 0.3M sucrose/Mg and the pellet extracted with 5 ml of ice cold 10% TCA. The supernatant of this extract, after centrifugation, was shaken three times with an equal volume of ether, and brought to pH 8 to 9 with NaOH, (Strominger, 1957).

UDP-N-acetyl muramic pentapeptide was purified by chromatography on a 12 by 1 cm column of Bio-Rad AG1-X2, 50-100 mesh, chloride form (Strominger, 1957). 10 ml fractions were collected. Optical density at 260 nm, radioactivity, and concentration of N-acetylamino sugar esters were determined. Tubes containing the intermediate were pooled and flash evaporated to a small volume. The salt used to elute the compound was removed by chromatography on Sephadex G-25. Optical density was again used to detect the intermediate and a spot test with silver nitrate to detect chloride ion. The specific activity of the intermediate varied between 1 to 5 μCi/μmole.

Cell-free homogenates. Cells at the desired stage of growth or sporulation were centrifuged, and suspended in 1/10 volume of 0.1M Tris-HCl buffer, pH 7.8. The suspension was passed twice through a French Pressure Cell operating at 10,000 lbs/sq in pressure. Almost total breakage of cells was obtained as observed by phase contrast

microscopy.

Actinomycin D treatment. Actinomycin D (Calbiochem.) was dissolved in a small volume of 35% ethanol and kept in a warmed (30 C) flask covered with black paper. At the required time of sporulation, sufficient volume of culture to give a final actinomycin D concentration of 10 µg/ml was added to the darkened flask and shaking was continued.

Scintillation counting. Samples were prepared for counting either by precipitating with trichloroacetic acid and placing on a membrane filter (Millipore Corp.), or in the case of lipid samples by dissolving directly in the scintillant: 4 g of 2, 5-diphenyloxazole per litre and 0.1 g of 1, 4-bis-2-(5-phenyloxazolyl)-benzene per litre in toluene. Water soluble samples were added to vials containing Aquasol (New England Nuclear Corp.). A Phillips liquid scintillation analyser was used.

Radioactive chemicals. Sodium acetate-1-^{14}C and ^{45}Calcium were obtained from the New England Nuclear Corp.; α, ε-diaminopimelic acid-T (G) dihydrochloride from the Amersham-Searle Corp.; and ^{32}P from Atomic Energy of Canada Limited. Uracil-2-^{14}C was obtained from New England Nuclear Corp.

IV. RESULTS

Characteristics of Sporulation in the Cortexless Mutant, A(-)1.

(a) Phase contrast microscopy. Vegetative cells of the mutant A(-)1 showed no morphological characteristics distinguishing them from the parent A(-). During the sporulation phase, the cells appeared less thickened and less granular and tended to remain in chains. Spore formation proceeded normally as seen by phase microscopy until the terminal stages. The spores were seen to whiten and become partially refractile (Fig. 2) as compared to the parent (Fig. 1), but even this submaximal degree of refractility was soon lost (Fig. 3). Drastic lysis of the spore and mother cell quickly followed, the only product of the lysis being resistant spore hulls (Fig. 4).

(b) Electron microscopy. The stages in spore formation of *B. cereus* have been extensively described elsewhere (Fitz-James, 1965; Young and Fitz-James, 1959a,b; Young and Fitz-James, 1962). In this mutant, stages 1, 2, and 3, that is axial-filament formation, septation, and engulfment of the forespore, appeared normal. However, the usual deposition of cortical material in stage 4 was observed to be completely lacking. The

Fig. 1-4. Phase-contrast photographs. Magnification as shown by the 5 µm marker in Fig. 1.

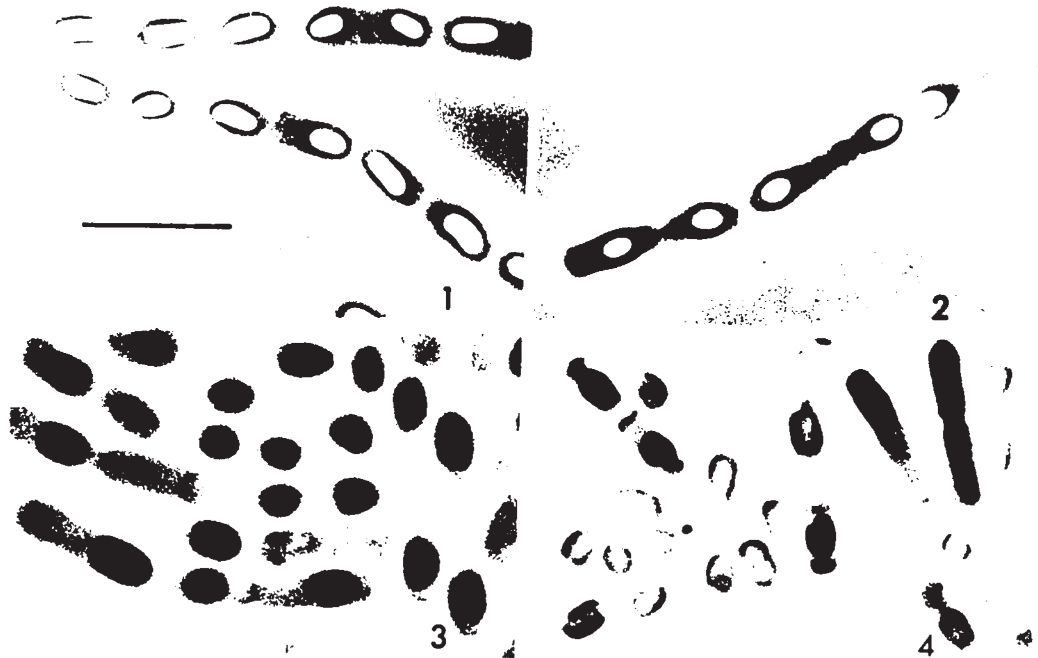
Fig. 1. Sporulation in the parent, *Bacillus cereus* var. *alesti* strain A(-), at maximum refractility.

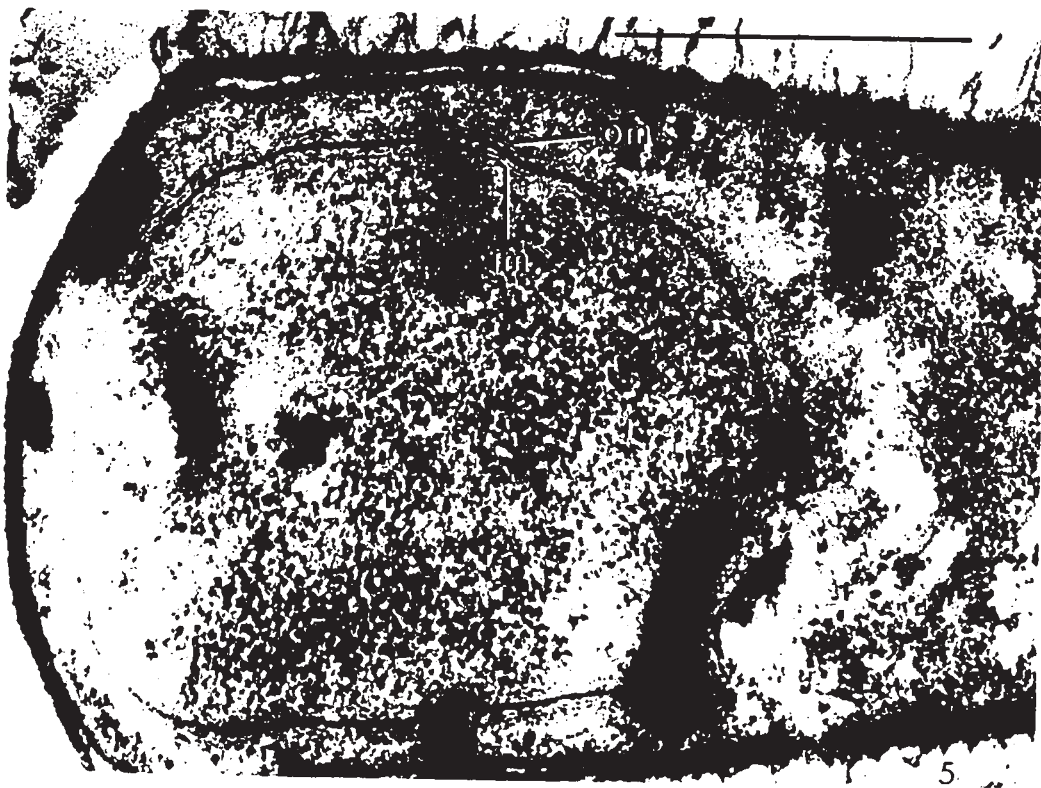
Fig. 2. The mutant, A(-)1, at its peak of phase whitening.

Fig. 3. Darkened spores of A(-)1, after peak whitening and just prior to lysis. Some spores appear larger due to flattening by the coverslip.

Fig. 4. Free spore hulls, the terminal product of lysis in A(-)1.

Fig. 5. Electron micrograph showing the dense narrow layer deposited by the mutant, A(-)1, between the forespore membranes after engulfment has been completed. Heavy lead staining was used to differentiate the inner (im) and outer (om) membranes. Magnification as shown by the 0.5 µm marker.


1
2
3
4


om
im
5

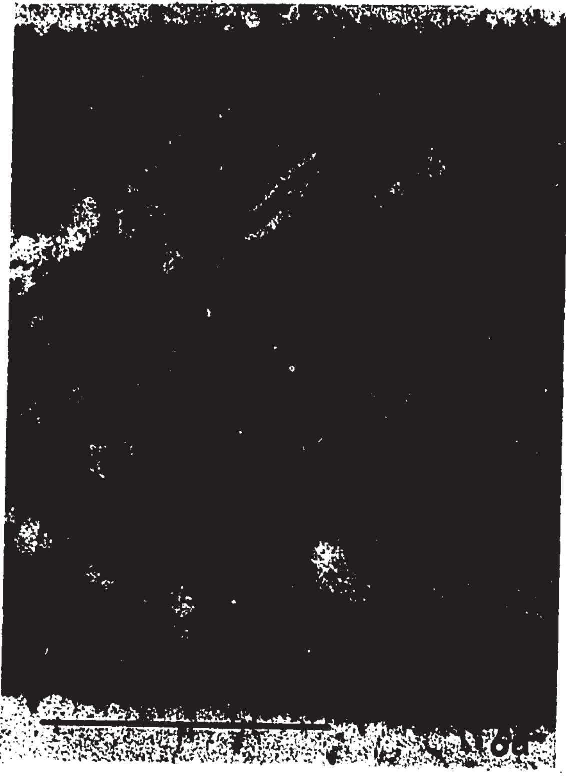
completed forespore possessed only a narrow dense profile between the inner and outer membranes (Fig. 5). This layer was similar to the parent germ-cell wall which is seen as a more electron-dense layer on the inner surface of the intact and isolated cortex (Warth *et al.*, 1963a; Warth *et al.*,1963b). Sometimes, excess of this layer was produced, apparently by the inner forespore membrane (Fig. 6a). Normal spore coats (stage 5) were deposited immediately in A(-)1 close to the dense profile (Fig. 6a,b). The structureless appearance of the spore protoplast typical of the refractile spore of stage 6 was not observed, although in some spore cores a slight glazing over of cytoplasmic detail and a condensation of the nuclear material occurred, characteristic of the early stages of refractility (Fig. 6a,b). With the drastic terminal lysis, the spore core was lysed, leaving the resistant coats and the exosporium broken but enveloping variable amounts of cell remnants (Fig. 7). Thus, this mutant produces spores lacking a cortex and consequently unable, in spite of the completion of spore coats, to resist digestion of their cores by the terminal lysis of sporulation.

(c) Chemical analysis. Because of the observed lack of full refractility in the spores of A(-)1, the ^{45}Ca uptake and DPA content of the mutant were compared with that of the parent. The total uptake of ^{45}Ca in

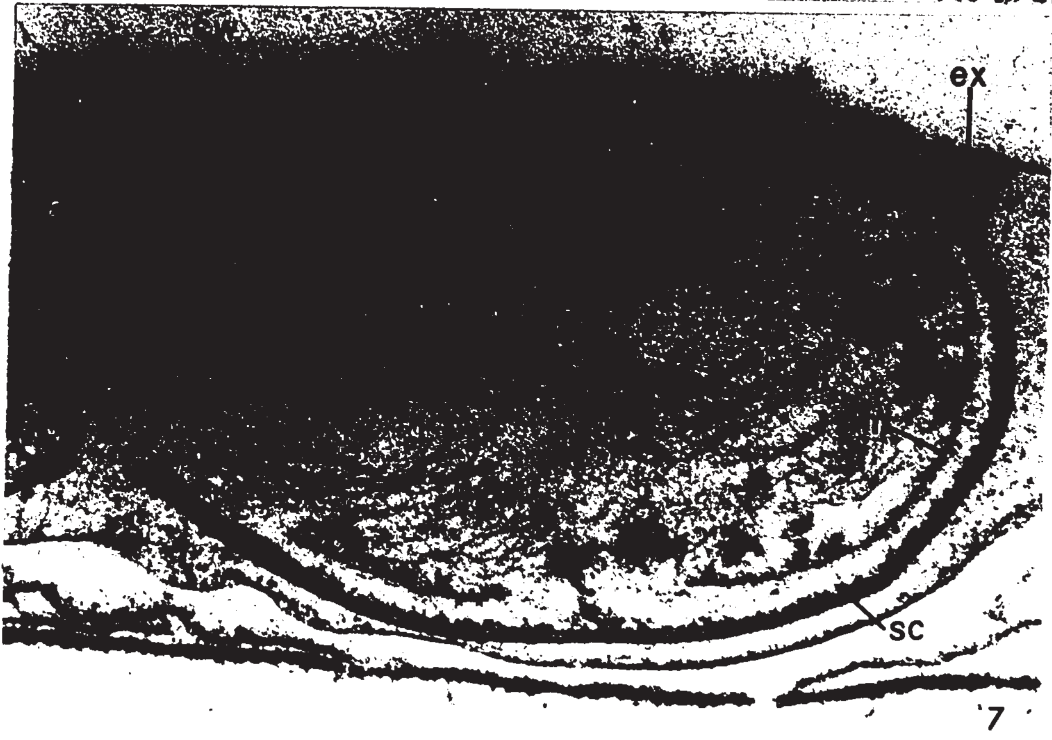
Fig. 6 (a,b). Electron micrographs of parts of two spores of A(-)1, at peak whitening. The spore coats (sc) have been deposited without intermediate cortex formation. The spore protoplast lacks detail and the nuclear material (n) has aggregated characteristic of the refractility reaction. In (a) excessive production of the initial dense layer (germ cell wall, g c w) apparently by the inner membrane (im) is shown.

Fig. 7. Electron micrograph of the lysed spore core of A(-)1, still partially within the sporangial cell wall (cw). The spore coats, although broken, and the exosporium (ex) are not destroyed. Part of the spore inner membrane has not yet broken down.

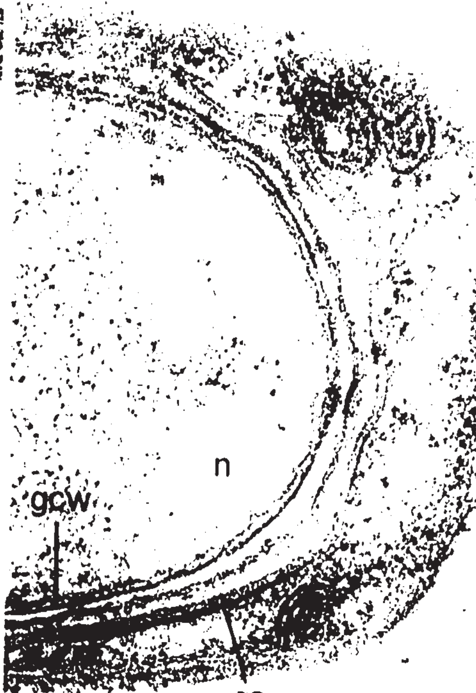
Magnification for both figures is indicated by the 0.5 μm marker in Fig. 6a.

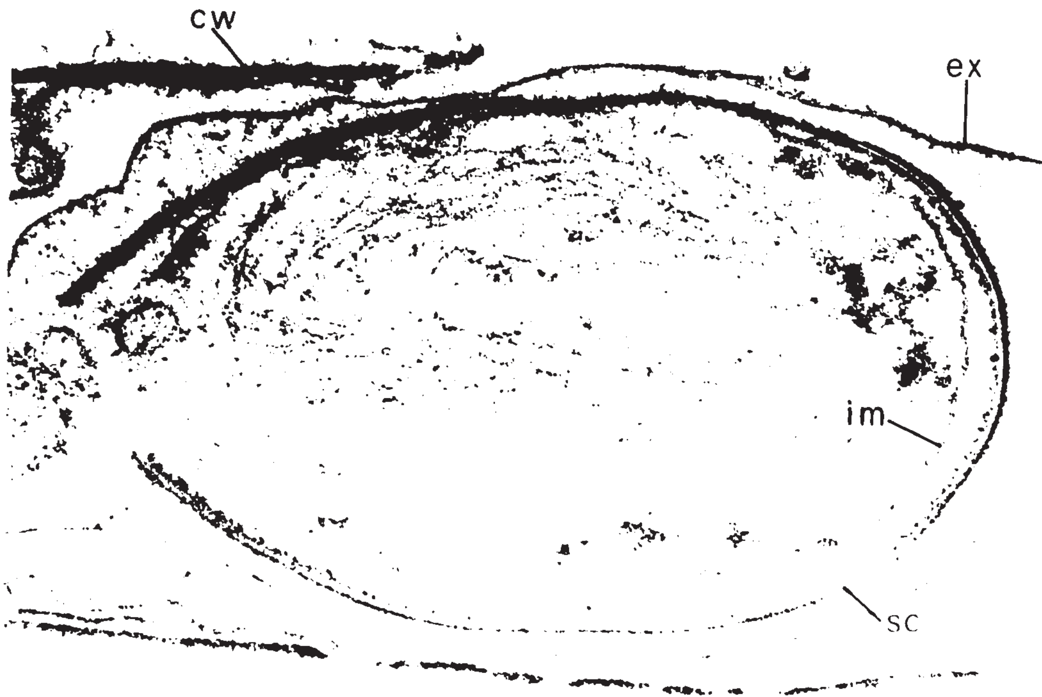

6a

sc
6b


ex
sc
7

gcw
im
6a


n
gcw
sc
6b


cw
ex
im
sc
7

A(-)1 approached the maximum uptake of A(-), but, coincident with the onset of terminal lysis, the ^{45}Ca was lost to the medium (Fig. 8). A similar pattern was observed with DPA formation (Fig. 8). The almost normal levels of Ca and DPA attained by the mutant followed by the rapid release of these compounds would account for the transient appearance of refractility. Often it was observed that the processes of uptake of Ca and synthesis of DPA occurred somewhat earlier in the mutant than in the parent, as if, with the lack of cortex formation, A(-)1 was able to move directly to the next steps in the sporulation sequence.

As DAP is one of the amino acids in the cell wall and cortical mucopeptides and since it has been shown by Vinter (1963a) that cell wall DAP is not utilized for spore formation, it is an ideal marker for synthesis of spore-specific mucopeptides. The uptake of ^{3}H-DAP throughout the sporulation process was investigated in both A(-) and A(-)1 (Fig. 9). In the parent, DAP uptake was seen to take place after engulfment and to increase to a maximum after phase whitening had occurred. In the mutant, a quite dramatic difference was observed. The initial uptake was similar but, instead of a further increase in incorporation, a rapid fall occurred (Fig. 9). Thus, there appeared to be two periods

Fig. 8. Comparison of Ca^{45} uptake and DPA synthesis in the parent, A(-), and the mutant, A(-)1. The arrow marks the beginning of lysis in the mutant. ■ Ca^{45} parent; □ Ca^{45} mutant; ● DPA parent; ○ DPA mutant.

