METABOLIC CHANGES DURING BACTERIAL SPOROGENESIS IN MUTANTS OF A *CLOSTRIDIUM SP.*

BY

AUGUSTINE CHIJIOKE EMERUWA

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To my wife and daughters, Nne and Israel

ABSTRACT

A highly sporogenic, MSp^+ and asporogenic RSpoIIIa mutants were derived from a wild type strain, Clostridium botulinum type E toxigenic ATCC 9564 which was treated with N-methyl-N-nitro-N'-nitrosoguanidine and clones were recovered on liver veal agar. In trypticase peptone glucose (TPG) broth, the MSp^+ mutant and the wild type strain yielded 90% and < 60% spores respectively after 48 h incubation at 30 C. Ultrastructural studies showed that the MSp^+ goes through the seven sequential stages of spore development, and accumulated intracellular granules by the prespore stage. On the other hand, the RSpoIIIa mutant showed septum formation and a nearly completed forespore as well as cytoplasmic granulation. The forespore membrane was defective at a few sites and a "germ cell wall" was not observed. Lysis of the cell cytoplasm of RSpoIIIa occurred during the laterstationary phase of growth. The

results indicate that the RSpoIIIa was blocked at stage III. Unlike the wild type, the mutants were nontoxigenic by mouse pathogenicity tests. The two mutants showed specific immunofluorescence and precipitin lines of identity with an antiserum prepared against the wild type strain, thus conforming their identity as type E. The isolation of the mutants, sporogenic and asporogenic, provided a basis for comparison in the analysis of the ultrastructural and biochemical changes which occur during the stages of spore formation.

The kinetics of growth in TPG broth was measured by absorbance at 600 nm which showed that at 10 h, maximum growth had been attained in cultures of both mutants and was designated "end of exponential growth". The pH of the cultures of MSp^+ and RSpoIIIa dropped rapidly by the end of exponential growth and did not change thereafter. The growth of the MSp^+ then decreased abruptly reaching minimum at the "prespore stage" or "early stationary phase", 15 h, but remained essentially unchanged thereafter during "endospore stage" or "late stationary phase" between 15 to 25 h. Free spores were detected after the "maturation stage" which occurred between 25 to 35 h. Dipicolinic acid (DPA) and refractile endospores

were first detected in cultures of MSp^+ at 16 h. As the DPA was synthesized, refractile endospores increased in number and reached a maximum of 90% at 30 h. DPA was not detected in cultures of RSpoIIIa and spores were not seen. The growth of the cultures of RSpoIIIa mutant remained constant for 10 h after the end of exponential growth before declining to its lowest level at 30 h due to autolysis.

Cultures of both mutants prepared in glucose-free trypticase peptone broth showed no significant changes in growth or pH and endospores were not produced.

In TPG, the two mutants catabolized glucose via the Embden-Meyerhof Parnas pathway during exponential growth. When glucose was exhausted in cultures of MSp^+, maximum accumulation of acetate, 8 mmoles/liter, CO_2, 60 mmoles/L and H_2, 80 mmoles/L was obtained at "prespore stage" and were reduced to 6 mmoles/L, 40 mmoles/L and 58 mmoles/L respectively during endospore formation. In the RSpoIIIa cultures, the levels of acetate, CO_2, H_2 and ethanol were lower than that of the MSp^+ and showed a slight increase throughout the growth cycle. The amount of ethanol produced by both mutants was identical up to the

endospore stage, then decreased in cultures of the MSp^+ but increased throughout the stationary phase of growth of the RSpoIIIa. These metabolic products appear to be derived solely from glucose breakdown since control cultures, without glucose did not contain any of the intermediates.

During spore formation of the MSp^+, butyrate was accumulating in significant amounts (0.5 to 3.5 mmoles/L). Thus, as the activity of acetokinase, phosphotransacetylase and butyryl-CoA dehydrogenase reached maximum, the butyrate content increased concurrently with endospore production in the culture. Simultaneously, acetate and intracellular granules decreased as butyrate was being produced. These findings indicate that the acetate metabolism may supply energy and carbon during spore formation. Butyrate biosynthesis was blocked in the asporogenic mutant and only traces were detected. As shown by isotopic assays, butyrate and acetate served as precursors of spore lipids. β-Phenethyl alcohol, fluoroacetic acid and 2-picolinic acid inhibited anaerobic sporogenesis almost completely, butyrate biosynthesis by > 87% and acetate accumulation by 50 to 62% showing a direct relationship between butyric type of fermentation and anaerobic sporulation.

The granules observed in cells of both MSp^+ and RSpoIIIa mutants were isolated at various developmental stages of growth and sporulation. Electron microscopy of thin sections and chemical analysis of cells showed that most of the granules were dispersed throughout the cytoplasm and that the granules were poly-β-hydroxybutyrate (PHB). The polymer began to accumulate after 8 h of growth reaching 9% and 13% of the cell dry weight in the sporogenic and asporogenic strains respectively, during early stationary phase. ^{14}C-acetate was readily incorporated into PHB and ^{14}C-butyric acid was not utilized to any significant extent. The rate of ^{14}C uptake paralleled the production and utilization of PHB indicating that the acetate served as its precursor. Most of the PHB which had accumulated in the sporogenic strain was catabolized during the development of the spore, suggesting that PHB catabolism may provide β-hydroxybutyrate, which undergoes dehydration and reduction via butyric acid type of fermentation to produce butyrate, energy for sporulation and spore-specific components. In contrast, the PHB in cells of RSpoIIIa mutant remained essentially undegraded and many granules were observed in the cytoplasm, indicating

an impaired mechanism of catabolism.