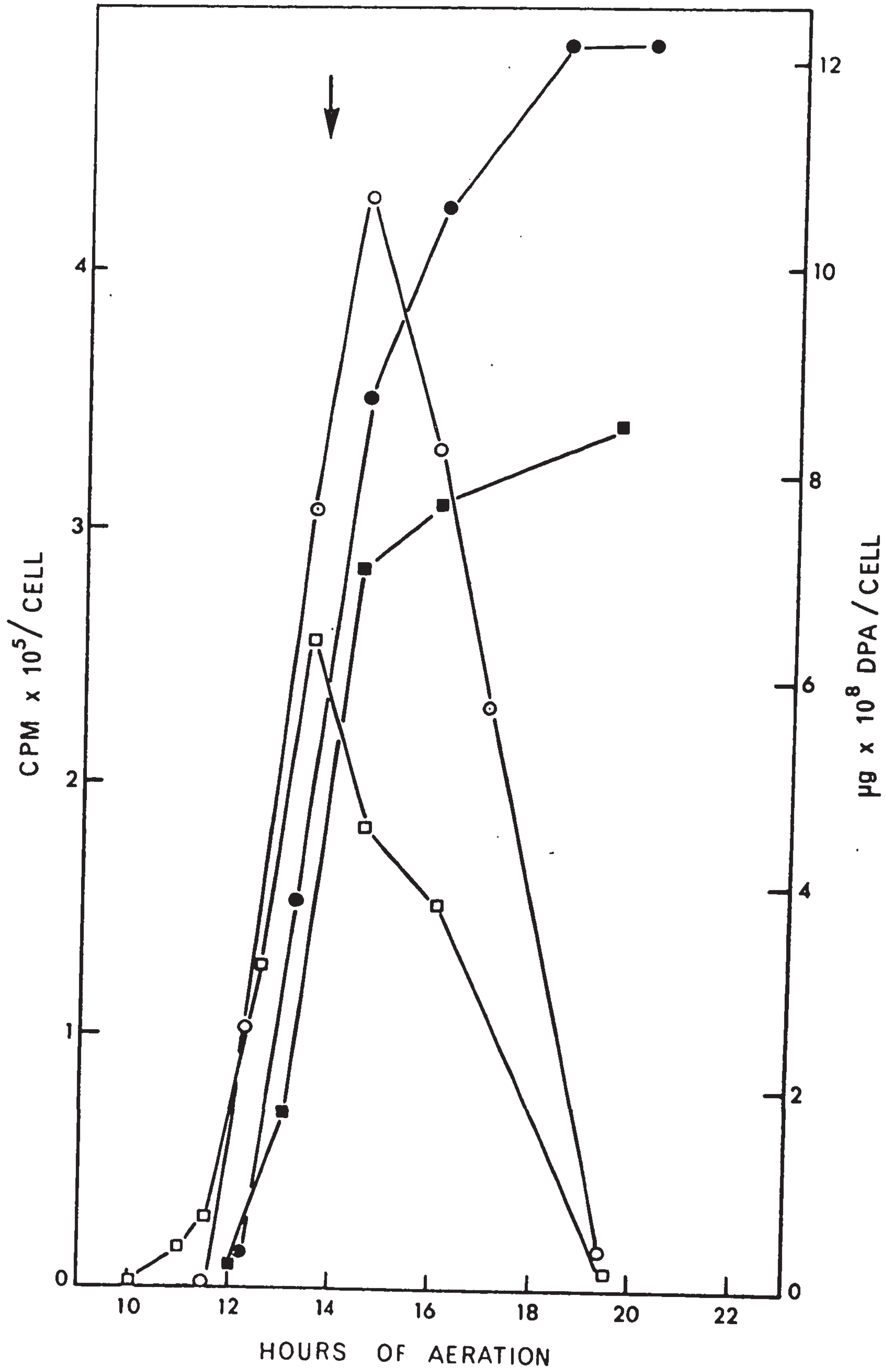
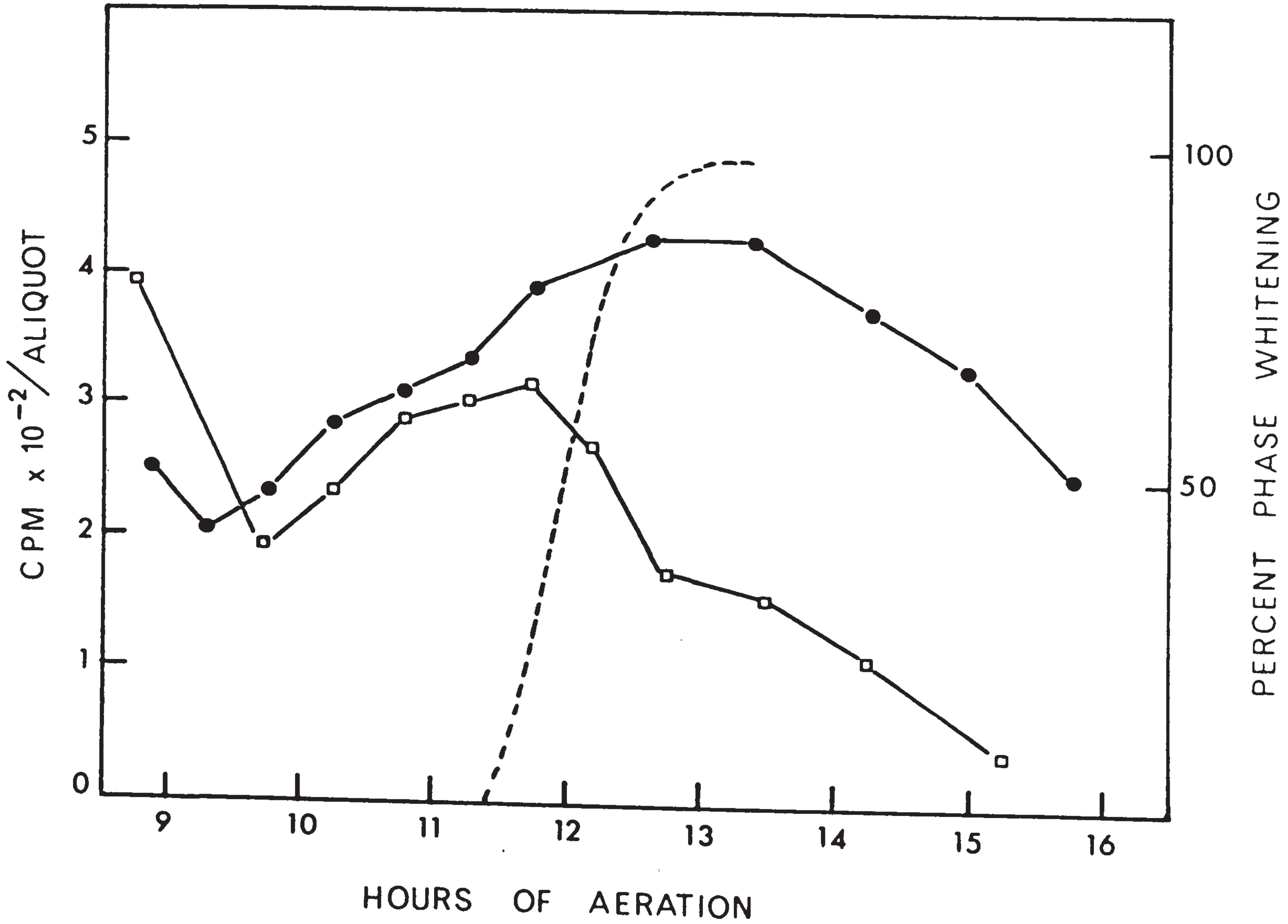

CPM x 10^5/CELL
0
1
2
3
4
μg x 10^8 DPA/CELL
0
2
4
6
8
10
12
10
12
14
16
18
20
22
HOURS OF AERATION

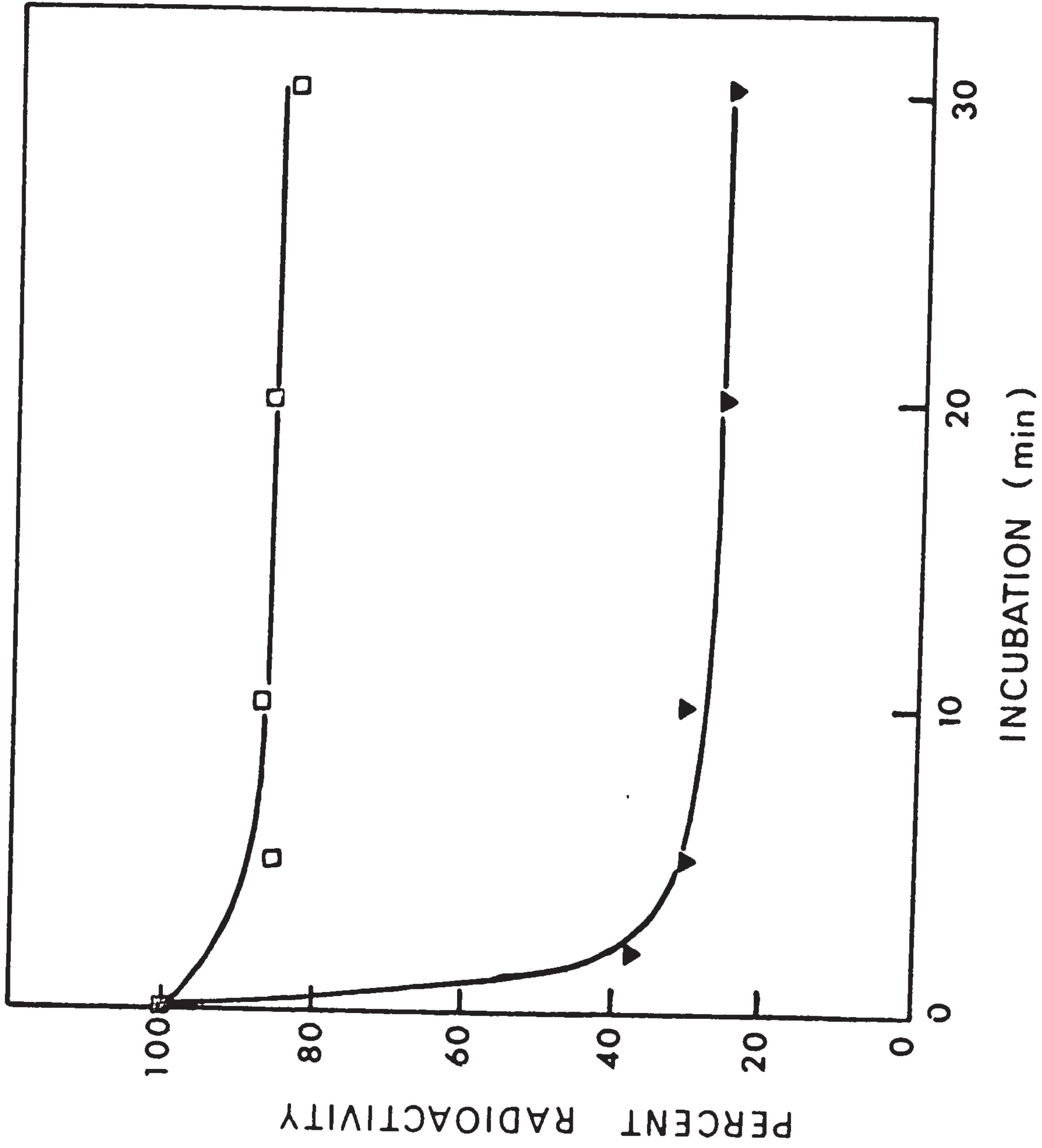
Fig. 9. Incorporation of H^3-DAP into mucopeptide of A(-), ● , and A(-)1, □ , during sporulation. Radioactivity was measured as cpm H^3-DAP in the mucopeptide extracted from 4 ml culture. Phase whitening occurred in both cultures as shown by the dotted line.


CPM x 10^{-2}/ALIQUOT
5
4
3
2
1
0
9
10
11
12
13
14
15
16
HOURS OF AERATION
100
50
PERCENT PHASE WHITENING

of mucopeptide synthesis in the parent, whereas the mutant seemed capable of only the first stage of synthesis.

Vinter (1965) has demonstrated two such stages of DAP incorporation for *B. cereus* NCIB 8122; he suggests that the first represents germ-cell wall synthesis; the second, synthesis of the cortical mucopeptide. By using similar techniques with the parent strain, A(-), the fate of mucopeptide material labelledwith ^{3}H-DAP after engulfment (early labelled) or after whitening (late labelled) was followed on germination. A major difference was that the radioactivity from early labelled spores remained mostly in the trichloroacetic acid precipitate, whereas that from late labelled spores was almost all lost to the supernatant (Fig. 10). Since the germ cell wall becomes the cell wall proper of the outgrowing cell and since the cortex is known to disintegrate on germination, these results showed clearly that in this system, as in that studied by Vinter, the germ cell wall is synthesised first.
A parallel series of experiments was carried out on the same labelled spores made coatless. These were digested with lysozyme which has been shown to break down the cortical mucopeptide but not the germ cell wall of *B. cereus* strains (Warth *et al.*, 1963b). A similar pattern to that found with normal germination

Fig. 10. Germination of A(-) spores, pulse labelled for 35 minutes with H^3-DAP, either after engulfment, (□), or after phase whitening, (▼). Radioactivity was measured as counts/min of H^3-DAP in the TCA precipitate from 2 ml of incubation mixture, and expressed as a percent of the counts in the resting spore suspensions.

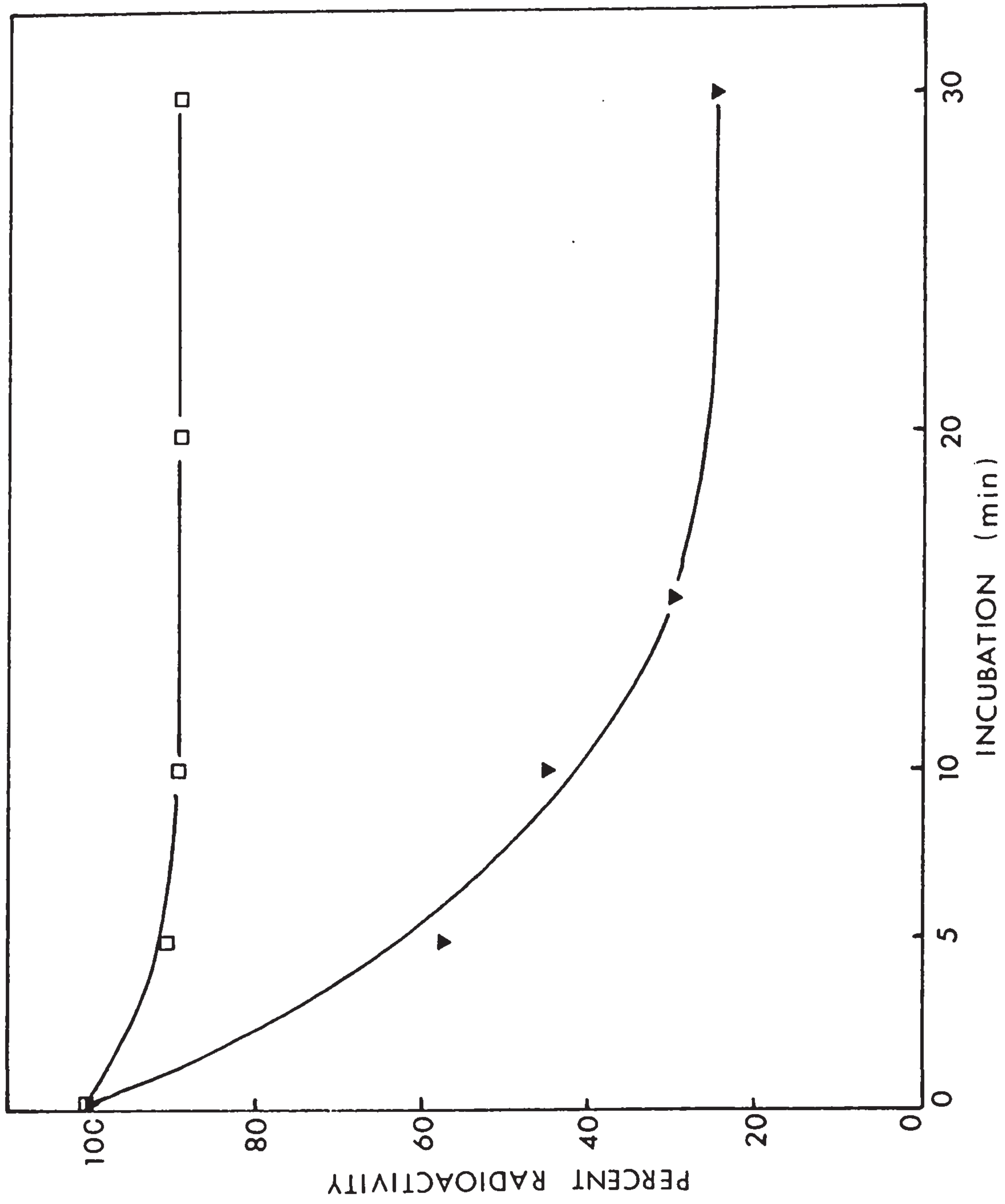

PERCENT RADIOACTIVITY
100
80
60
40
20
0
0
10
20
30
INCUBATION (min)

was observed (Fig. 11). The mutant A(-)1 could not be directly tested as free spores were never produced. However, as the time of uptake of ^{3}H-DAP in A(-)1 was seen to coincide with that of germ-cell wall formation in A(-) and from the electron microscopy studies, the dense line formed between the forespore membranes of the mutant was seen to be similar to the germ cell wall of the parent; A(-)1 appeared capable of synthesising germ cell wall and not an elementary cortex.

(d) Site of the lesion. Since cortical precursors must be carried through the forespore membrane, with probable involvement of several steps if the process resembles cell wall formation, this membrane could be a likely site for the biochemical lesion. The comparative uptake and turnover of ^{32}P into lipids of A(-) and A(-)1 during mid-sporulation were investigated, since membrane activity in some biological systems has been related to changes in phospholipid turnover (Cunningham and Pardee, 1969; Hokin, 1967; Hokin and Hokin, 1960).

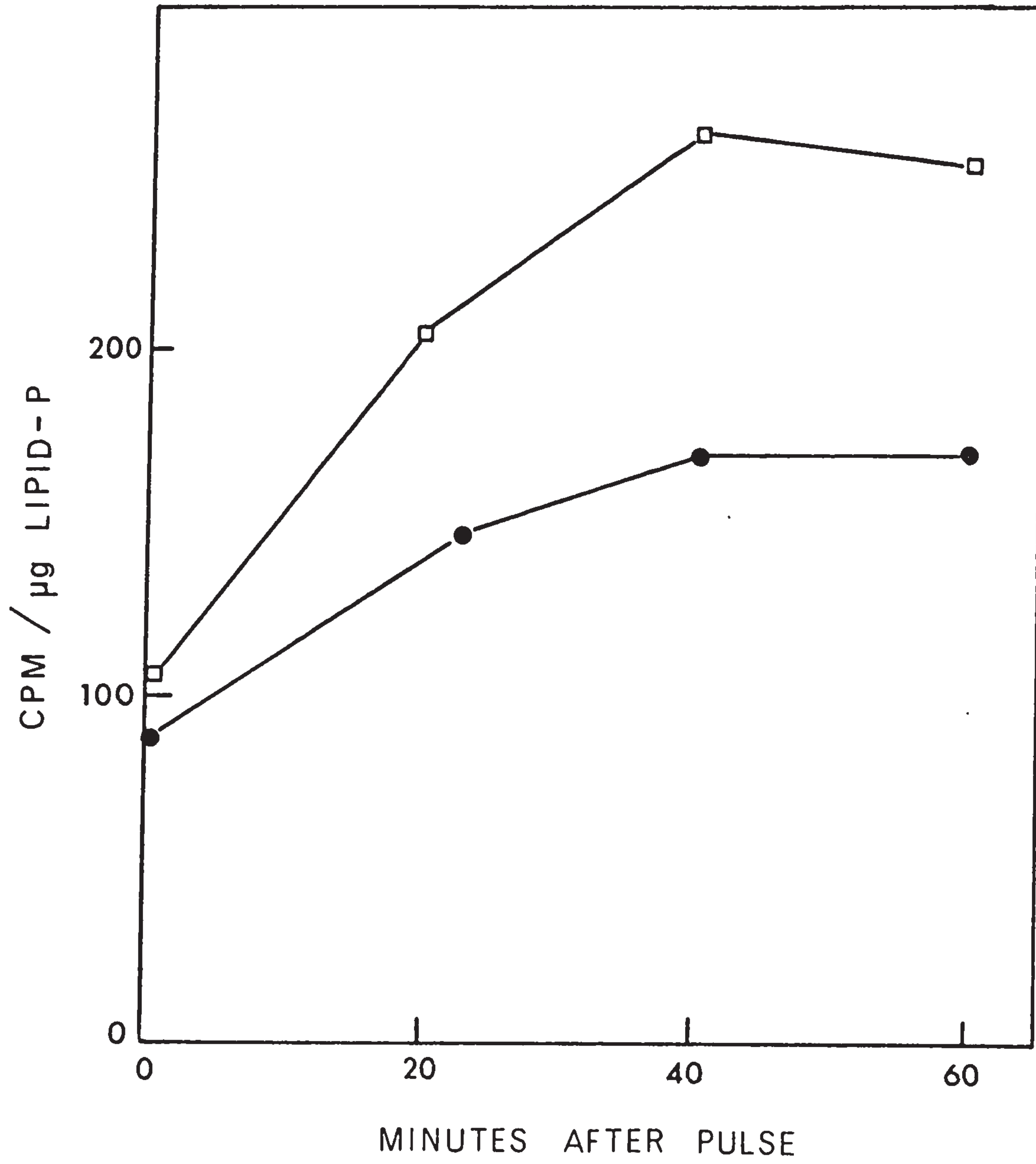
Unexpectedly, in all of five experiments the specific activity of the lipids of A(-)1 was increased by 40 to 80% over that of the parent. A typical result is shown in Fig. 12. The size of the soluble phosphorus pool during sporulation stages 4 and 5 remained the same in both

Fig. 11. Lysozyme digestion of coatless spores of A(-), pulse labelled for 35 min with ^{3}H-DAP either after engulfment, (□), or after phase whitening (▼). Radioactivity was measured as counts/min of ^{3}H-DAP from 2 ml of incubation mixture and expressed as a percent of the counts in the resting spore suspensions.



PERCENT RADIOACTIVITY
10C
80
60
40
20
0
0
5
10
20
30
INCUBATION (min)

Fig. 12. Incorporation of ^{32}P into total phospholipids of the parent, (●), and the mutant, (□), following a 5 minute pulse during early cortex formation.


CPM / µg LIPID-P
200
100
0
0
20
40
60
MINUTES AFTER PULSE

parent and mutant. Moreover, such an increase in specific activity of the lipids was not observed during vegetative growth (Fig. 13 and 14). The increase in specific activity of ^{32}P-labelled phospholipids in A(-)1 seemed to be a general phenomenon not related to any one lipid species. Fractionation of the lipids on a DEAE-cellulose column showed an increase in incorporation of ^{32}P into all fractions containing the major phospholipid species, that is into fractions 2, 4, and 6 (Table 1).

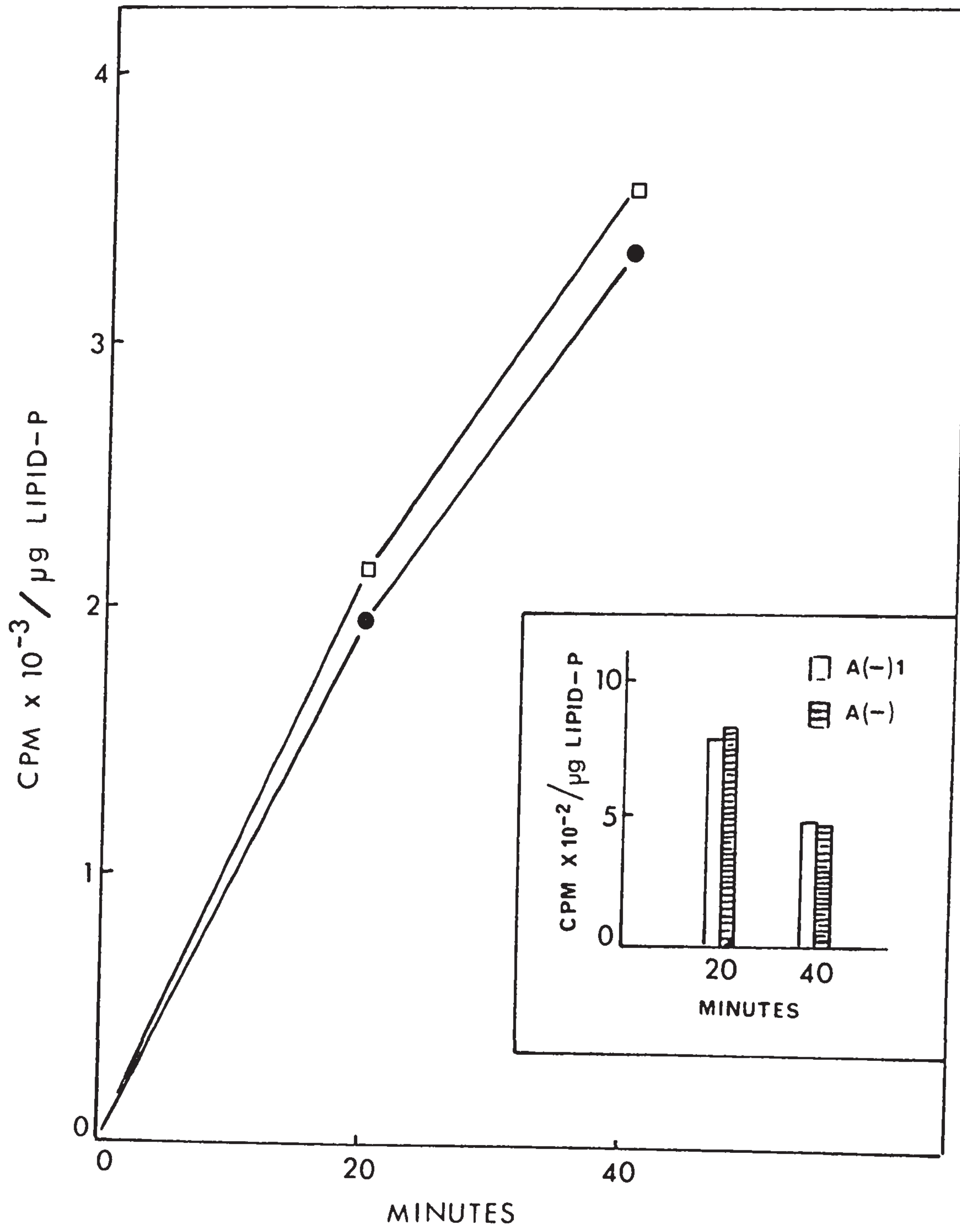
In three experiments sodium acetate-1-^{14}C incorporation was also dramatically increased in the mutant during mid-sporulation. One of these is shown in Fig. 15.

Fatty acid analysis of the parent showed a slight increase in 15:0 iso fatty acid content and a decrease in 14:0 iso, 16:0 iso and 16:1 fatty acids of sporulating cells compared to vegetative cells. In the mutant, during vegetative growth, the fatty acid composition was similar to that of the parent; during sporulation, the usual changes in composition were accentuated, that is 15:0 iso fatty acid content increased further than in the parent and 14:0 iso, 16:0 iso and 16:1 decreased to a greater extent. These results are shown in Table 2.

As the lipid metabolism of the mutant was shown to be abnormal compared to the parent, freeze-etching

Fig. 13. Uptake of ^{32}P into lipids of vegetative cells of A(-), (●), and A(-)1, (□).

Fig. 14. (Inset). Incorporation of ^{32}P into lipids of A(-) and A(-)1 at times of chase after a 10 min pulse during vegetative growth.



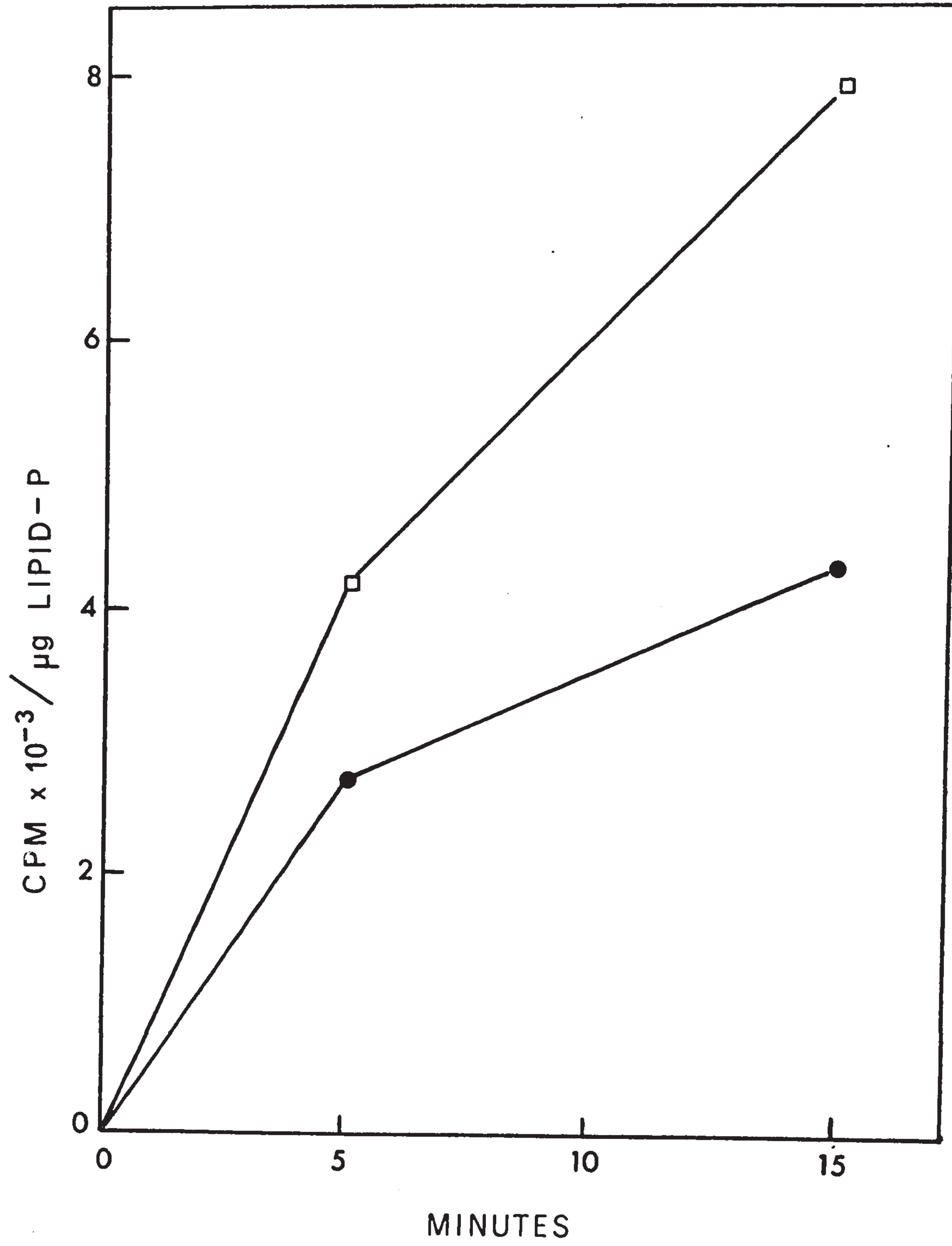
CPM x 10^{-3}/µg LIPID-P
0
1
2
3
4
0
20
40
MINUTES
CPM X 10^{-2}/µg LIPID-P
0
5
10
20
40
MINUTES
A(−)1
A(−)

TABLE I

Fractionation of ^{32}P-labelled lipids on a DEAE-cellulose column (10 cm x 1.25 cm diam.)

	Solvent	Volume (ml.)	Lipids eluted	cpm A(-)	cpm A(-)1
1.	Chloroform:methanol (9:1)	55	Neutrals	50	53
2.	Chloroform:methanol (7:3)	65	Phosphatidyl ethanolamine	340	748
3.	Methanol	40	wash	16	21
4.	Acetic acid	60	Phosphatidyl serine	174	252
5.	Methanol	60	wash	28	22
6.	Chloroform:methanol (4:1) containing 20 ml NH_3/1 and 0.01 M ammonium acetate	90	Phosphatidyl glycerol, phosphatidyl inositol, phosphatidic acid	2266	2926

Fig. 15. Uptake of sodium acetate-1-^{14}C into lipids of the parent, (●), and the mutant, (□), during early cortex formation.



CPM x 10⁻³/µg LIPID-P
8
6
4
2
0
0
5
10
15
MINUTES

TABLE 2

Fatty acid analysis of the parent A(-) and the mutant A(-)1 during vegetative growth and during mid-sporulation (60% phase white)

Fatty acid	Per cent of total fatty acids			
	A(-) vegetative	A(-)1 vegetative	A(-) sporulation	A(-)1 sporulation
12:0	trace	trace	trace	trace
13:0 iso	5.7	5.4	6.2	6.3
14:0 iso	3.1	3.2	1.8	1.5
14:0	2.7	2.8	2.9	2.9
15:0 iso	38.5	39.4	46.1	50.5
15:0	0.8	0.8	0.8	1.0
16:0 iso	7.9	7.8	5.2	2.6
16:0	6.5	6.0	7.4	5.1
16:1	15.1	14.0	9.9	9.3
17:0 iso	15.2	16.4	15.5	15.6
17:0	4.4	4.2	4.2	4.9

of A(-) and A(-)1 at varied stages throughout sporulation was carried out, and membrane surfaces were examined. At all times, in the parent, the outer surface of the outer spore membrane appeared lightly scattered with small granules (Fig. 16) similar to those described by Remsen (1966). In the mutant, this same appearance was observed in samples taken before whitening, that is when the mutant was functioning normally (Fig. 18). However, at the time of whitening when the mutant had been shown no longer capable of incorporating ^{3}H-DAP into mucopeptide, a severe change in the appearance of this surface was noticed. Rounded lumps and ridges had formed as if by bulging of the membrane outwards by excess membrane-bound material (Fig. 19a,b). This appearance remained until lysis began, when the whole structure began to break down (Fig. 20).

The inner surface of this outer membrane in both the parent and the mutant retained a lightly granulated appearance throughout (Fig. 17 and 21) again similar to that reported by Remsen (1966). Neither surface of the inner spore membrane appeared altered in the mutant compared to the parent at any time.

The effect of inhibition of the membrane-associated stages of normal cell wall mucopeptide synthesis on plasma membrane morphology was examined. Vancomycin (5µg/ml) and bacitracin (100µg/ml) were added to

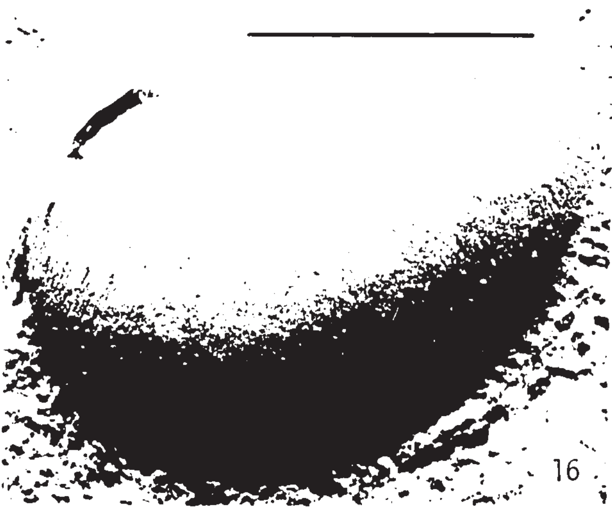
Fig. 16-19. Freeze-etchings of the parent, A(-), and the mutant A(-)1. In all figures the direction of metal shadowing is from the bottom. Magnification is shown by the 0.5 μm marker except in Fig. 19 in which a 0.1 μm marker is used.

Fig. 16. The outer, (cytoplasmic) surface of the outer forespore membrane of the control, A(-), during phase whitening.

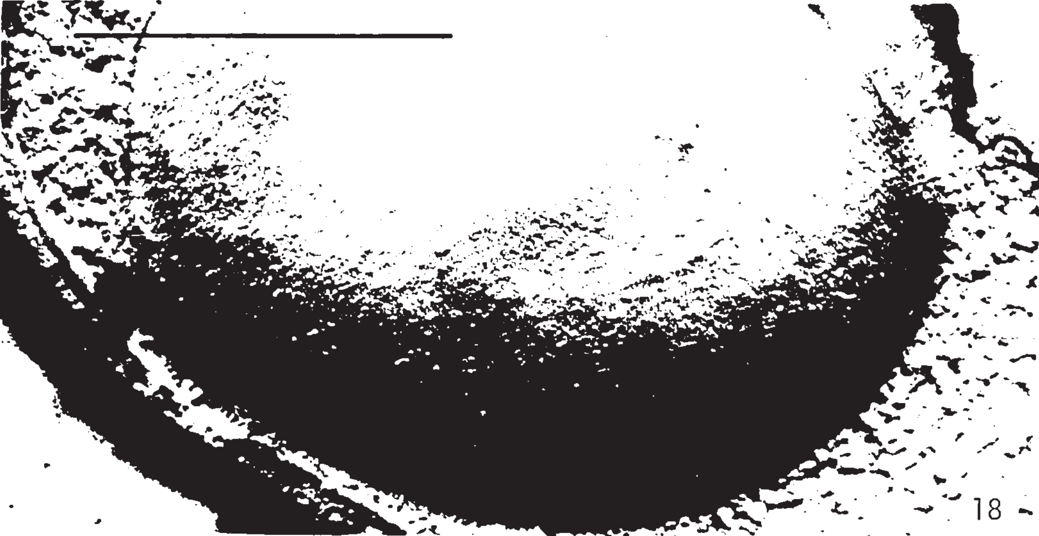
Fig. 17. The inner surface of the outer forespore membrane of A(-) during phase whitening.

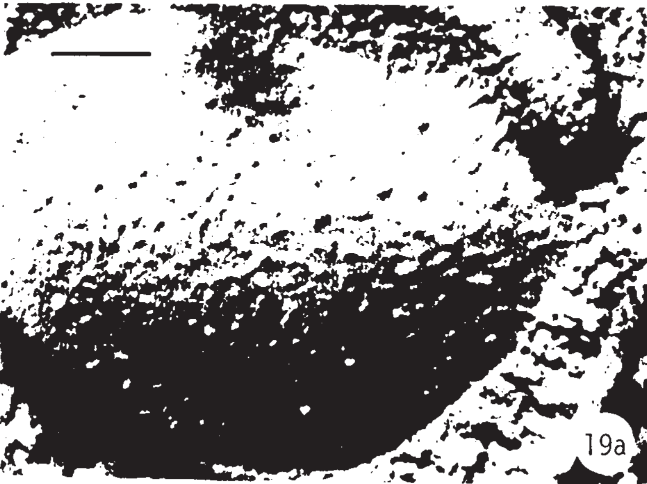
Fig. 18. The outer surface of the outer membrane of A(-)1 prior to phase whitening. Note the similarity to the control A(-), in Fig. 16.

Fig. 19(a,b) Two areas of the outer surface of the outer membrane of A(-)1 at the end of phase whitening, showing the lumps and ridges suggesting a surface accumulation.


16

17


18

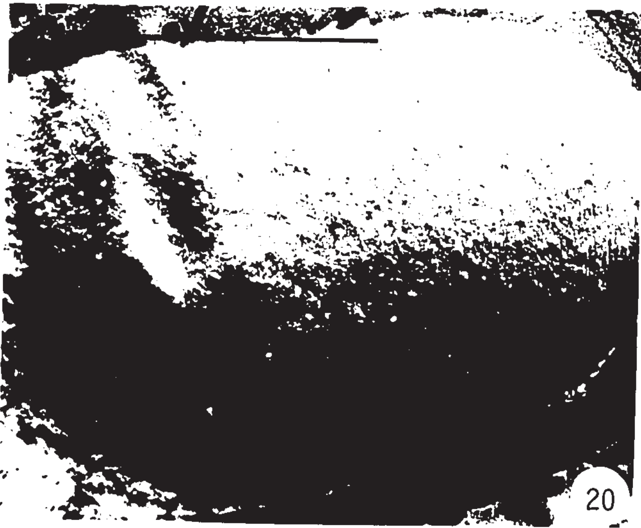

19a

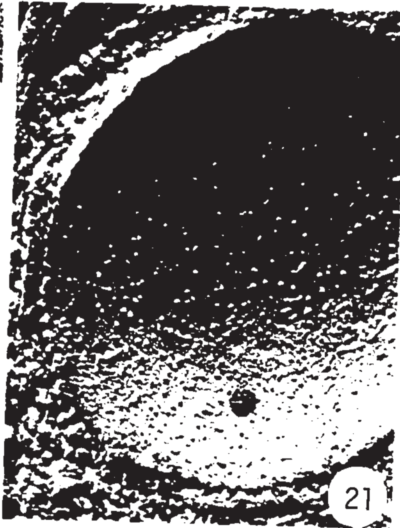
19b

Fig. 20-21. Freeze etchings of the mutant, A(-)1. In both figures, the direction of metal shadowing is from the bottom. Magnification is shown by the 0.5 μm marker in Fig. 20.