The sporulation of the MSp^+ was reduced to < 30% in excess glucose, 0.27 M, indicating that anaerobic sporulation is under catabolite repression. In addition, cells of RSpoIIIa in excess glucose, 0.135 M, were stabilized at stage II and lysis was inhibited for up to 36 h. Cyclic AMP or MB-cyclic AMP, 10^{-5} or 10^{-4} M reversed the repression of MSp^+ sporulation from 30 > 80% without affecting the growth rate. The addition of either of the cyclic nucleotides enhanced glucose consumption and corresponding changes in pH were observed. The catabolite repression by glucose was reversed also by ATP or ADP. Except for GTP, guanine nucleotides were not effective. The intracellular cyclic AMP levels were high in vegetative, sporulating and derepressed cells, but low in glucose-repressed cells. The findings appear to suggest that the intracellular content of cAMP fluctuates so as to mediate in the catabolite derepression of anaerobic sporulation. The reversion is achieved through enhancement of glycolytic activities and the accumulation of metabolic products such as acetate which are stimulatory to anaerobic sporulation metabolism.

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ABBREVIATIONS

ADP - adenosine 5'-diphosphate

AMP - adenosine 5'-monophosphate

ATCC - American Type Culture Collection

ATP - adenosine 5'-triphosphate

C_1 - CO_2 or carbon dioxide

COASH - Coenzyme A

CPM - counts per minute

cAMP (cyclic AMP) - adenosine 3',5'-cyclic monophosphate

cGMP (cyclic GMP) - guanosine 3',5'-cyclic monophosphate

DPA - dipicolinic acid

DPM - disintegration per minute

DPNH - diphosphopyridine nucleotide (reduced)

E_{235} - extinction at 235 nm

EMP - Embden-Meyer-Parnas pathway

GMP - guanosine 5'-monophosphate

GTP - guanosine 5'-triphosphate

ID - internal diameter

LVA - liver veal agar

MB-cAMP (MB-cyclic AMP) - monobutyrate adenosine 3',5'-cyclic monophosphate

MB-cGMP (MB-cyclic GMP) - monobutyrate guanosine 3',5'-cyclic monophosphate

MSp^+ - sporogenic mutant of C. botulinum type E

NTG - N-methyl-N'-nitro-N-nitroso-guanidine

OD - optical density

O/R - oxidation / reduction

OSP - oligosporogenous mutant, that sporulates at low frequency

PHB - poly-β-hydroxybutyrate

RSpoIIIa - asporogenic mutant of C. botulinum type E

Sp^- - asporogenous mutant, that will not sporulate under any condition

Sp^+ - sporogenic mutant, that sporulates at high frequency

t_o - end of exponential growth

t_1 to t_8 - development time-scale of sporulation (1 h intervals)

TCA cycle - Tricarboxylic acid cycle

TPG - Tripticase peptone glucose broth

TPGe - Trypticase peptone with excess glucose 0.27 M

TTZ-pyocyanine - triphenyltetrazolium chloride-pyocyanine

VL agar - liver veal agar

V/V - volume per volume

W/V - weight per volume

INTRODUCTION

INTRODUCTION

The development of bacterial endospores follows a well-defined time course which is essentially identical in various Bacillus species (6) and has been divided into seven morphological stages (117).

A variety of asporogenic mutants of Bacillus spp, blocked at defined stages of the spore cycle, has been studied by groups such as Schaeffer, et al (124, 125), Mandelstram and Waites (34, 85, 155) and others (47, 86, 93) to elucidate the sequence of biochemical and morphological changes which occur during sporulation of the Bacillus spp. A few asporogenic mutants of C. histolyticum and C. perfringens have been examined (25, 127, 128), but in general, studies of sporulation of Clostridium spp have been deterred because of the technical problems inherent in anaerobic growth.

The ultrastructural changes in sporulating cells have been described for a number of species of Bacillus and Clostridium, and, the sequence of endospore development is, for the most part, the same in both genera. Recent studies of a few species of Clostridium indicated that the spore coat synthesis may precede cortex formation and cytoplasmic granules have been observed

in some species (81, 115).

Some morphological changes observed during sporulation have been associated with simultaneously occurring biochemical events in the Bacillus spp. (66, 85, 92), however, except for a few studies (9, 127, 128) a similar kind of correlation in the Clostridium spp. has been impeded because sporulation of the anaerobic species is often irregular and difficult to control. The isolation of mutants of Clostridium spp. is complicated by the spreading growth and the structural abnormalities exhibited by some asporogenic mutants (8).

The biochemical changes occurring during spore formation in Bacillus have been studied extensively whereas only a limited number of reports have been published on Clostridium species. Clostridium botulinum, a "saccharolytic bacterium" ferments carbohydrates via the butyric acid type of fermentation (158) with end products such as acetate and butyrate (3, 113). Hence Day and Costilow (32) suggested that the acetate, which accumulated during glucose catabolism, was utilized during early sporulation, which is reminiscent of the fate of acetate during aerobic spore formation (20, 55). The amount of glucose or acetate has been shown to regulate the synthesis of PHB (36, 131) which accumulates in

stationary cells and disappears during sporulation in Bacillus (74).

Schaeffer et al (122) reported that sporogenesis of B. subtilis is under catabolite repression and since cyclic AMP (cyclic 3',5'-adenosine monophosphate) is a mediator in metabolic regulation of both plants and animals as well as microorganisms (65, 100, 111), it has been suggested that cAMP may reverse the repression of sporulation (61, 152).

Some of the methods used in the study of metabolic changes included gas chromatography (3), which was modified by selecting a sensitive column so as to quantitize the assay of volatile organic acids. The radioactive method assisted in the study of the translocation and distribution of substrates in the cells. Enzyme activities have also been utilized as a parameter to establish metabolic pathway(s) operational in any system and consequently, the activity of certain enzymes has been found to increase during sporulation (56) and to be lacking in asporogenic mutants (144).

This work was undertaken to study the metabolic changes which occur during anaerobic sporulation using an asporogenic mutant as a control. At the onset, stable mutants comprising of a high frequency sporogenic, MSp^{+} and an asporogenic, RSpoIIIa were

isolated and characterised. The sequence of the ultra-structural changes occurring during growth of the RSpoIIIa mutant was investigated. The catabolic activities of both mutants were measured including growth response, the rate of accumulation and the utilization of various intermediates of glucose metabolism via the butyric acid type of fermentation. The role of the butyric type of pathway during anaerobic sporulation and the contribution of poly-β-hydroxybutyrate (PHB) to the pathway is discussed.

The cytoplasmic granules observed in the cells of both mutants during the stationary phase were isolated, identified by chemical analysis and by electron microscopy.

The metabolism of PHB was monitored during growth and sporulation and acetate and butyrate were examined for precursor roles. The work also included the study of the effect of glucose on sporulation and the dere-pression by cyclic AMP.

H I S T O R I C A L

HISTORICAL

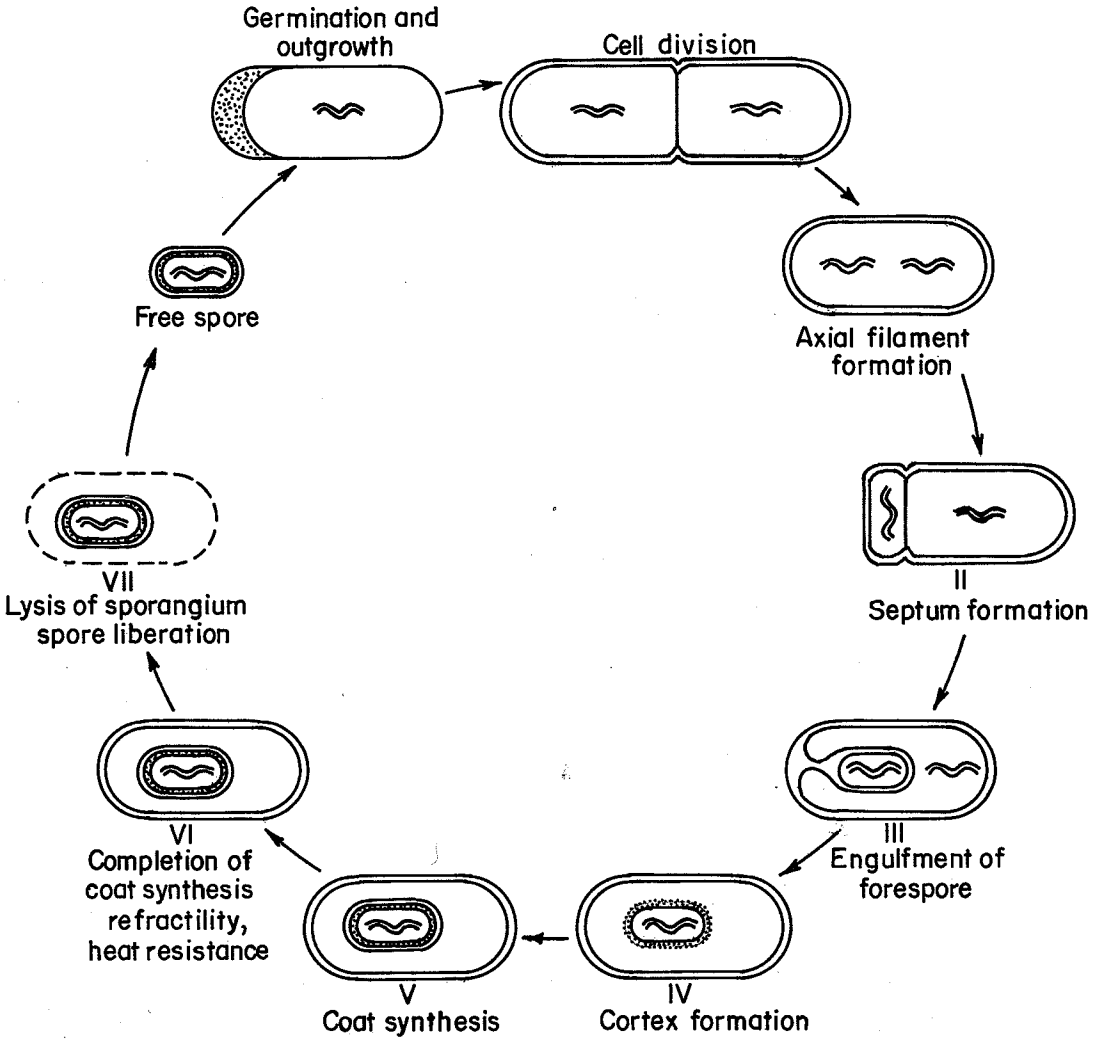
In the family Bacillaceae endospore formation is a distinctive feature in the life cycle of the aerobic genera of Bacillus and Sporosarcina, as well as, the anaerobic genus Clostridium.