Fig. 20. The outer surface of the outer membrane at early lysis showing slight folding, and the now less lumpy surface.

Fig. 21. The inner surface of the outer forespore membrane during phase whitening shows no abnormality compared with the parent, A(-), in Fig. 17.


20


21

cells in mid-logarithmic phase of growth. After 30 min and 45 min respectively, samples were centrifuged and quick frozen for freeze etching. Similar defects to that in the mutant were not seen, but marked changes in the inner surface of the plasma membrane of bacitracin-treated cells (Fig. 22) compared to the normal membrane (Fig. 23) were observed. Thus, interference with mucopeptide synthesis can give rise to altered membrane morphology. The vancomycin-treated cells lysed rapidly; in those remaining intact at the time of freeze etching, the mesosomes appeared to stand out more clearly than usual, but no overall change in membrane structure was visible.

The results in this section indicate that the outer forespore membrane is the site of the lesion in A(-)1.

Further Biochemical Localisation of the Lesion in A(-)1.

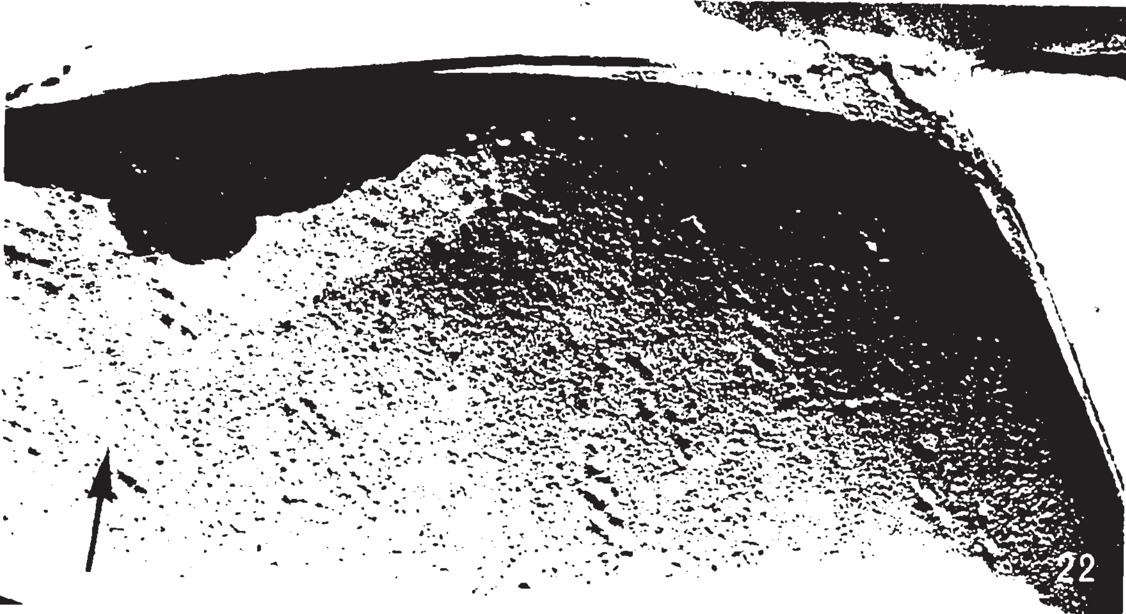
(a) Effect of the lesion on soluble intermediates of cortical mucopeptide synthesis.

Strominger (1957) has used the estimation of N-acetylamino sugar esters as an indication of the amount of soluble precursors of cell wall mucopeptide in normal and antibiotic treated cells of *Staphylococcus aureus* and related organisms. With this procedure, the total amount of these intermediates (mainly UDP-N-acetylmuramic pentapeptide and

Fig. 22-23. Freeze etchings of the parent, A(-). The direction of metal shadowing is shown by the arrows. Magnification is shown by the 0.5 μm marker in Fig. 23.

Fig. 22. The inner surface of the plasma membrane treated with bacitracin (100 μg/ml). Note the abnormal ridges and depressions.

Fig. 23. The inner surface of the plasma membrane untreated.


22

23

tri-peptides) was estimated on 200 ml of culture of A(-) and A(-)1 during mid-cortex formation (70% phase white), and on 400 ml of cultures after engulfment. Results are shown in Table 3 and the ratio of intermediates in A(-)1 to A(-) calculated. An accumulation of these precursors occurs in the mutant at the time of cortex formation but not during germ cell wall formation (after engulfment).

The slight increase in A(-) over A(-)1 in the latter case is probably due to the beginnings of cortex formation since this process overlaps germ cell wall synthesis in A(-), (see Fig. 9).

Although the total amount of precursors varies, within each experiment the total growth of A(-) and A(-)1 is comparable since the same batch of mixed medium was used.

(b) *In vitro* studies on mucopeptide synthesis.

Precursors of both cell wall and cortical mucopeptide synthesis have to be transported through a membrane barrier to the site of polymerisation. The chemistry of the lipid-bound intermediates of cell wall synthesis has been well documented (Anderson and Strominger, 1965; Anderson *et al.*, 1965; Anderson *et al.*, 1967). To investigate the nature of such

TABLE 3

N-acetylamino sugar ester content of A(-) and A(-)1 during germ cell wall formation (after engulfment) and cortex formation (70% phase white).

	μmoles of N-acetylamino sugar esters/200 ml culture.		
	A(-)	A(-)1	Ratio $\frac{A(-)1}{A(-)}$
Exp. 1 (70% phase white).	0.35	0.816	2.33
Exp. 2 (70% phase white).	0.236	0.57	2.41
Exp. 3 (after engulfment).	0.075	0.06	0.8
Exp. 4 (after engulfment).	0.122	0.103	0.84

intermediates in cortex synthesis in A(-) and to determine if A(-)1 is capable of their formation, a cell free system for synthesis of both vegetative and spore mucopeptide was developed. Such a system was necessary, since addition of ^{3}H-DAP to whole cells adapted to its uptake, did not allow sufficient incorporation of label for detection of such lipid-intermediates, (see section C for further details).

Vegetative mucopeptide synthesis. An homogenate of cells from 80 ml of culture in mid-logarithmic growth (0.8 O.D. at 650 nm), suspended in 10 ml of 0.1M Tris-HCl buffer, pH 7.8, was added to a flask containing sufficient of the following compounds to give final concentrations as indicated:

10^{-3}M lysine	10^{-5}M UTP
10^{-5}M alanine	10^{-3}M $MgCl_2$
10^{-5}M glutamic acid	10^{-4}M $MnCl_2$
10^{-5}M N-acetylglucosamine	5×10^{-4}M ATP

0.5 μCi ^{3}H-DAP/ml

This incubation mixture was adapted from a variety of sources dealing with cell wall chemistry appearing in Methods in Enzymology, vol. VIII, Academic Press, New York.

The mixture was shaken at room temperature for 30 min. 2.5 ml of cold 40% TCA was added and the flask left for one hour in the cold. Mucopeptide was

extracted by the method of Park and Hancock (1960), made up to 10 ml volume and 0.05 ml aliquots filtered and counted for radioactivity.

Total incorporation of ^{3}H-DAP was 73,800 cpm. indicating some synthesis of mucopeptide.

Spore mucopeptide synthesis. Homogenates (20 ml volume) were prepared from 200 ml cultures of A(-) and A(-)1containing 70% phase white forespores. 17 ml of these were added to flasks containing additives to the final concentrations shown in the previous section. The total volume in the flasks was 20 ml. After 25 min incubation at room temperature, 5 ml of cold 40% TCA was added to each, and mucopeptide extracted and radioactivity estimated.

One ml of the original homogenate was kept for total protein estimations and 2 ml were centrifuged and the supernatant retained for protein assay. The amount of protein released to the supernatant indicates the relative efficiency of the cell disruption.

Results are seen in Table 4, Exp. 1. The parent strain, A(-), is capable of extensive incorporation into mucopeptide, while the mutant, A(-)1, incorporates very little. These results parallel those obtained *in vivo*. The slight incorporation in A(-)1 is probably due to residual germ cell wall synthesis. The extent of this was variable in three experiments, due to differences in synchrony and sampling time of the cultures.

To ensure that the lowered incorporation of A(-)1 was due to the expression of the biochemical lesion only at the time of normal cortex synthesis, a similar experiment was carried out on cells of both strains during germ cell wall formation, i.e. 30 min after engulfment in the majority of the population. *In vivo* studies had shown a similar synthesis of mucopeptide in A(-)1 at this time compared to the parent.

The results are shown in Table 4, Exp. II. Now, incorporation of ^{3}H-DAP into mucopeptide of A(-)1 was of the same order as that into A(-). The relatively slight difference in total incorporation could be due to difficulty in timing of the cultures to the same stage of sporulation since observation by phase contrast microscopy is rather imprecise at this period of sporulation. Some cortex formation may also have been initiated by A(-) as the processes of cortex formation and germ cell wall synthesis overlap.

The possibility that the mutant's inability to form cortex was due to the production of an inhibitor at the time of cortex formation was next investigated, although the marked changes in lipid metabolism and membrane structure of the mutant made this seem unlikely. Equal volumes of homogenates of A(-) and A(-)1 cultures (at 70% phase whitening) were mixed and added to the test system for incorporation of

TABLE 4 Incorporation of ^{3}H-DAP into mucopeptide by homogenates of A(-) and A(-)1 individually and mixed (1:1, v/v).

	System	Total cpm	Total protein in homogenate mg	cpm/mg of homogenate protein	Total protein in supernatant mg	cpm/mg of supernatant protein	A(-)/A(-)1 cpm/mg protein
I.	A(-) at 70% phase white	231,000	65.62	3,670	48.25	4,830	63.2
	A(-)1 at 70% phase white	3,800	65.62	58	49.48	76	
II.	A(-) after engulfment	11,900	61.9	192	53.7	221	1.44
	A(-)1 after engulfment	8,250	61.9	133	53.1	155	
III.	A(-) at 70% phase white	30,710	26.9	1,140	24.48	1,250	23.1
	A(-)1 at 70% phase white	1,160	24.48	47	19.38	54	
	A(-) plus A(-)1 at 70% phase white (1:1, v/v)	26,860	25.69	1,045	21.93	1,224	

Concentration of ^{3}H-DAP used: Exp. I, 2.5 µCi/ml; Exp. II, 0.5 µCi/ml; Exp. III, 1.25 µCi/ml.

^{3}H-DAP into mucopeptide as described. The two homogenates were also assayed and protein was estimated in each. If a soluble inhibitor were present in the A(-)1 homogenate, it is feasible that it would inhibit the A(-) system to some extent. Results are shown in Table 4, Exp. III.

Again the marked difference between incorporation into A(-) and A(-)1 was observed. If no interaction took place between the two systems, then the mixture should have incorporated the sum of half the total incorporations of A(-) and A(-)1, that is, 15,965 cpm. No inhibition was found; on the contrary, a striking increase (69%) over the theoretical value was observed. This was probably due to production by the mutant of soluble precursors which would increase the pool size of these intermediates available for use by the complete system of A(-) for mucopeptide synthesis. That the mutant can produce such precursors has already been demonstrated.

It is clear from the previous experiments with the mutant, that when 70% of forespores have become phase white, the germ cell wall synthesising system contributes little to the total incorporation of ^{3}H-DAP into mucopeptide. Hence, this system should allow a study of cortical mucopeptide synthesis alone. The effects of antibiotics in this cell free

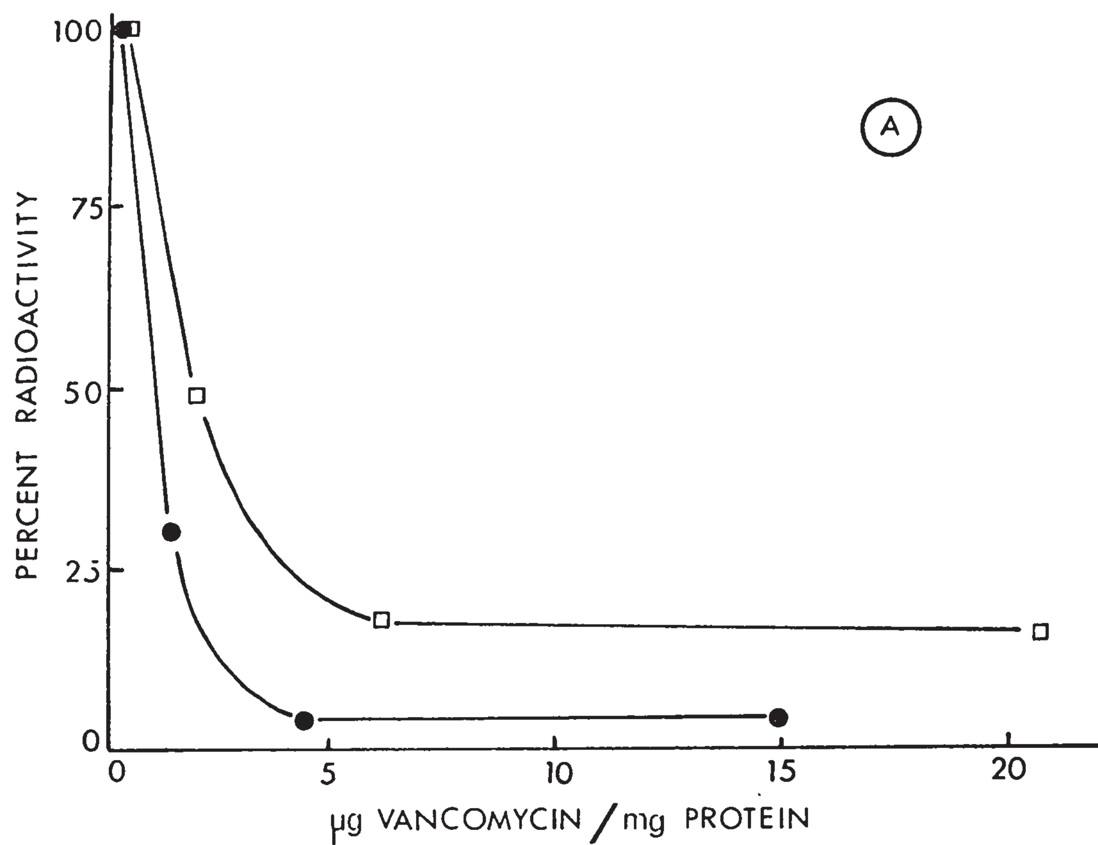
system were compared with those in cell wall mucopeptide synthesis also in the same cell free system.

A range of concentrations of vancomycin, bacitracin and methicillin (rather than penicillin since *B. cereus* produces a potent penicillinase; Pollock, 1950) was used, and ability of homogenates to incorporate ^{3}H-DAP into mucopeptide assayed as described previously. Results are plotted as percent of activity of untreated control against µg antibiotic/mg protein (Fig. 24 and 25). The total amount of vegetative cells in mid-logarithmic growth was such that the amount of protein in the homogenate was similar to that in homogenates from the sporulating cells.

A slight difference in sensitivity to vancomycin was seen, whereas sensitivity to bacitracin was almost identical in both systems. However, methicillin caused a marked reduction in activity of the cortical system but cell wall synthesis as one would expect was virtually unaffected at low concentrations of antibiotic. Some inhibition was seen at higher concentrations.

This difference in sensitivity to methicillin of the cell wall and cortical mucopeptide synthesising systems suggested a study of the effect of methicillin on germ cell wall mucopeptide synthesis. For this,

Fig. 24. Effect of concentration of (a) vancomycin and (b) bacitracin on incorporation of ^{3}H-DAP into mucopeptide *in vitro*. □ , cell wall mucopeptide synthesis; ● , cortical mucopeptide synthesis.


100
75
50
25
0
PERCENT RADIOACTIVITY
A
0
5
10
15
20
μg VANCOMYCIN / mg PROTEIN

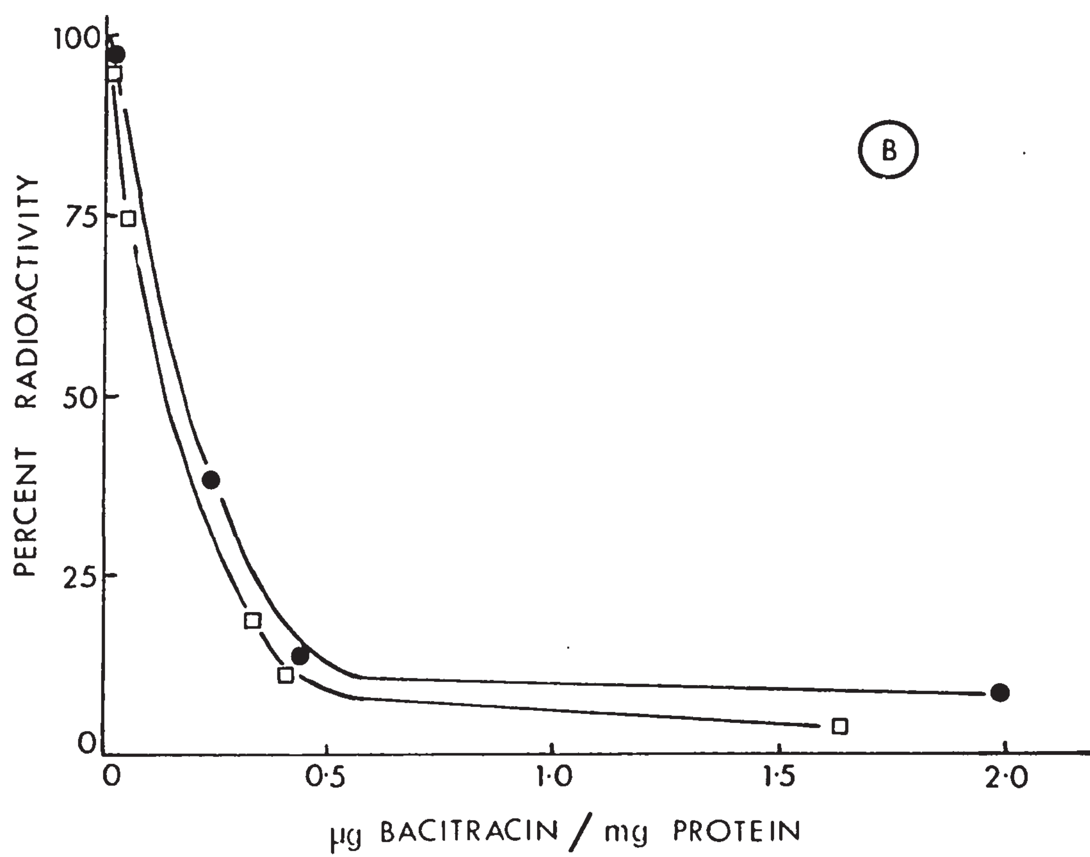
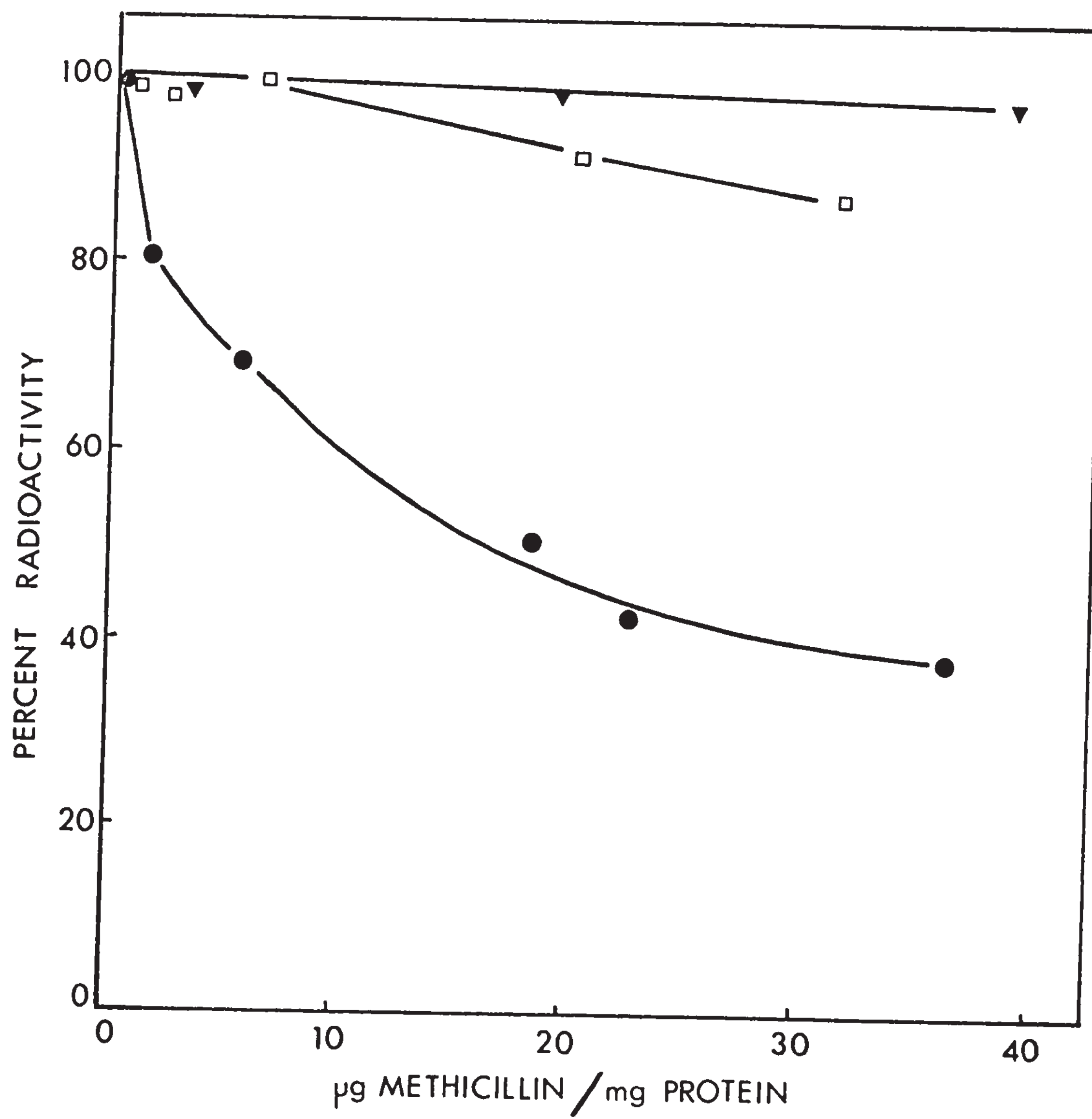