The sequence of ultrastructural changes characterizing sporulation was recognized and described for Bacillus cereus by Young and Fitz-James (160, 161) and for Clostridium pectinovorum by Fitz-James (44).

On the basis of anatomical changes, Fitz-James (45) and Schaeffer (119) defined seven stages of the sporulation of Bacillus. Murrell (92) has reviewed the biochemical events which have been correlated to the time-scale of the morphological stages. Stage I begins at t_o, the end of exponential growth and ends when the chromatin body of the cells is visible as a single axial filament (Fig. 1) rather than the two or three fragments observed in dividing cells (116). Stage II begins 2 h later (t_2) with an invagination of the cytoplasmic membrane and ends with the completion of a double membrane spore septum which enclosed a portion of the nuclear material at one pole. The next stage

results in the completion of an intracytoplasmic forespore "carved out" of the mother cell (Stage III at t_4). At this period, numerous poly-β-hydroxybutyrate granules can be seen in the cytoplasm of the sporulating cells of Bacillus spp. The next two stages (t_6 and t_8) are characterized by the formation of new envelops around the forespore: the cortex, is deposited between the layers of the forespore membrane at stage IV and as the cortex undergoes maturation, the spore coat layers can be seen exterior to the cortex (Stage V). A change in the structure of cortex then occurs which results in the mature endospore becoming thermoresistant, refractile and impenetrable to physical and chemical agents (Stage VI at t_{10}). Lysis of the mother cell eventually releases the free spore (Stage VII). A schematic representation of the life cycle and morphological stages as observed in Bacillus species is presented in Fig. 1. Sporogenesis in Clostridium followed a similar life cycle and morphological changes except that the spore coat appears as discontinuous masses of material simultaneously with the formation of cortex (81, 115) whereas the coat usually spreads as a continuous layer (160) in the Bacillus spp.

Figure 15. Life cycle of sporulating *Bacillus spp.* and morphological stages (Modified drawing from Kornberg et al (71).


Germination and outgrowth
Cell division
Free spore
Axial filament formation
VII
Lysis of sporangium spore liberation
II
Septum formation
VI
Completion of coat synthesis refractility, heat resistance
III
Engulfment of forespore
V
Coat synthesis
IV
Cortex formation

Some biochemical events have been correlated to specific growth phases. Nutrients are utilized before the end of exponential growth phase (three to four cell generations) and metabolic products accumulate (91). During the early exponential phase, the products are not in sufficient quantity to affect growth or metabolism. However, by the stationary phase, the medium becomes depleted of > 75 percent of the main energy and nitrogen sources and the cells begin to adapt to some of the end products. It is under these conditions that sporogenesis occurs, resulting in an overall change in the metabolic pattern; synthesis of new enzymes and eventually new integuments of spore protoplast.

In Bacillus species (91), glucose has been the carbon source usually studied during sporulation and a culture may not sporulate if the glucose concentration is too high. The hexose is converted to organic acids, and the degree of pH decrease depends on the amount of glucose and the buffering capacity of the medium. The depletion of the carbohydrate ends exponential growth and the utilization of the organic acids commences, the pH value rises and the cells sporulate. If the pH falls to < 5.0 the cells do not sporulate (11). This pattern of events is fairly general among Bacillus species (53, 91, 154).

Vegetative cells of Bacillus and Clostridium species metabolize glucose almost exclusively via the Embden-Meyerhof pathway and only a small portion of the available glucose is catabolized via the hexose monophosphate pathway (51). Goldman and Blumethal (52) reported the presence of enzymes of glycolysis in spores of B. cereus but Doi et al (35) could not confirm their findings.

Martin and Foster (86) suggested the involvement of the TCA cycle in sporulation of Bacillus spp. and the evidence for the pathway was provided by Hanson et al (55). They found that when the pH of a culture was at its lowest, only the TCA cycle operated and acetate is converted to CO_2 and poly-β-hydroxybutyrate. A number of enzymes of TCA cycle are present in Bacillus cereus during sporulation but only in minute quantities during vegetative growth, for example, condensing enzyme, aconitase, fumarase, and malic dehydrogenase. The formation of these enzymes and the oxidation of glucose to CO_2 was detected prior to or during spore formation (52, 55). Krebs' cycle enzymes play an important role during sporogenesis of other species (57, 58) as shown by a genetic block of aconitase synthesis in B. subtilis in the presence of glucose, glycerol or glutamate which resulted in inhibition of sporulation (146).

Hanson et al (55) showed that the activities of several enzymes of the TCA cycle are repressed during exponential growth in media containing glucose alone or with glutamate, arginine and yeast extract. They also reported that enzyme activities increased at times t_1 and t_3 in glucose-containing medium following exhaustion of glucose and that in the presence of acetate or lactate, glutamate did not cause repression of aconitate hydratase. The involvement of the TCA cycle is therefore supported by the evidence of an increased activity of TCA cycle enzymes at the time of sporulation, the derepression of the enzymes when glucose is exhausted and the occurrence of asporogenic mutants lacking activities. Szulmajster and Schaeffer (144) reported that the transformation of the Sp^- mutants led to the recovery of both the enzyme activity and the ability to sporulate.