100
75
50
25
0
PERCENT RADIOACTIVITY
B
0
0·5
1·0
1·5
2·0
μg BACITRACIN / mg PROTEIN

Fig. 25. Effect of concentration of methicillin on incorporation of ^{3}H-DAP into mucopeptide *in vitro*.

□ , cell wall mucopeptide synthesis;

● , cortical mucopeptide synthesis;

▼ , germ cell wall mucopeptide synthesis.

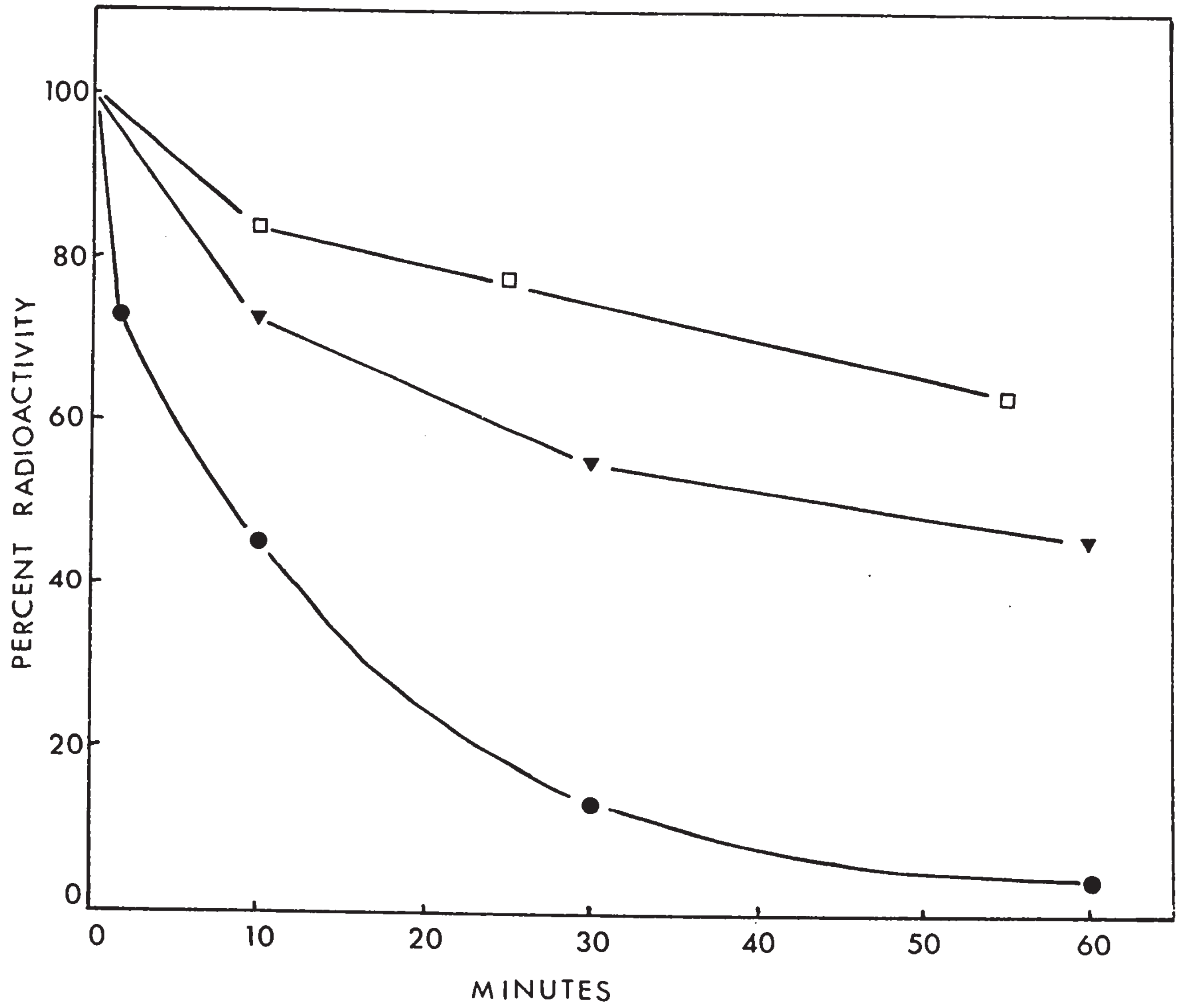


PERCENT RADIOACTIVITY
100
80
60
40
20
0
0
10
20
30
40
μg METHICILLIN / mg PROTEIN

an homogenate from A(-)1 cells at early sporulation stage 4, was used since, in this mutant, there is no interference by cortical mucopeptide formation. Little effect by methicillin on incorporation of ^{3}H-DAP into mucopeptide by this system was found (Fig. 25).

Since it is known that in *B. cereus* strains cell wall and germ cell wall are resistant to lysozyme digestion, while the cortex is sensitive (Warth *et al.*, 1963b), the lysozyme sensitivity of the radioactive products of the *in vitro* mucopeptide synthesising systems was investigated. In these experiments, homogenates (5 ml final volume) were allowed to incorporate ^{3}H-DAP for 30 min and were precipitated with TCA as usual. The pellet from the TCA precipitate was washed twice with water to remove TCA and was suspended in 5 ml of 0.1M Tris-HCl buffer, pH 7.8. Samples were run through filters, before and after incubation with lysozyme (100 μg/ml) at 37 C. The amount of radioactivity remaining in insoluble material after lysozyme digestion is expressed as a percent of that present before addition of lysozyme. The cortical product is seen to be quickly and almost completely digested, while those of the cell wall and germ cell wall systems are only partially digested at a much slower rate (Fig. 26).

Fig. 26. Digestion by lysozyme (100 μg/ml) of the ^{3}H-DAP labelled products of *in vitro* systems for the synthesis of cell wall mucopeptide, □ , cortical mucopeptide, ● , and germ cell wall mucopeptide, ▼ .


PERCENT RADIOACTIVITY
100
80
60
40
20
0
0
10
20
30
40
50
60
MINUTES

Thus, from the difference in both sensitivity to methicillin and susceptibility to digestion by lysozyme, these *in vitro* systems appear to be capable of producing mucopeptides of specific characteristics.

These cell wall and cortical mucopeptide synthesising systems were used in attempts to detect the lipid intermediates of mucopeptide synthesis as described in the next section of this project.

(c) Studies on lipid intermediates of mucopeptide synthesis.

Because of the altered lipid metabolism of the mutant during cortex formation, and the abnormal appearance of the outer forespore membrane, the lesion appeared to be manifested in this membrane system. The mutant cannot form polymerised cortical mucopeptide either *in vivo* or *in vitro*, yet it does have a supply of soluble precursors again indicating the membrane barrier as the site. Hence, a more detailed study was attempted on the nature of the lipid intermediates in A(-) and A(-)1. It was hoped that some differences would be found between the two strains, thus allowing more precise definition of the lesion in A(-)1, and giving information on the nature of these intermediates in normal cortical synthesis.

Since cell wall mucopeptide synthesis has been well

studied, attempts were first made to detect these lipid intermediates in vegetative cells.

As mentioned in the previous section, in the first experiment of this type, ^{3}H-DAP was added to a suspension of whole cells. In order to increase the relative concentration of label, 100 ml of cells in logarithmic growth (0.9 O.D. at 650 nm), previously trained for uptake of ^{3}H-DAP, were centrifuged, suspended in 10 ml of the supernatant and added to a warmed flask (30 C) containing 100 µCi of ^{3}H-DAP (10 µCi/ml final concentration). After 25 min incubation with shaking, the suspension was centrifuged, the cells washed once with Grelet salts, and the pellet extracted for total lipids. These were fractionated on a DEAE-cellulose column. After evaporation of solvent, the lipid from each fraction was made up to 5 ml volume with chloroform: methanol (2:1), and 1 ml aliquots counted for radioactivity.

Fraction 8 should contain the lipid intermediate (Strominger *et al.*, 1966). Radioactivity was found in all fractions containing the major classes of lipids: i.e. fraction 1,2,4,5,6, and 8, (Table 5).

To see whether the ^{3}H-DAP in fraction 8 was incorporated into a lipid intermediate compound, the remaining lipid of this fraction was run through a silicic

TABLE 5

Fractionation on a DEAE-cellulose column (8 cm x 1.25 cm diam.) of lipids from vegetative cells of A(-), after 25 min incubation with ^{3}H-DAP (10µCi/ml).

	Fraction	Volume of solvent (ml)	Total cpm.
1.	chloroform: methanol (7:1)	44	3,600
2.	chloroform: methanol (7:3)	55	3,200
3.	methanol	35	650
4.	chloroform: acetic acid (3:1) containing 1.0 mM potassium acetate	50	4,950
5.	acetic acid	50	1,175
6.	chloroform: methanol (4:1) containing 10 ml NH_3/l	75	3,050
7.	methanol: 1M pyridine acetate, pH 4.2 (1:1)	60	270
8.	methanol: 6M pyridine acetate, pH 4.2 (1:1)	60	10,000

acid column (1.25 x 10 cm). Cell wall lipid intermediates should be eluted with chloroform: methanol, 1:1, v/v (Strominger *et al.*, 1966). Radioactivity was not found in this fraction. Only in the final polar fraction eluted by 80% methanol containing some acetic acid and water, did radioactivity appear, indicating that the counts appearing in fraction 8 from the DEAE-cellulose column were probably due to non-specific binding of ^{3}H-DAP to lipid.

To avoid the problem of dilution of radioactive label due to the presence of excess unlabelled DAP used to train the cells for uptake of ^{3}H-DAP, the above study was repeated using a cell-free system for synthesis of mucopeptide. This has been described in detail in the previous section. For detection of lipid intermediates, the concentration of ^{3}H-DAP was increased to 10 μCi/ml. After 25 min incubation at room temperature, two volumes of butanol: 6M pyridine acetate, pH 4.2, (2:1) were added and lipids extracted into the butanol phase.

Again DEAE- cellulose chromatography was used to fractionate the lipids, and silicic acid chromatography of fraction 8 was carried out. The results were again negative, as in the previous experiment, in that some radioactivity was found in fraction 8 of the DEAE-cellulose column, but on elution from the

silicic acid, it did not appear in the chloroform: methanol (1:1) fraction.

Thus, use of ^{3}H-DAP as a label proved unsatisfactory for detection of these intermediates, either in an *in vivo* or *in vitro* system.

In final attempts to label the lipid intermediates of mucopeptide synthesis, the soluble precursor, UDP-N-acetyl muramic pentapeptide, labelled in the peptide side chain with ^{3}H-DAP was prepared and added to the cell-free system. The intermediate was obtained from vegetative cells grown in the presence of ^{3}H-DAP under conditions in which build-up of the soluble intermediate occurred. Details of the actual preparation are given in the Methods. However, the precise conditions for maximum accumulation had first to be determined.

Accumulation of soluble intermediates of cell wall synthesis in *S. aureus* in the presence of penicillin had been observed by Park and Johnson (1949), and the compounds characterised by Park (1952a,b,c). Strominger (1957) further investigated the biological parameters of this accumulation in *S. aureus* and other organisms. In this present study, no such accumulation was found in *B. cereus*, A(-). Methicillin (50 μg/ml) added to the culture in mid-logarithmic growth, caused lysis

within 30 min. If cells were centrifuged and suspended in the same volume of Grelet salts containing 0.3% glucose, 0.05% casamino acids, 0.3M sucrose and 10^{-3}M $MgCl_2$, then addition of 50 μg of methicillin did not cause lysis for at least 90 min. However an insignificant accumulation of intermediates was found (Table 6). The optical density of a control culture diluted to the same density but lacking sucrose was used for calculating results in this experiment and those following.

As methicillin was unsuccessful in producing the desired build-up of soluble cell wall intermediates, vancomycin which inhibits the utilisation of the lipid intermediates of cell wall synthesis (Anderson *et al.*, 1967) and causes an accumulation of these soluble precursors in *S. aureus* (Reynolds, 1961), was next used. As in the methicillin experiments, cells from mid-logarithmic growth were centrifuged and suspended in Grelet salts containing 0.3% glucose, 0.05% casamino acids, 0.3M sucrose and 10^{-3}M $MgCl_2$.

The effect of vancomycin concentration on the amount of N-acetyl amino sugar esters accumulated is shown in Table 7. A 30 min incubation at 30 C with aeration was used. A 5-fold increase of precursors was observed with 5 μg of vancomycin/ml.

The time course of this accumulation was studied

TABLE 6

Effect of methicillin on accumulation of N-acetylamino sugar esters. A 30 min incubation at 30 C was used. Results are expressed as µmoles/l at O.D. of 1.0.

	Control	50 µg methicillin/ml
Exp. I	4.07	5.07
Exp. II	4.9	5.1

TABLE 7

Effect of vancomycin concentration on accumulation of N-acetylamino sugar esters.

Vancomycin µg/ml	µmoles of N-acetylamino sugar esters/litre at O.D. of 1.0.
0	4.2 6.0
1	15.0
5	25.8 25.2
20	24.2
50	23.5

TABLE 8

Effect of time of incubation with vancomycin on accumulation of N-acetylamino sugar esters.

Time of incubation with vancomycin (5 µg/ml) in min.	µmoles of N-acetylamino sugar esters/litre at O.D. of 1.0.
0	5.2
30	19.3
60	22.3
90	24.6

(Table 8).

For the preparation of radioactively labelled precursor, 60 min incubation with 5 μg of vancomycin/ml was used (see Methods).

As the amount of labelled precursor prepared was limited, it was used directly in the spore system rather than in that of vegetative cells. In the first experiment, about 1 μCi of precursor (1 μmole) was added to homogenates from 200 ml of cultures of A(-) and A(-)1 at 70% phase whitening, in a final volume of 20 ml and containing 5 x 10^{-4}M ATP, 10^{-5}M UTP, 10^{-5}M N-acetylglucosamine, 10^{-3}M $MgCl_2$, and 10^{-4}M $MnCl_2$. After 5 min at room temperature, lipids were extracted, chromatographed on DEAE-cellulose, and fractions counted for radioactivity. Radioactivity was scarcely above background in any fraction. In a second experiment, about 10 μCi of precursor (2 μmoles) were added to each test flask as above, and an incubation time of 30 min at 30 C was used. Fraction 8 from DEAE-cellulose chromatography was found to contain 20,000 cpm from the A(-) extract and only 5000 cpm from the A(-)1 extract. After silicic acid chromatography, for the first time in these lipid experiments, some radioactivity appeared in fractions other than the final polar fraction (Table 9) whereas previously radioactivity had appeared only in this fraction. This indicates that

TABLE 9

Silicic acid chromatography of fraction 8 from a DEAE-cellulose column, after incorporation of UDP-N-acetyl muramic pentapeptide (labelled with ^{3}H-DAP) into lipids of A(-) and A(-)1.

Fraction	cpm A(-)	cpm A(-)1
1. 30% methanol 70% chloroform	147	86
2. 42.5% methanol 57.5% chloroform	207	45
3. 55% methanol 45% chloroform	302	54
4. 65% methanol 35% chloroform	109	20
5. 80% methanol 10% chloroform 5% acetic acid 5% water	110	104

radioactivity from the labelled precursor had been incorporated into relatively nonpolar lipid-like material. Only 10% of the total applied to the column was recovered. The remaining activity must have been tightly bound to the silicic acid.

More of this lipid-like material was formed by the parent, A(-), than the mutant, A(-)1, suggesting that A(-)1 may partially lack the ability to form mucopeptide lipid intermediates. The low incorporation of labelled soluble precursor did not allow further detailed chemical analysis to be carried out.

Refractility in Cortexless Spores of *B. cereus* var. *alesti*.

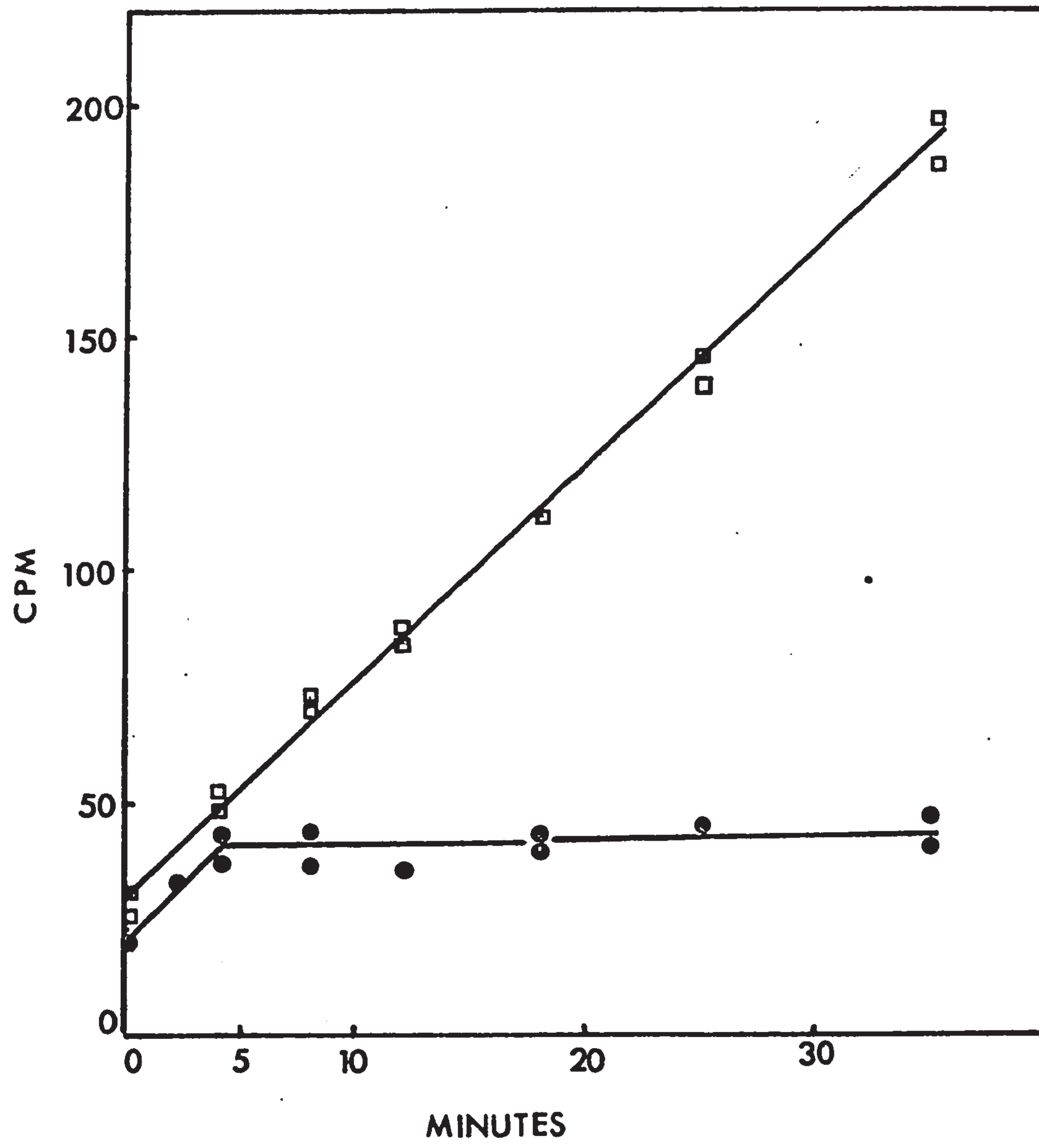
(a) Studies on the mutant, A(-)1.

The cortex of bacterial spores has been implicated in the mechanism of dehydration of the spore core, and consequently in the acquisition of refractility and heat resistance (Murrell, 1967; Lewis, 1969; Vinter, 1969). Since a transient refractility terminated by the onset of lysis, had been observed in the cortexless mutant, A(-)1, the process of refractility in this organism was further investigated. Actinomycin D was added at times through sporulation stages 4 and 5 to prevent synthesis of lytic enzyme, thus halting the terminal lysis and hopefully allowing the

full expression and maintenance of the refractile state. That actinomycin D at 10 μg/ml does inhibit RNA synthesis was first determined. Uptake of uracil-2-^{14}C in the presence and absence of actinomycin D was followed (Fig. 27).

Morphology. Fig. 28 at 21 hr aeration shows the effect of addition of actinomycin D (10 μg/ml) at 30% phase whitening (11½ hr aeration). Many of the spore forms are held in a phase bright stage. The control spores (Fig. 29) have undergone terminal lysis with lysed spore hulls as the final product of sporulation. In most cultures of A(-)1 treated with actinomycin D at times varying from prior to any phase whitening in the culture to 75% phase whitening, only 50-60% of the spore structures remained in a phase bright state. This presumably is due to lack of complete synchrony in the culture. At any time of addition there would be only a proportion of cells sufficiently advanced to attain phase refractility yet not so far advanced that lysis would occur. Use of the nigrosin smear technique to test for refractility showed that those spores maintained in a whole state by actinomycin D appeared quite refractile (Fig. 30). They also appear smaller and rounder than the normal A(-)1 spore. If addition was delayed until most of the culture had phase whitened, an actual decrease in size of the spore forms

Fig. 27. The effect of actinomycin D (10 μg/ml) on incorporation of uracil-2-^{14}C by A(-) during mid-sporulation (10% phase white forespores). Actinomycin D treated cells, ● ; untreated control, □ .



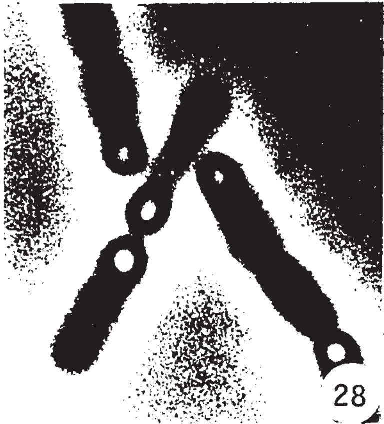
200
150
100
50
0
CPM
0
5
10
20
30
MINUTES

Fig. 28-30 Magnification as shown by the 5-μm marker in Fig. 28.

Fig. 28. Phase contrast photograph of A(-)1 10 hr after treatment with actinomycin D at 30% phase whitening of the culture. The fore-spores have remained phase bright.

Fig. 29. Phase contrast photograph of A(-)1 untreated, taken at the same time as Fig. 28. Mainly lysed spore hulls are seen.

Fig. 30. Nigrosin smear of A(-)1 photographed 8 hr after addition of actinomycin D at 1% phase whitening in the culture, showing bright refractile forespores in many cells.


28

29

30

resulted.

In electron micrographs of thin sections, refractility is characterised by a lack of detail within the spore core. Concurrently the DNA condenses into semi-crystalline masses at the periphery. In the mutant A(-)1, at peak phase whitening some refractility is seen (Fig. 6a,6b). Here, by the addition of actinomycin D, the degree of refractility was increased and maintained as synthesis of the lytic enzyme was prevented thus halting terminal lysis. Fig. 31 shows a dense, presumably dehydrated spore core within the sporangium which is seen in some areas to be lysing internally. The germ cell wall is seen as a narrow band between the forespore membranes. Freeze fracture etching also revealed the characteristic dense appearance of refractile spore core cytoplasm compared to the more granular "spongy" sporangial cytoplasm (Fig. 32). Fig. 33 shows a cell quick frozen at a later time than that in Fig. 32. The sporangial cytoplasm has lysed leaving the refractile spore core intact. A freeze etching of a fully coated refractile spore of the parent strain A(-) is shown for comparison of the degree of refractility obtained (Fig. 40).

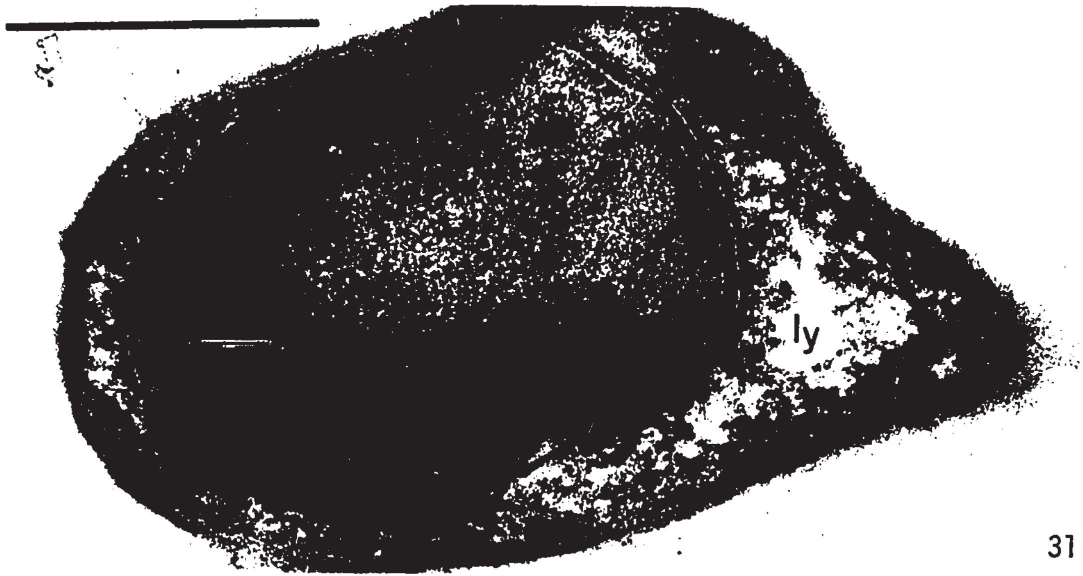
<u>^{45}Calcium uptake and DPA content</u>. DPA synthesis and ^{45}Ca uptake in A(-)1 approached that of the parent

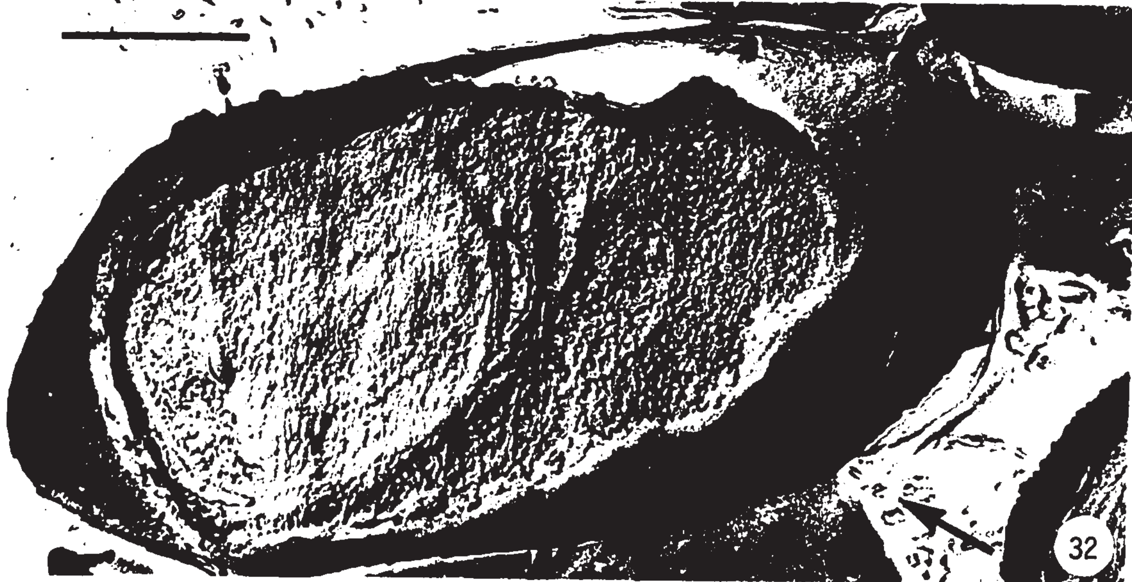
Fig. 31-33. Magnification as shown by the 0.5 μm markers.

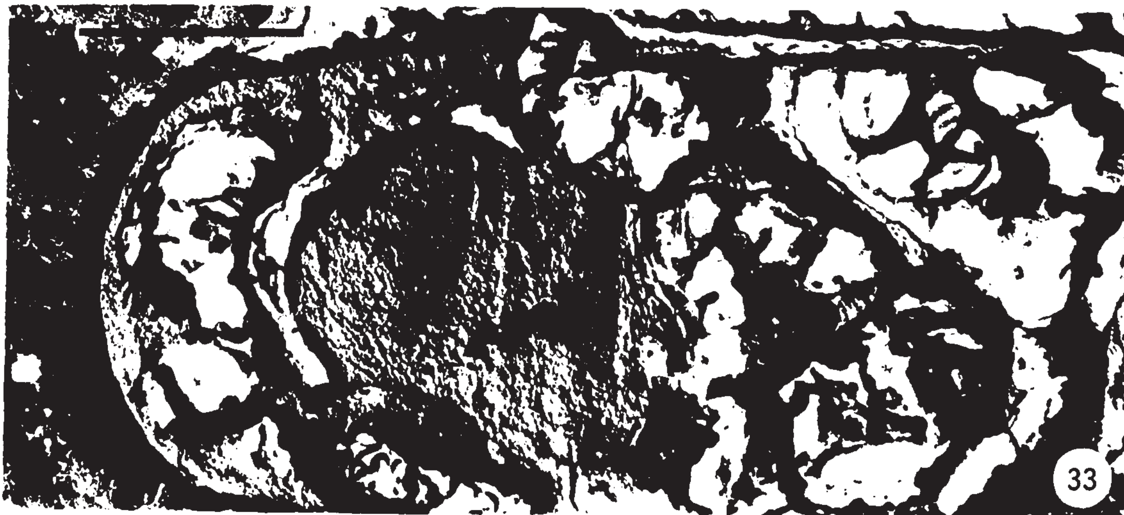
Fig. 31. Thin section electron micrograph of A(-)1 9 hr after addition of actinomycin D at 5% phase whitening. The spore core has become refractile. The germ wall (gcw) is seen lying between the inner (im) and outer (om) forespore membranes. The sporangial cytoplasm has partly lysed (ly).

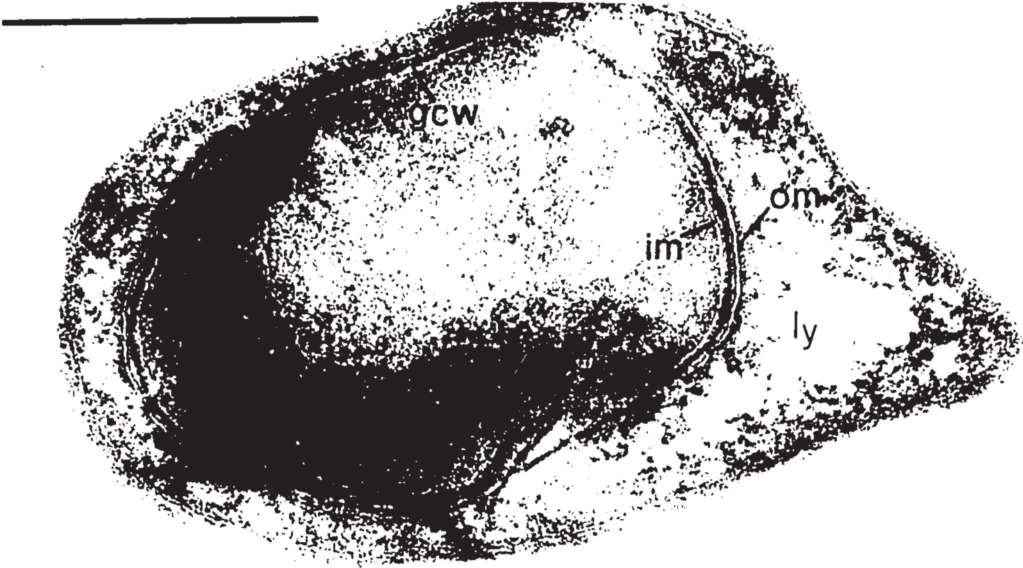
Fig. 32. Freeze-etching of A(-)1 7 hr after actinomycin D addition at mid-phase whitening. The spore core appears more dense in texture than the sporangial cytoplasm. The direction of shadowing is shown by the arrow.

Fig. 33. Freeze-etching of A(-)1 12 hr after actinomycin D addition just prior to phase whitening. The sporangial cytoplasm has lysed while the spore core remains intact. The direction of shadowing is shown by the arrow.

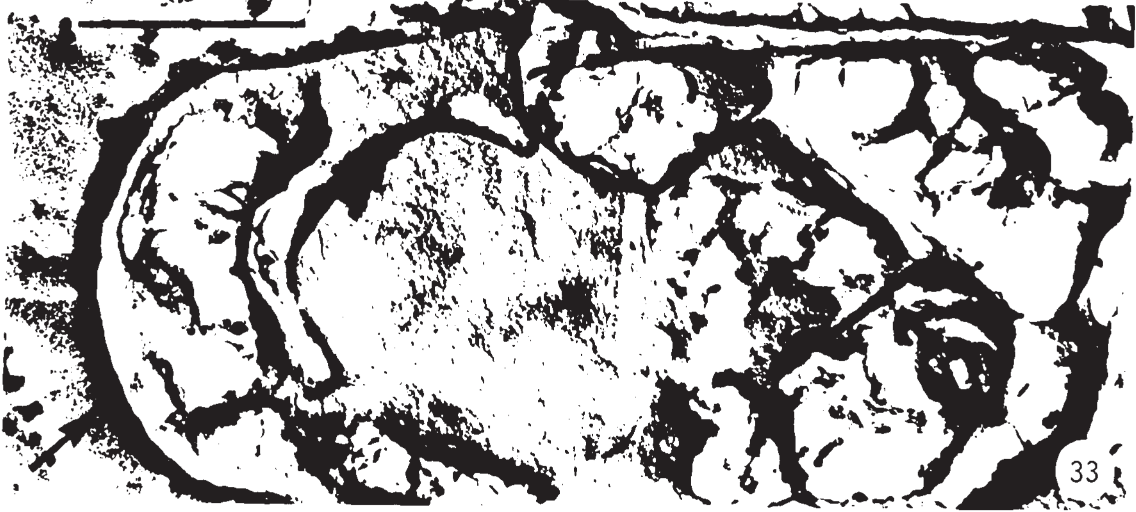

ly
31


32


33


gcw
om
im
ly
31

32


33

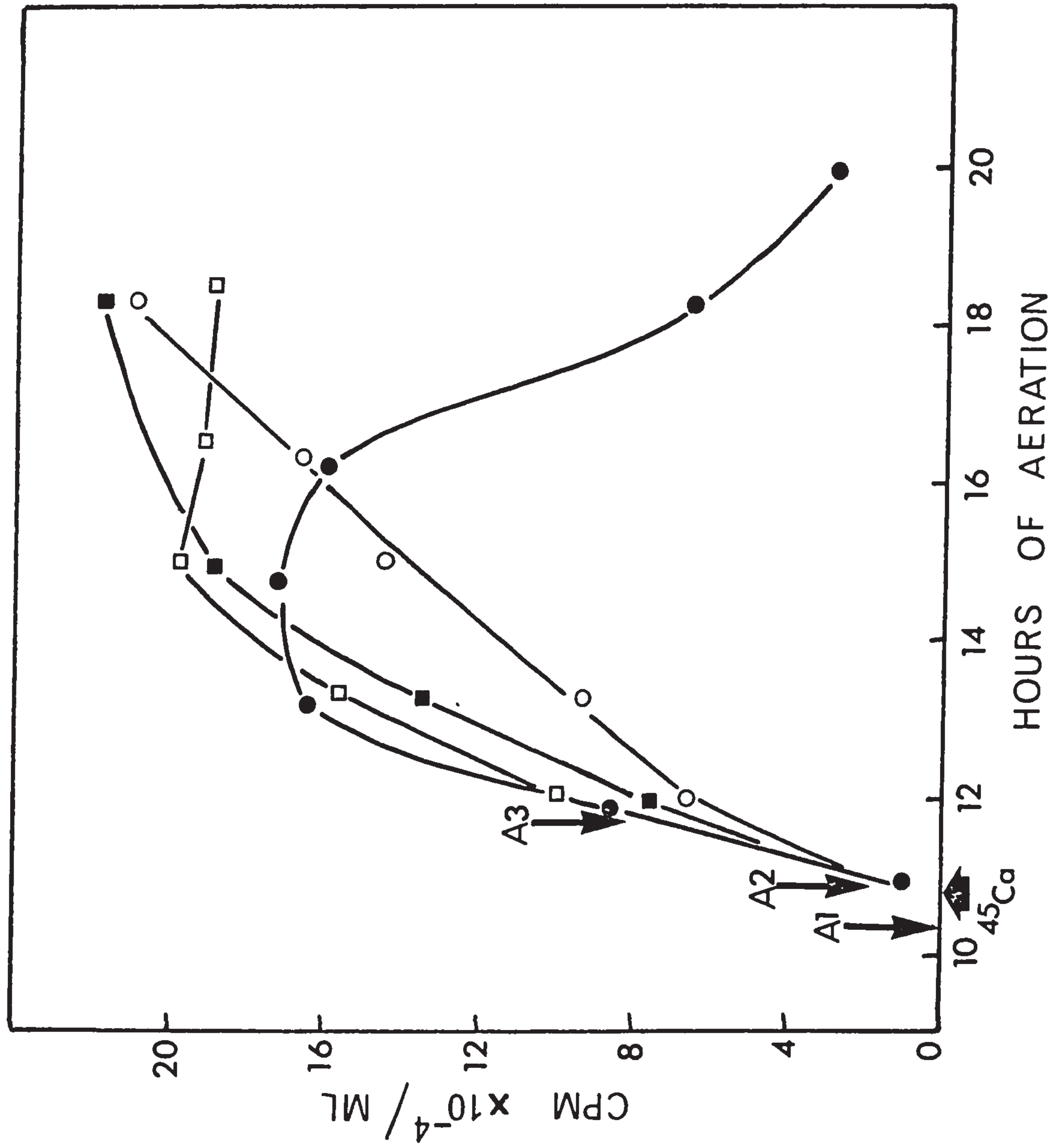
A(-), but these components were lost during the terminal lysis (Fig. 8). In the present study, ^{45}Ca levels were maintained and sometimes increased by the addition of actinomycin D at appropriate times during the sporulation process (Fig. 34). However, even at addition A3 (35% phase white), some lysis does occur causing a slight drop in the ^{45}Ca level. This effect becomes more marked if actinomycin addition is further delayed. The effect of actinomycin D on DPA synthesis was much more marked in that synthesis was inhibited within 90 min or less after addition (Fig. 35), whereas Ca uptake continued for many hours (Figs. 34 and 35). However, the levels of DPA attained were held for many hours. Higher levels of DPA than those shown in Fig. 35 could not be obtained by delaying actinomycin D addition. Such later additions allowed some synthesis of lytic enzyme which led to a drop in DPA and ^{45}Ca content and to a decline in numbers of phase-bright spore bodies.