Other enzymes have been found which appear de novo or increase in activity or amount during sporulation for example protease in B. licheniformis (14), B. subtilis (136) and B. cereus (75). Genetic studies of asporogenous mutants indicate a functional relation or genetic linkage between the capacity to form the enzyme and the ability to sporulate. From the effect of amino acids on protease production, the hypothesis adopted has been that the enzyme is con-

trolled by the level of a repressor which is directly or indirectly an intermediate in many pathways and may be the same compound functioning in the regulation of the synthesis of spore structural compounds (107). Wall-lytic enzymes have been reported in Bacillus spp. and although the physiological significance is not yet understood, one function of these enzymes might be in differentiation or transformation (163). Wall-lytic enzymes have also been detected by Schaeffer (123) and mutants of B. subtilis lacking these enzymes were asporogenous. The amino acid-degrading enzymes found in B. licheniformis may provide energy and building blocks for sporulation via amino acid degradation (14, 46). Amino acid degradation probably occurs in all sporeformers and must be carried out by specific enzymes synthesized during sporulation and absent during growth. Several examples of these degradative enzymes have been studied in Bacillus (62, 108).

Bacillus species which sporulate in minimal replacement media use intracellular reserves of the mother cell as the main supply of carbon and energy (101, 149). The reserves comprise three groups, namely, proteins and ribosomal particles, low molecular weight solutes in the intracellular pool and poly β-hydroxybutyrate (PHB).

Much of the protein is in the form of enzymes no longer required by mother cell (59). Considerable protein turnover occurs (88) with proteolytic enzyme activities increasing several folds (14) and these are associated with the physiological expression of spore formation. The rate and quantity of turnover of the vegetative-cell protein and of the amino acid pool indicated extensive reorganization of the cell protein. RNA also undergoes turnover and is re-utilized in the synthesis of spore RNA.

The assimilation of low molecular weight solutes, such as amino acids, purines, pyrimidines and nucleotides, present in the intracellular pool as reserve, occurs during sporulation. Metabolite analogue inhibition and reversal studies (54) indicated that these preformed metabolites are essential for sporulation. Ninety percent of the amino acids present in the intracellular pool of Bacillus spp. is glutamate and alanine which accumulate and are utilized during sporulation.

In B. licheniformis (14), the acid-soluble nucleotides increased during exponential growth phase reaching maximum at stage III and decreasing during later stages. The relative proportion of each nucleotide remained fairly constant throughout the stages

of sporulation. Intracellular ribonuclease activity and a decline in the total nucleic acid content (74) suggested that the increase in the pool constituents comes from polymer breakdown. Poly β-hydroxybutyrate accumulated in the stationary-phase cells of many Bacillus species and was degraded during sporulation (72). The rate of synthesis and utilization of PHB in relation to sporulation is dependent on the type of strain and environmental conditions such as degree of aeration, amount of glucose and acetate in the medium and effect of pH. Synthesis usually begins before or by the time t_o, reaches maximum 3-5 h later and is depleted by the end of sporulation. High concentrations of glucose and acetate enhanced PHB formation (141).

C Cytoplasmic granulation has been observed in some cells of Clostridium spp. and the granules have been considered to be carbohydrate in nature and were designated as polyside (115), amylopectin (143) or granulose (81). PHB was not detected in cells of C. botulinum assayed by Day and Costilow (32) and besides cytoplasmic granulation was not observed in these cells.

Oxygen has been reported to be critical for the optimal sporulation of Bacillus species (131, 149) and may be required for the oxidation of the accumulated organic acids via TCA cycle and the utilization of the intracellular poly-β-hydroxybutyrate prior to spore formation. Optimum aeration resulted in increased PHB catabolism and a higher degree of sporulation. A large proportion, about 70% of acetate-2-^{14}C was incorporated into PHB in B. cereus (93), thus acetate and subsequently PHB provided carbon precursors and energy for sporulation.

Studies of glucose metabolism during growth and sporulation of Clostridium species have been sparse but published reports (32, 34, 153) indicate some fundamental differences from those of the Bacillus spp. (92). The mutants used in this study belong to the group designated "saccharolytic clostridia" which show limited proteolytic activity. Unlike Bacillus species, most species of saccharolytic Clostridium ferment glucose via the butyric type of fermentation pathway (153). The metabolic products generally accumulated include organic acids such as acetate, butyrate, butanol, acetone and isopropanol. Day and Costilow (32) have suggested that the acetate was utilized during early part of spore formation.

In the presence of large amounts of glucose, 75 g/l, C. butyricum (12) unlike Bacillus spp. does not continue to grow vegetatively. Only 15% of the initial glucose is fermented during exponential growth, 55% after time t_o and about 1.5 g/l remained at 21 h when endospores are formed.

Excess glucose had little effect on the growth response, the number of endospores, the final pH or the amount of glucose fermented. From these findings, Bergere and Hermier (12) concluded that glucose provided energy for growth and sporulation and that carbon metabolism of the sporangium and the vegetative cell was similar. In addition they observed that in the presence of excess glucose, the degree of growth was not affected by other nutrients since increased amounts of these did not augment the number of cells or spores formed. Although induction of sporulation was associated with substrate depletion in C. botulinum, an unknown growth retardation factor has been reported (12) for C. butyricum.

Some Clostridium species require a single amino acid such as arginine for spore formation (102). Therefore, it has been suggested that in C. botulinum, the energy for sporulation may be derived via the amino acid metabolism or from the organic acids which

accumulate during glucose breakdown (32). In C. butyricum the energy for sporulation is derived from glucose catabolism (12).

In Bacillus and in Clostridium species the analyses of various spore extracts have revealed considerable differences in the chemical composition of the free spore from that of the vegetative cell. In 1953 Powell showed the presence of dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA) in spore extracts (105) and in 1954, Strange and Powell identified muramic acid in germinating spore extracts 142). In 1955, Tinelli showed that spores of B. megaterium were rich in protein and low in carbohydrate and β-hydroxybutyrate as compared to vegetative cells (104).

Recent findings suggest that in the early stages of sporulation of both Bacillus and Clostridium species dipicolinic acid (DPA) is synthesized mainly in the mother cell (70). The synthesis commences during the forespore stage (stage III), and continues through stages IV, V, and possibly stage VI in parallel with cortex formation (103, 106). One hour after the start of DPA synthesis, the number of refractile endospores increases from 0 to 100% in C. roseum (53), a sequence similar to that found in C. botulinum (32). Heat resistance is

developed simultaneously with DPA synthesis for example when 40% of DPA was synthesized only about 1% of the sporulating cells were viable.

The onset of Ca^{++} uptake and DPA formation occurs concurrently with forespore formation in B. cereus var. Alesti (161) and the rate of Ca^{++} uptake and DPA synthesis is practically identical.

Mutants blocked at specific morphological stages are widely used for the study of sequential events and the cellular regulation which occur during sporulation. Various asporogenous mutants obtained by chemical mutagenesis (26, 53, 68, 79, 95) have facilitated the establishment of the genetic map and specific reactions of the sporulation process in Bacillus species. Schaeffer (120) has divided these mutants into two groups, viz, asporogenous sp^-, are those that will not sporulate under any condition and the oligosporogenous OSp, which sporulate at low frequency. Temperature-sensitive mutants have been isolated (18, 30) and have proven extremely useful in studies of activating enzymes (95), DNA synthesis (132) and other aspects of macromolecular synthesis in prokaryotic organisms.

Sporulation is controlled by genetic elements which are non-functional during vegetative growth and the phenotypic expression of the genes is observed only under certain environmental conditions. In

1958 and 1961, Spizizen (135) presented evidence for three genetic loci controlling sporulation from studies on transformation of asporogenic mutants of B. subtilis. However asporogenic mutants of B. subtilis, studied by transformation and transduction in different laboratories indicated a greater number of genetic loci (estimated to be several hundred) at which mutation can affect sporulation (125, 147, 148). These genes are widely scattered along the B. subtilis chromosome and some of them seem to be clustered in operon-like units, however, no operator typical mutation has been reported. Some are structural genes and code for specific enzymes but most serve as regulatory genes (145) which may control the synthesis and function of the products of the structural genes.

Jacob et al (63) suggested that sporulation of Bacillus species was controlled by episomal genetic elements which may occur in an autonomous or integrated form. Schaeffer et al (121) did not favor the episomal control of sporulation on the basis of genetic data. However, the idea persisted because acridine dyes can eliminate the episomal factors in E. coli and can produce asporogenic mutants in B. subtilis (111). The other reason for the continued interest in episomal control of sporulation is the finding of satellite DNA in germinating spores which may have an effect on

spore formation.

A factor called "Sporogen" present in the non-protein fraction of broken cells (137) of B. cereus, was speculated to have a hormone-like activity by Murrell (90). Up to now "sporogen" has not been shown to play a role in cultures sporulating normally, and it is possible that its activity may be secondary. Two peptide factors isolated from Clostridium roseum (159) may be similar to the "sporogen". A growth-retarding factor from C. roseum has been reported (12) which allows sporulation to occur in growing cells, but it may have an indirect effect of releasing catabolite repression.

The sporulation process examplifies a developmental system which involves a sequential gene expression. In 1969, Losick et al (76) showed that one of the earliest biochemical changes occurring during sporulation of Bacillus subtilis is an alteration in the transcribing specificity of the DNA-dependent RNA polymerase. Subsequently, it was reported that the normal sigma activity was lost during sporulation and that the β subunits of the RNA polymerases were altered and replaced by lower molecular weight polypeptides (77, 84). At the time, the modification was considered to be essential for spore formation but recent evidence indicates that the alteration of core enzyme is an

artifact (99). Murray et al (89) have reported that the changes resulting from the loss of sigma activity, which wereppreviously observed, did not occur in a defined sporulation medium although sporulation was normal.

Studies of nucleic acid changes during sporulation (160) show that growth and net synthesis had occurred before $t_{\frac{1}{2}} - t_2$ in B. cereus and that synthesis of DNA ceased during the axial filament stage (approximately t_1). At the time of forespore development secondary synthesis of DNA occurred at a linear rate and ceased when each sporulating cell contained three times the amount of DNA found in the free spore. The DNA content of the cells remained constant at this period and then decreased as lysis of the mother cell began.

The antibiotics produced by Bacillus species has been implicated in an early step of the initiation of sporulation (5, 85, 125). Balassa (6) suggested that early in sporulation, the antibiotic is a possible candidate for one of the first products of transcription of the spore genome. This hypothesis was extended by Halvorson (54) who postulated that the synthesis of vegetative cell mRNA is selectively blocked by the antibacterial agent. However a number of mutants have been found which produce small amounts of

antibiotics and sporulate normally. From preliminary work on Bacillus species three distinct antibacterial agents such as edeine, gramicidin and tyrocidine (118) have been reported. The work of Bernlohr and Novelli (13) indicated that the antibiotic, bacitracin may be a primary structuraluunit of the spore coat of B. licheniformis but this could not be confirmed by Snoke (132).

Antibiotic production has been reportedin Clostridium species but it is not known whether antibiotics similar to those of Bacillus species occur during anaerobic sporulation.