Heat resistance of these spore forms could not be tested as germination of A(-)1, or of A(-)1 treated with actinomycin D could not be accomplished.

(b) Studies on the parent strain, A(-).

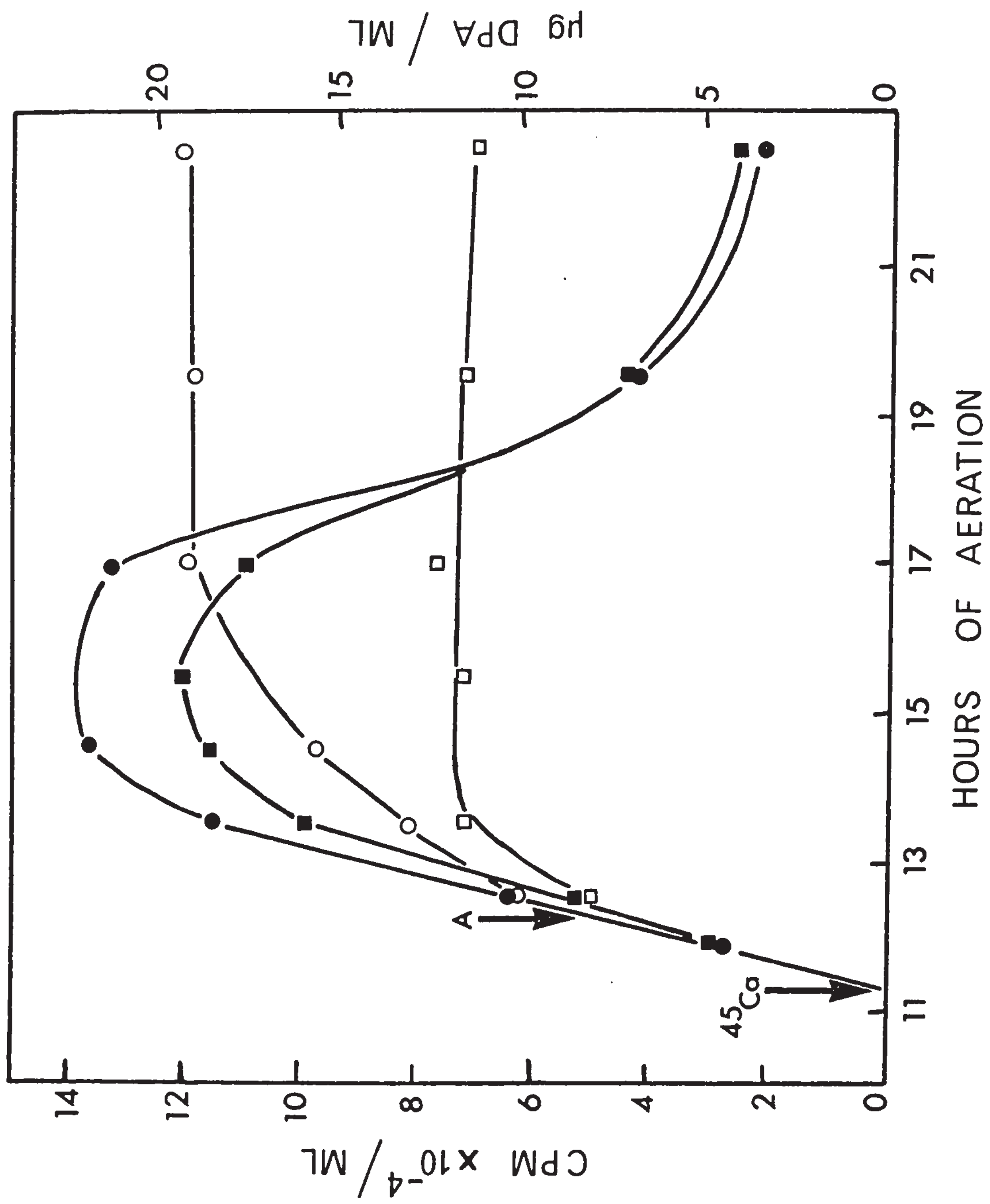
As A(-) does germinate, cortexless spores of this strain were prepared by actinomycin D addition at

Fig. 34. Uptake of ^{45}Ca by A(-)1 in control, ●, and in portions of the stock culture treated with actinomycin D. ○ represents addition at time indicated by arrow A1, (30 min prior to phase whitening); ■, arrow A2 (5% whitened); □, arrow A3 (35% whitened).



HOURS OF AERATION
10
12
14
16
18
20
CPM x10-4/ ML
0
4
8
12
16
20
A1
A2
A3
45Ca

Fig. 35. Uptake of ^{45}Ca by A(-)1 in control culture, ● , and in portion treated at point A with actinomycin D, ○ . DPA content is shown for control, ■ , and actinomycin D treated culture, □ .


HOURS OF AERATION
CPM x10^-4 / ML
µg DPA / ML
A
45Ca
11
13
15
17
19
21
0
2
4
6
8
10
12
14
5
10
15
20

early phase whitening, (Fitz-James, 1965), and the relationships between heat resistance, refractility, and lack of cortex further investigated.

Morphology. The addition of actinomycin D at various times during stage 4 produced spores similar in phase microscopy to those previously described (Fitz-James, 1965). Again in thin section electron micrographs cores similar in appearance to those of refractile spores were seen without a surrounding cortical layer if actinomycin D was added prior to or during early phase whitening in up to 20% or so of the culture (Fig. 36). The germ cell wall was formed but coat deposition was prevented. A(-) so treated, looks very similar to the cortexless mutant at this stage of formation (see Fig. 31). If actinomycin D is added after further whitening has occurred, the most advanced spores proceeded to form some cortex and coats (Fig. 37). In a limited number of forespores, an unusual and uneven thickening of apparently germ cell wall was seen (Fig. 38). The significance of this is not known. A similar appearance was also seen occasionally in actinomycin D treated cells of A(-)1. Freeze fracture etching revealed the expected smooth textured cytoplasm of the spore core (Fig. 39), as in the untreated mature spore (Fig. 40).

^{45}Calcium uptake and DPA content. ^{45}Ca uptake and DPA

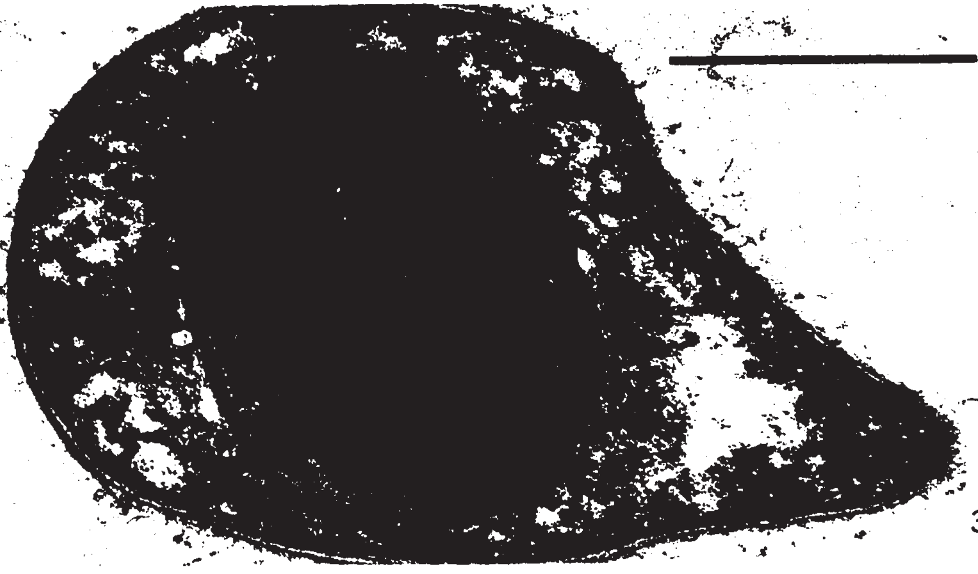
Fig. 36-39. Magnification as indicated by the 0.5 μm markers.

Fig. 36. Electron micrograph of the parent strain A(-) embedded 9 hr after actinomycin D addition at 1% phase whitening. The germ cell wall has formed but the cortex has not been synthesised. Note the similarity to the cortexless mutant A(-)1 in Fig. 31.

Fig. 37. Electron micrograph of A(-) treated with actinomycin D when 25% of forespores had become phase white. Cortex (co) and some spore coat (sc) have developed.

Fig. 38. Electron micrograph of A(-) treated with actinomycin D showing an unusual thickening of what appears to be germ cell wall material.

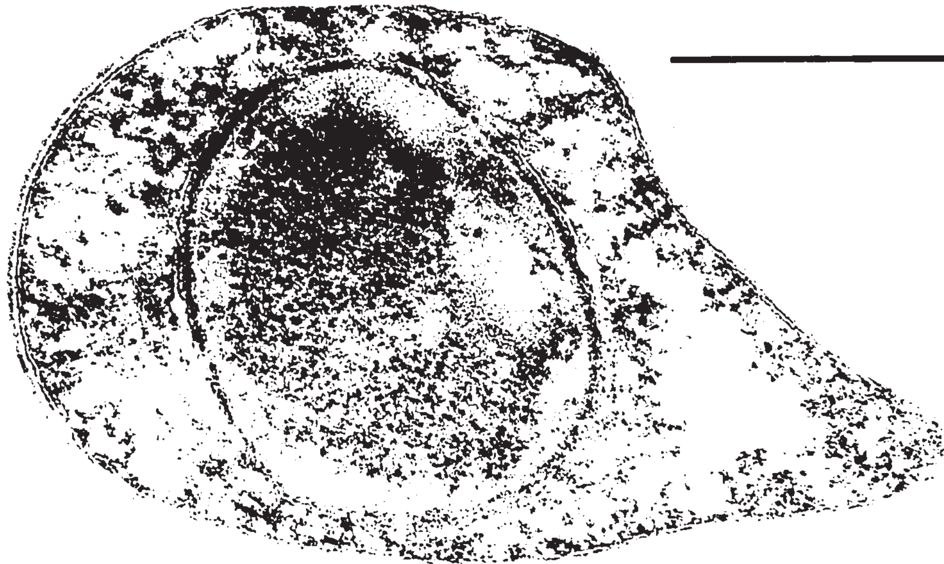
Fig. 39. Freeze-etching of A(-) 9 hr after actinomycin D addition at 15% phase whitening. The spore core is dense and fine compared to the sporangial cytoplasm. The direction of shadowing is shown by the arrow.

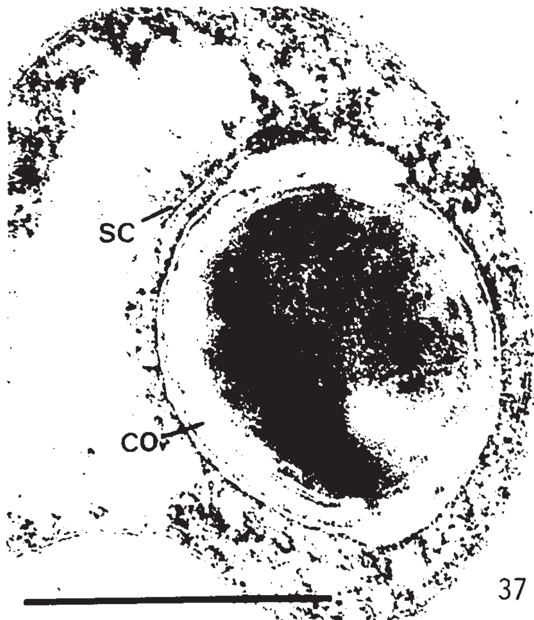

36

sc
c
37

38

39


36


sc
co
37

38

39

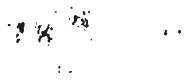


Fig. 40. Freeze-etching of a mature spore of A(-) showing the typical dense appearance of refractile spore cytoplasm. Magnification is shown by the 0.5 μm marker. The direction of shadowing is shown by the arrow.

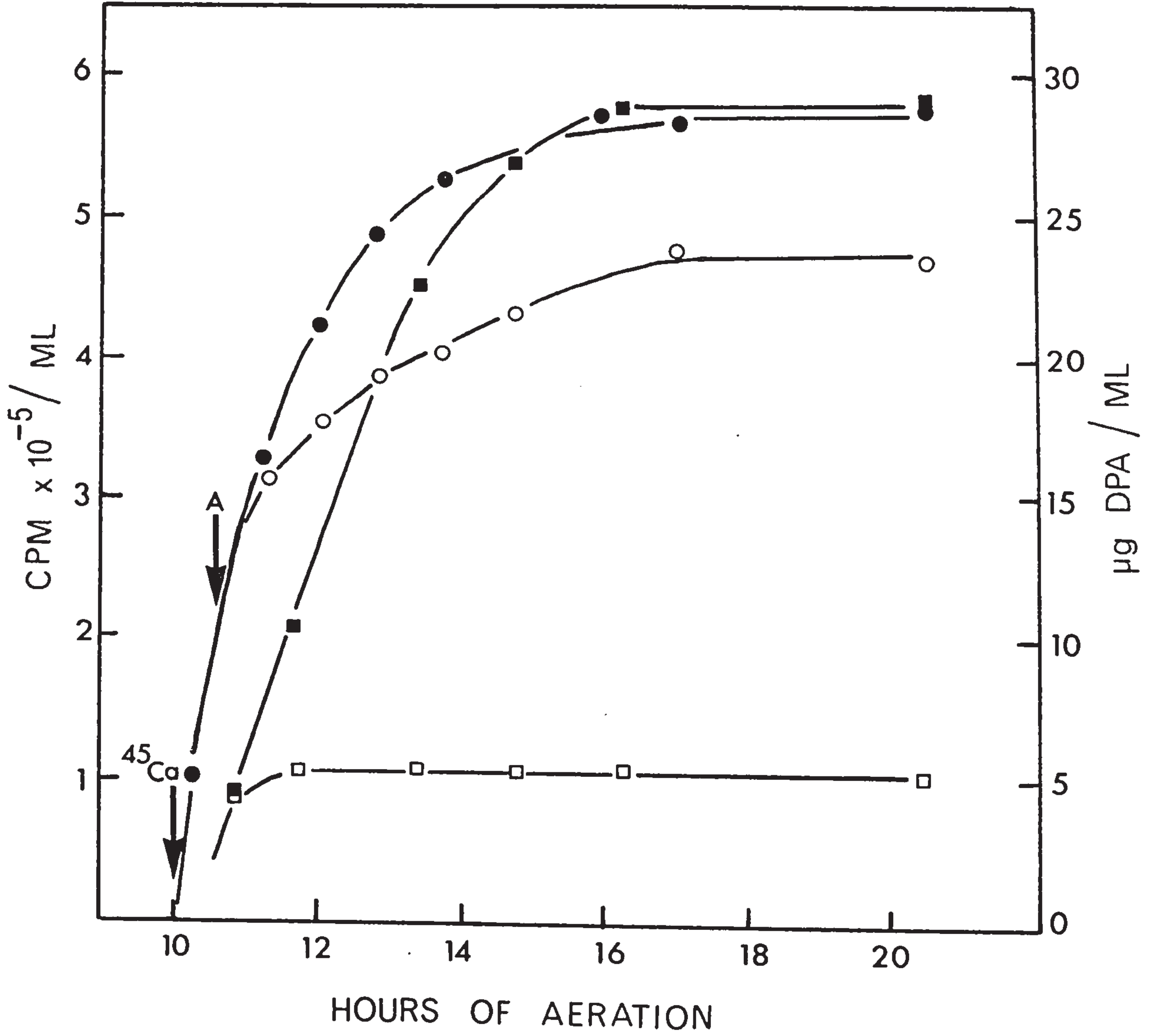
40

synthesis were studied in A(-) under conditions in which little or no cortex was formed, due to actinomycin D addition. Fig. 41 shows the effect of addition at approximately 8% phase whitening on these processes. ^{45}Ca uptake reached over 80% of the control level while DPA levels remained very low. Other experiments showed that addition of actinomycin D just prior to phase whitening still allowed ^{45}Ca uptake while DPA content was negligible. To obtain higher levels of DPA, addition had to be considerably delayed and thus some cortical synthesis occurred.

Heat resistance. If actinomycin D was added prior to the stage where 20% of the cells were whitening, heat resistance ranged up to 30% of the control. The number of actinomycin D treated spores that germinated before heating was much lower than that of control spores. Of those capable of germinating after actinomycin D treatment, usually at least 50% were heat resistant. The higher figures reported previously (Fitz-James, 1965) could not be obtained if actinomycin D addition was kept to early phase whitening to ensure little cortex formation.

Addition of exogenous DPA (50 µg/ml and 250 µg/ml) to cultures at 1% phase whitening, and 10 min before addition of actinomycin D did not enhance heat resistance.

Fig. 41. Uptake of ^{45}Ca and synthesis of DPA by A(-) untreated, and treated with actinomycin D at time indicated by arrow A. ^{45}Ca uptake in control, ● ; in treated cells, ○ . DPA synthesis in control; ■ ; in treated cells, □ .



CPM x 10^{-5} / ML
µg DPA / ML
HOURS OF AERATION
^{45}Ca
A
6
5
4
3
2
1
30
25
20
15
10
5
0
10
12
14
16
18
20

V. DISCUSSION

Morphological and Biochemical Characterisation of the Cortexless Mutant, A(-)1.

The spore protoplast is surrounded by a series of layers, all of which have specific roles in sporulation. The inner membrane must be an intact viable entity since, on germination, it becomes the plasma membrane of the outgrowing cell, while the adjacent germ cell wall becomes the new cell wall. The outer membrane, during sporulation, has many roles including those of ion transport, organisation of coat deposition and exosporium formation, and the transport of mucopeptide precursors into the cortical space.

Between these membrane layers, lies the relatively wide cortex - a most critical layer for the maintenance of the dormant spore state, and the subject of interest in this study. The instability of spores formed in the presence of penicillin(Vinter, 1962; Fitz-James, 1965), or cycloserine (Murrell, 1969), which inhibit cross-linking of the mucopeptide, clearly demonstrate this essential role of the cortex. The failure of the mutant, A(-)1,to give rise to free, resting spores, confirms this role.

Presumably, one function of the cortex is protection of the germ cell wall against the action of lytic enzyme during terminal lysis of the sporangium. The coat layers may help in this function but are obviously insufficient in themselves, since the mutant possesses normal coat and exosporium. Spores with incomplete coats due to addition of chloramphenicol during coat formation, soon autogerminate and lyse (Fitz-James, 1965). The coats also prevent mechanical damage to the cortex, act as a barrier to premature germination, and prevent clumping of spores (Fitz-James, 1971). The lack of retention of calcium and DPA shown here in the mutant, and demonstrated previously in spores formed in the presence of penicillin (Fitz-James, 1965), and the failure of the mutant to attain full refractility also point out the importance of the cortex for normal sporulation.

It is significant that in this mutant, the block in sporulation is not complete; late reactions such as calcium uptake, DPA synthesis, and coat deposition are unaffected. Thus formation of the cortex is not obligatory for the triggering of succeeding reactions. Studies on glucosamine-requiring mutants in *B. subtilis*, in which spore-like particles with minimal or no cortex but with normal coats are formed, also indicate this (Freese *et al.*, 1970), as do the mutants blocked in DPA formation (Halvorson and Swanson, 1969). It appears that branched sequences of reactions probably exist in the sporulation process rather than a strictly dependent sequence as suggested by Waites *et al.* (1970) from studies on mutants of *B. subtilis*.

The studies on germination of A(-) spores pulse-labelled with ^{3}H-DAP, in which ^{3}H-DAP incorporated early after engulfment, remains in a TCA-insoluble form, indicate that in this organism, as in that studied by Vinter (1965), the germ cell wall mucopeptide is synthesised prior to the cortex. The similarity in appearance of this layer and that formed between the forespore membranes in the mutant, and the parallel time of incorporation of ^{3}H-DAP in the mutant with that into germ cell wall mucopeptide in the parent, show that A(-)1is capable of synthesising germ cell wall. Thus, the fact that the mutant has apparently normal cell wall, and germ cell wall formation but is completely lacking the ability to form cortex, suggests a difference in the enzymes involved in these mucopeptide syntheses. Warth and Strominger (1969) have shown differences in the structure of the cortical and cell wall mucopeptides, and propose that a different set of enzymes may be required for the synthesis of each mucopeptide species. Certainly, the formation of the spore-specific lactam ring, and the shortening of the peptide side chain on many muramic residues to one alanine only, an arrangement not found in cell wall, would require sporulation-specific enzymes. The present studies indicate that a more basic difference also occurs, at a stage earlier than polymerisation; presumably a sporulation-specific enzyme(s) may be required here as well.

The morphological characteristics of the mutant could be accounted for by a biochemical lesion at a number of sites: first, formation of specific small molecular weight precursors; second, transport of intermediates across the spore membrane; third, polymerisation in the cortical space.

The general increase in specific activity of the phospholipids of A(-)1 compared to the parent during mid-sporulation indicates some alteration in membrane activity and suggests that the second proposed site is the most likely. The phospholipid labelling pattern agrees with the results of Lang and Lundgren (1970) with *B. cereus*, in that, although phosphatidyl ethanolamine represents 60% of the total phospholipid, it accounts for only 20% of the labelling during sporulation, while phosphatidyl glycerol accounts for 70% of the phospholipid labelling.

The changes in the fatty acid pattern of the parent, A(-), in mid-sporulation compared to vegetative growth, are different from those observed in *B. subtilis* by Scandella and Kornberg (1969). However, the fatty acid composition of *B. cereus* strains, in itself, differs from that of other *Bacilli*: C12 and C13 fatty acids and a significant amount of an unsaturated C16:1 fatty acid are present unlike other *Bacillus* species (Kaneda, 1967). The more marked changes on sporulation in the mutant, are probably due to the increased turnover of the membrane as

shown by the phospholipid labelling studies, and thus, the increased incorporation of sporulation-type fatty acids.

Attempts were made to demonstrate a missing or altered membrane protein by two systems of polyacrylamide gel electrophoresis: the acetic acid : urea system of Razin and Rottem (1967), and the sodium dodecyl sulphate system of Inouye and Guthrie (1969). Unfortunately, reproducible patterns were not obtained in either system, nor was separation of protein bands sufficient for detection of a missing component by the double labelling technique of Inouye and Guthrie (1969). In this technique, proteins of the parent are labelled with ^{14}C-amino acids, those of the mutant with ^{3}H-amino acids, the proteins mixed, run together on the one gel, and the ratio of $^{14}C/^{3}H$ measured in consecutive small slices of the gel. However, even if the gels had been satisfactory, there would for technical reasons, have to be a major band missing or altered greatly for it to be noticed. For one thing, this system is particularly complex, and, in order to examine the mutant at the time of expression of the lesion, the whole sporangium plus developing spore with cortex and some protein coats has to be disrupted, and the proteins extracted from the total particulate residue. The sporangial membrane proteins, which are similar in both parent and mutant, would contribute to the total protein applied to the gel,and would hinder the detection of a subtle change in the forespore membrane.

The markedly changed appearance of the outer forespore membrane of A(-)1, with its pronounced lumps and ridges, occurring at the time that normal cortex synthesis should take place, confirms the membrane as the site of the biochemical lesion, as indicated by the lipid studies. This is supported by the studies on the plasma membrane, in which inhibition of normal cell wall mucopeptide synthesis by bacitracin also gives rise to altered membrane morphology.

In freeze etch studies of spore membranes the precise plane of fracture is difficult to state. The interpretations of Moor and Muhlethaler (1963), Remsen (1966) and Nanninga (1970) have been followed in that the actual outer and inner surfaces of the membranes were considered to be exposed, rather than surfaces resulting from a splitting within the membrane. Da Silva and Branton (1970) and Tilloch and Marchesi (1970) have shown recently that this splitting does occur in red blood cell ghost membranes. However, the plane of fracture in a multicomponent system (coats, membranes, cortex, germ cell wall) may differ significantly from that found in the free membrane system of red blood cell ghosts. If the plasma membrane is indeed fractured in bacteria, as now seems indicated by the very recent studies of VanGool and Nanninga (1971), then the excessive lumps and ridges observed in the mutant, and the changes occurring in the bacitracin-treated plasma membranes, will actually be in the hydrophobic membrane interior.

The difference in phospholipid metabolism, and the alteration in membrane morphology of the mutant may be accounted for by a lack of a specific membrane component. In other systems, such changes have been observed. Estes and Lombardi (1969) have shown alterations in the Golgi apparatus, and fragmentation and dilation of the endoplasmic reticulum cisternae in the livers of rats on a choline-deficient diet. They also quote unpublished results of Pani and Lombardi, that the lethicin: protein ratio of microsomes of these livers are distinctly different from those from rats on a choline-supplemented diet. Wilson and Leduc (1963) have demonstrated that mitochondria from the livers of essential fatty acid-deficient mice are enlarged and have greatly increased numbers of cristae. They postulate that the increased number may be due to an ineffective attempt at compensation for the lack of functional cristae for the production of ATP. The increased phospholipid turnover and incorporation of sporulation-type fatty acids observed in the present study, could also be similar attempts at compensation by the membrane for the lack of or gross alteration of some normal component.

The alteration in morphology of the outer forespore membrane only, and just during the period when normal cortex formation should be taking place, indicates a sporangial source of cortical precursors. The germ cell wall may be formed by both inner and outer forespore

membranes, although, in electron micrographs the redundant folds of germ cell wall material appear to follow the inner membrane (Fig. 6a). Murrell (1967) has also suggested that germ cell wall formation is a function of the inner membrane, and that cortex synthesis is a function of the outer fore-spore membrane, although he has no direct evidence.

Further biochemical support for the hypothesis that the site of the lesion is at the membrane level of muco-peptide synthesis, comes from studies on the accumulation of soluble precursors. During the time of cortex formation, but not during germ cell wall formation when the mutant is functioning normally, N-acetylamino sugar esters were ac-cumulated by the mutant. This is to be expected if cortical synthesis is indeed blocked in the membrane stages, since accumulation of precursors has been shown to occur when cell wall mucopeptide formation is inhibited by antibiotics acting either at the membrane or polymerisation stages (Reynolds, 1961; Anderson *et al.*; 1967); Strominger, 1957).

The comparison of incorporation of ^{3}H-DAP by homo-genates of the parent and mutant, during cortex formation, indicated that cell free synthesis of cortical mucopeptide was possible. These cell free studies on cortical muco-peptide synthesis agree with the *in vivo* studies: A(-)1 homogenates incorporate relatively little ^{3}H-DAP into muco-peptide compared to A(-) during the time of cortex formation, whilst extracts prepared during the period of germ cell wall formation show almost normal capability for mucopeptide

synthesis. The presence of some inhibitor of cortical synthesis in the mutant is excluded by the experiment in which homogenates from both A(-) and A(-)1 were mixed in equal amounts and their combined ability to synthesise mucopeptide examined. The increased incorporation of ^{3}H-DAP over that expected had there been no interaction between the two systems, is probably due simply to the increased pool size of soluble precursors available to the intact A(-) system, since the mutant has been shown capable of synthesising these precursors, and actually of accumulating them.

This cell free system for cortical mucopeptide synthesis is, itself, of interest, in that it should allow further chemical studies on the process of cortex formation. The cortexless mutant provides a necessary control in that it shows that germ cell wall synthesis is contributing very little to incorporation of ^{3}H-DAP if cells are smashed at 70% phase whitening or later. Indeed, without the mutant, it would be difficult to be certain that germ cell wall synthesis is not continuing.

As discussed earlier, it seems possible that one or more of the enzymes for cortical mucopeptide synthesis may be a sporulation specific gene product. In the cell free mucopeptide synthesising system, the effects of antibiotics on both cell wall and cortex synthesis were compared to see if differences in the sensitivity of these

two systems could be observed.

From the vancomycin study no conclusion as to differences in the enzymes involved, can be drawn, although a slight difference in sensivity was noticed, since vancomycin has been shown to bind to terminal D-ala-D-ala residues (Perkins, 1969). Hence in a cell free system with no permeability barriers, any precursors ending in this peptide sequence, either soluble or membrane-associated, and any yet uncross-linked side chains on the mucopeptide polymer, would be available for binding by this antibiotic. As the relative amounts of precursors present in logarithmically growing cells and sporulatingcells may differ, a difference in the amount of vancomycin required for inhibition is not unexpected.

Bacitracin has been shown to inhibit the dephosphorylation of carrier lipid pyrophosphate (Siewert and Strominger, 1967), and thus inhibit regeneration of the carrier lipid monophosphate for the next round of synthesis. The carrier lipid of cell wall synthesis has been identified as a derivative of a C_{55} polyisoprenoid alcohol (Higashi *et al.*, 1967). Similar derivatives have been shown to be involved in other polymer forming systems; the O-antigen system (Wright *et al.*, 1967); mannan synthesis (Scher *et al.*, 1968); and synthesis of capsular polysaccharide (Frerman *et al.*, 1971). It seems likely that a similar carrier is involved in cortex synthesis, and possibly

the enzymes involved in its metabolism will also be similar to those in the cell wall cycle. Certainly, no difference was observed here in the study of bacitracin sensitivity.

However, the difference observed in sensitivity to methicillin was unexpected and striking. Since penicillin inhibits cross linking of mucopeptide side chains, it should not have any effect on incorporation of ^{3}H-DAP into the mucopeptide itself. This was the case with the cell wall system at low concentrations of antibiotic, and the germ cell wall system. The marked inhibition of this DAP incorporation into cortical mucopeptide is puzzling but may reflect a difference in secondary and tertiary organisation of the cortex compared to cell wall. Certainly, in thin sections of acid treated spores stained with lanthanum, the cortex is seen as concentric layers of fine fibrils (Mayall and Robinow, 1957; Fitz-James and Young, 1969), unlike the structure of cell wall mucopeptide. Perhaps, in the organisation of the cortex, polymers of smaller sugar chain length are used, and thus, there may be some dependence on peptide cross-linking to form the trichloroacetic acid insoluble mucopeptide studied in these experiments. If such were the case, methicillin would interfere with the total incorporation of ^{3}H-DAP into the acid precipitable mucopeptide. Otherwise, methicillin may have a completely unidentified mode of action in this system. These results are also surprising in that *in vivo* some increased incorporation of ^{14}C-DAP in the presence of penicillin has been

reported (Vinter, 1963b), and the cortex of spores produced in the presence of this antibiotic appears thickened and more pliable (Fitz-James, 1965). The latter effect may simply be the result of improper organisation of the mucopeptide.

The lack of effect of methicillin on germ cell wall mucopeptide formation *in vitro* in A(-)1 suggests that synthesis of germ cell wall is more like that of cell wall proper, than of cortex.

The marked difference in sensitivity to lysozyme of the ^{3}H-DAP labelled products of these *in vitro* systems prepared from cells synthesising either cell wall, germ cell wall, or cortical mucopeptide, further supports the idea that these products are specific mucopeptides with at least some characteristics of the corresponding mucopeptide produced *in vivo*. The germ cell wall again acts more like cell wall mucopeptide product than the cortical product, in its rate of digestion by lysozyme.

Thus, from these results with both antibiotics and lysozyme, plus the fact that the mutant cannot form the cortical product, it appears that an *in vitro* system specific for cortical mucopeptide synthesis has been established. The final proof of this would require identification of the chemical components characteristic of cortex synthesis, that is, the lactam ring structure, or a free ^{3}H-DAP labelled tetrapeptide proposed to be released by

an endopeptidase to leave the single L-ala substitution found on many muramic residues (Warth and Strominger, 1969).

The small extent of radioactive labelling of presumed cortical mucopeptide intermediates was disappointing, although there is evidence that the mutant cannot form the full complement of these components compared to the parent. It was hoped that a marked difference would be observed and that the lipid material would be sufficiently highly labelled to allow further definition of the biochemical lesion in the mutant and make possible identification of the lipid intermediates of normal cortex synthesis. If the process of cortical mucopeptide synthesis is analogous to that of cell wall, there would be two steps at which the mutant could be blocked: first, at the transfer of N-acetyl muramic pentapeptide from its UDP precursor to the phospholipid acceptor in the membrane; second, at the addition of N-acetyl glucosamine to the lipid-N-acetyl muramic pentapeptide to form the lipid-disaccharide intermediate. The lack of label incorporated in these studies made any detailed chemistry of these intermediates impossible. The mode of transport of precursors through the membrane barrier, and the chemistry and enzymology of these membrane-bound intermediates are major topics to be investigated. These problems will probably only be solved by the use of soluble intermediates

of very high specific activity as synthesised by Strominger *et al.*, (1966) for studies on cell wall chemistry. Such syntheses were not feasible in this laboratory. For this work, a cell free system for the specific synthesis of cortical mucopeptide such as the one described is a necessity.

The present investigation makes no attempt to describe the genetic defect in the mutant. It does indicate that the biochemical lesion is manifested in the outer forespore membrane. This block in cortex synthesis could be the result of several possible defects: a sporulation specific enzyme may be altered in some way and thus be non-functional; it may be missing entirely due either to a deletion in part of the genome, or to a failure in the control system such that the gene is not transcribed.

Spore Refractility and the Role of the Cortex.

The precise roles of the cortex, calcium and DPA in the formation of refractile, heat resistant spores are not clearly understood. The "contractile cortex" hypothesis (Lewis *et al.*, 1960) proposes that pressure is exerted on the spore core by a contraction of the cortical mucopeptide, caused by neutralisation of the electrostatic repulsions of the carboxyl groups by calcium or calcium dipicolinate. This contraction would dehydrate the spore protoplast. The hypothesis has received support from morphological and chemical studies on the cortex, (Warth *et al.*, 1963b; Hitchins and Gould,

1964; Murrell and Warth, 1965) and from the reversion of germination by basic peptides (Vinter and Stastna, 1967). The results presented here on the cortexless mutant A(-)1, treated with actinomycin D to prevent the terminal lysis, show that refractility with its associated changes in the spore cytoplasm presumably due to dehydration, can occur in spite of the lack of a cortex. These spores are typically bright when viewed by phase contrast microscopy and typically refractile when examined by bright field microscopy in air dried and air mounted smears negatively stained with nigrosin. Moreover, the lack of detail in the spore core and the semicrystalline form of the spore DNA seen in electron micrographs of thin sections plus the "hard" appearance of the fractured spore cytoplasm in the freeze-etch studies bear this out.

A similar development is seen with the parent A(-) treated with actinomycin D during early stage 4 to prevent normal cortical synthesis. Phase contrast studies (Fitz-James, 1965), and the present freeze-etch and thin section electron micrographs again show characteristics associated with refractility although in all specimens the corresponding thin section micrographs show that the cortex is absent. Thus, the mechanism for refractility is not dependent on the presence of a cortex, and is set up early in the sporulation process since it is resistant to additions of actinomycin D during sporulation stage 4.

Walker (1969) has demonstrated that at this same time changes occur in the character of the spore ribosomes making them resistant to digestion from ultrathin sections by ribonuclease. These changes are suggested to be due to core dehydration again indicative of initiation of the dehydration mechanism early in cortex development.

The studies of ^{45}Ca uptake show that even with addition of actinomycin D to cultures of both A(-) and A(-)1 before phase whitening begins, uptake of ^{45}Ca proceeds. Indeed, since usually only 50% of A(-)1 spores treated with actinomycin D become phase bright, these spores would have a greater content of calcium than that normally accumulated. The process of Ca uptake can be separated from that of DPA synthesis using actinomycin D addition (Fig. 41). The Ca transporting system must be organised early in stage 4 and be no longer dependent on continued RNA formation, whereas DPA synthesis appears to be inhibited comparatively soon after actinomycin D addition, showing a dependence on continued mRNA production. A similar inhibition of DPA synthesis by actinomycin D has been described by Chasin and Szulmajster (1969). It should be noted that in these cultures with low DPA levels, refractility is still seen (Fig. 36). Earlier work with chloramphenicol (Fitz-James, 1965) led to similar conclusions as to the time of initiation of the refractility mechanism. These studies are in accord

with the recent hypothesis of Fitz-James (1971) that some of the spore Ca^{++} is required to bridge and stabilise ionic phosphate groups of the spore protoplast membrane when dehydration occurs, in that Ca uptake would be necessary for refractility to be maintained, whereas DPA would not be required at this time. Instead, DPA would function mainly by removing Ca from its sites on the inner membrane at the time of germination.

Heat resistance cannot be studied in the mutant A(-)1, as it is incapable of germinating. Studies of the parent strain show that some heat resistance (up to 30% of the control) can be obtained in cultures treated with actinomycin D at early phase whitening. However, it is difficult from these results to distinguish the possible need for some cortical development from the definite need for DPA for normal heat resistance (Halvorson and Swanson, 1969) as these early additions severely depress the synthesis of DPA (see Fig. 41). To obtain higher levels of DPA and consequently higher levels of heat resistance addition of actinomycin D has to be delayed. This also allows the synthesis of more cortical material. It would appear that heat resistance can be associated with spores possessing limited cortex provided they have sufficient DPA.

The location of DPA in the spore is indefinite although it is generally thought to be associated with

calcium (Murrell, 1967). Proponents of the contractile cortex hypothesis tend to place it with calcium as the Ca-DPA chelate in the cortex (Vinter, 1969; Warth *et al.*, 1963b; Murrell, *et al.*, 1969) although other work suggests a core location (Donellan and Setlow, 1965). These results with the cortexless mutant indicate a core location, since the DPA levels attained in the presence of actinomycin D are maintained for many hours, even at a time when it is known from freeze-etching and thin section electron micrographs that the sporangial cytoplasm has lysed (Fig. 31 and 33). Presumably the DPA and calcium are held within the spore-like particle. The actual state of DPA in the core is not known but it may be in association with amino acids as suggested by Young (1959) and Tang *et al.*, (1968).

The precise mechanism for dehydration of the spore core remains unknown. Marshall and Murrell (1970) have proposed that metal ions, particularly calcium, which have been shown to be effective in the formation and contraction of certain gels, may act in causing similar gelation in the spore protoplast with the subsequent development of crystal-like structures and the exclusion of water. These authors suggest that a developing contractile cortex would help in this dehydration by exerting pressure on the spore core and would prevent, by mechanical constriction, uptake of water by the core. Such a concept

is in keeping with the results presented here.

It appears that the cortex is not essential for producing the initial dehydration and consequent refractility and heat resistance, and that it is not the major location of DPA and Ca^{++}. Rather, its role appears to be that of maintaining and protecting the dehydrated stable state of the resting spore protoplast.

In conclusion, this combination of morphological and biochemical approaches have proved successful in characterising a most interesting sporulation mutant - one completely lacking a critical spore layer, the cortex - and in clarifying the role of this layer.

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