Other factors which have contributed to our knowledge of sporogenesis of both Bacillus and Clostridium spp. include the development of cultural methods which permit synchronous sporulation (29, 165), and electron microscopy used by various investigators to reveal the ultrastructural changes of sporulating cells (81, 119, 161, 162).

Methods for the synchronization of nuclear and cellular division of microorganisms include single or multiple temperature shifts (80) medium changes (165) and mechanical selection (23). During sporogenesis a high degree of synchrony is readily achieved in Bacillus species whereas clostridial cells invariably show a mixed population. Collier (29) introduced a method

consisting of serial sequential transfers of an actively growing culture and obtained a pseudo-synchronous population of C. roseum. This technique was later used for sporulation of Bacillus species (22, 120, 137), a putrefactive anaerobe and for a detailed study of the development of heat resistance of spores of C. roseum in relation to the intracellular calcium and DPA content (159). In Bacillus, synchronous cultures have been used to study the sequence of morphological (120), biochemical (54) and physiological changes (153) and the effects of specific inhibitors (62, 116, 139) on the biosynthetic activities during the stages of sporulation.

The temperature of incubation of Bacillus and Clostridium species affects the growth response, the yield and properties of spores. Elevated temperatures have resulted in increased heat resistance of spores of B. subtilis (73) and B. coagulans (97) but not of B. stearothermophilus (41). In B. cereus (91), a shift above or below the optimum growth temperature, 30 C resulted in a lowered heat resistance and the reduction of the number of spores.

The term "endotrophic sporogenesis" refers to spore formation occurring under extreme conditions of starvation after transfer of the vegetative cells

to a replacement medium such as water, saline etc. Endotrophic sporogenesis of Bacillus species is independent of exogenous nutrition (19).

Few data are available on the endotrophic sporogenesis of Clostridium species. Hsu and Ordal (60) have shown that C. thermosaccharolyticum does not sporulate endotrophically but required an exogenous carbon and energy. Experiments with C. botulinum (32) indicate that even though the cells are committed to sporulate, they are not able to complete the process endotrophically because a few specific amino acids are essential for forespore maturation. B

Bacterial (27, 122) and yeast (150) sporogenesis is under the control of catabolite repression and has been shown to be derepressed by cyclic AMP in yeast (43). In bacteria, cyclic AMP has been established as a mediator in catabolite repression (99) involving the control of the synthesis of sporulation-related antibiotics (125) and proteases (16) as well as the regulation of a number of catabolic pathways.

A plausible model of the control of sporulation in Bacillus presented by Schaeffer (120) and Halvorson (54) has now been extended to include new data. The DNA of the cell can be transcribed at all times during growth and sporulation (28). Thus, mRNA can be produced

when required, and the amount of mRNA is controlled via the feedback type mechanism proposed by Jacob and Monod (63) and Stent (140). The control of the phenotype of the cell is at the translation level.

"Spore genes" are distributed throughout the genome of Bacillus and some close linkages are known. It is probable that there are many spore gene sites on the whole genome and thus many controlling operator sites. The mechanisms of regulation throughout all stages of the cycle are: repression of enzyme synthesis (160), feedback inhibition of enzyme activity (161), induction-catabolite repression of spore enzymes and catabolite enzymes (122). The model presents a picture consistent with the controls in effect when cells are growing either on a relatively high level of readily utilizable carbohydrate, such as glucose, or on a glucose-free rich medium that also produces a high growth rate, such as nutrient broth.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organisms

Non-toxigenic mutants, sporogenic, MSp^+ and asporogenic, RSpoIIIa, derived from Clostridium botulinum type E, ATCC 9564, a toxigenic wild type were used in this study.

Media

The media used included Trypticase peptone glucose broth (TPG) which contained 5% Trypticase (BBL), 0.5% peptone (Difco), 0.4% glucose and 0.2% mercapto-acetate (Matheson, Coleman and Bell) as reducing agent (126). The standard volume used for growth was 10 ml except when otherwise specified. Solid media such as Liver veal agar, LVA (Difco), brain heart infusion agar (BBL), potato dextrose agar (Oxoid), nutrient agar (Difco) and blood agar containing 5% human blood were used for pure culture studies. Each agar medium contained 0.13 mM $MnSO_4$ and 0.2% mercaptoacetate and was adjusted to pH 7.2. Cultures were incubated at 30 C for 48 h in a GasPak anaerobic system (BBL).

Isolation of mutants

The wild type strain was grown in TPG broth for 10 h and washed in 0.05 M tris-maleic acid (TM) buffer pH 6.0 (1). The cells were then treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG), 0.15 mg/ml. After treatment for 20 min at 30 C, the cells were collected by centrifugation, washed in TPG broth and diluted before plating on liver veal agar. Clones of mutants were isolated after 48 h anaerobic growth, and transferred to 5 ml TPG.

Acriflavin or quinacrine was added to 10 h cultures of the wild type strain, toaa concentration of 6 ng/ml (112) and incubated for 14 h. Mutants were isolated from clones on LVA after 48 h.

An oligosporogenous mutant population was selected by replicate sampling of broth cultures after 30 transfers in TPG.

A variant of an oligosporogenous strain was also obtained by mixing an equal vol of the supernatant fluid of the MSp^{+}culture containing 90% refractile spores and a 10 h culture of the wild type strain and inoculated into fresh TPG broth in a 1:10 dilution.

The toxicity was determined by mouse protection tests (38) using untreated and trypsinized filtrates of 72 h cultures. Control groups of mice received type E antitoxin (10,000 IU/mouse) 30 min prior to

injection of filtrates.

The strains were stained with fluorescein isothiocyanate conjugates of type specific E antiserum of C. botulinum and the extracts were also tested by immunodiffusion with type E antiserum (41).

Cultural methods

The cells of the sporogenic, MSp^+ and the asporogenic, RSpoIIIa mutants were prepared in Trypticase peptone glucose (TPG). Initial cultures were grown for 10 h in TPG with the sporogenic culture being derived from spores activated at 75 C for 15 min. After three successive 10 h transfers, the cells were of the same physiological age and the MSp^+ cells sporulated nearly synchronously. This procedure was used throughout the study at a final cell density of 10^6 cells/ml media. Samples of actively growing cultures were withdrawn with a syringe at 5 h intervals during incubation at 30 C. Growth was measured by optical density at A_{600}. Sporulation was estimated as percent of refractile spores in 1000 cells examined by phase contrast microscopy. Viable spore counts were carried out on cultures which were preheated at 75 C for 15 min, then plated on liver veal agar and incubated in a GasPak anaerobic system.

Cultures grown in excess glucose

The cells from nearly synchronous 10 h "active cultures" prepared as described, in TPG broth were harvested by centrifugation and washed three times with trypticase peptone broth containing 0.2% mercaptoacetate. The washed cells were inoculated into 10 ml TPG, 22 mM glucose or 10 ml Trypticase peptone containing excess glucose, 0.27 M (TPGe) to give cell density of 10^6 cells/ml. Samples were tested for growth, pH, glucose consumption and degree of sporulation and ultrastructure of RSpoIIIa.

Carbon sources

Selected sugars and organic acids, 0.2 - 0.4% were used to replace glucose as the main carbon source in trypticase peptone broth (Tables 4 and 5). The standard procedure was followed and control cultures were grown without glucose. Samples were withdrawn at intervals and analyzed for growth, pH, granulation (133), degree of sporulation and cell morphology.

Electron microscopy

Five ml samples of cultures of the asporogenic mutant, RSpoIIIa, were removed at 2 h intervals, centrifuged and fixed by the method of Kellenberger et al. (67). The pellet of cells was dehydrated in

graded concentrations of ethanol (30%, 50%, 70%, 90% and absolute) and infiltrated with araldite (81). Sections were mounted on a 400-mesh uncoated grid, stained with uranyl acetate (25%) and lead citrate (0.3%) and examined with a Hitachi HU-11 electron microscope.

Detection of exoprotease and antibiotic

Samples of the supernatants fluids of 10 h cultures of MSp^+ and RSpoIIIa were screened for proteinase and antibiotic activities. Serum albumin plates containing one part sterile bovine albumin, 2%, Fraction 5 (Nutritional Biochemicals Corp.) in 0.1 M potassium-phosphate buffer, pH 7.2 and one part agar, 2.2% (47) were spotted randomly with the supernatant fluid of each mutant. Proteinase activity was ascertained by zones of clearing (2-6 mm in diam) after 48 h incubation. Antibiotic activity was tested by the cup-plate assay method with Staphylococcus aureus, strain p209 as the indicator organism (155).

DPA Assay

The method of Janssen et al (64) was used with 40 ml samples.

Gas chromatography

Batch cultures of MSp^+ and RSpoIIIa were grown in air-tight flasks (100 ml TPG) fitted with a sampling assembly. Gas samples were withdrawn with Hamilton gas tight syringes and analyzed with a Beckman thermo-conductivity gas chromatograph by injection of 50 nl samples into a 12 ft copper column (¼" outside diam) filled with 100-200 mesh Poropak Q (10) (Waters Assoc. Inc., Framingham, Mass., U.S.A.). The column was maintained at 260 C and a bridge current of 81 mA applied. Helium at a flow rate of 30 ml/min was used as a carrier gas. Samples of authentic mercaptoethanol vapour, and CO_2, N_2O and NO gases were similarly analyzed. The relative retention and per cent composition of the gases were calculated.

Isolation and estimation of poly-β-hydroxybutyrate (PHB)

Cells were collected by centrifugation from 15 ml samples of TPG cultures and suspended in 9ml alkaline hypochlorite reagent (157). After 24 hr at 37 C lysis was confirmed by phase contrast microscopy. The reaction mixture was centrifuged at 5500 x g for 30 min and the solid pellet was washed successively with water, acetone, and ether and dissolved in hot chloroform. After evaporation, the sample was treated with acetone-ether (2:1 v/v) and the precipitate was dissolved in

1 ml hot chloroform. The sample was then precipitated with 2.5 ml ether before drying for 12 h at 110 C. The white powder was dissolved in 0.5 ml hot chloroform and heated for 10 min at 100 C after the addition of 10 ml H_2SO_4. The solution was cooled and read at 235 nm against an H_2SO_4 blank (72). Purified extracts of PHB and sodium DL-β-hydroxybutyric acid (Sigma) were used to prepare a calibration curve (E_{235} of 1.0 was given by 8.2 μg poly-β-hydroxybutyrate/ml). Duplicate samples were assayed twice and the averages of four determinations were calculated. Purified granules were examined by electron microscope using carbon (156) for the shadow casting.

Incorporation of acetate and butyrate into PHB

^{14}C-Acetic acid (U) (specific activity 16.5 mCi/mmol) or butyric acid 1-^{14}C (specific activity 57 mCi/mmol), (Amersham Searle Corp. Arlington Heights, Illinois) was added to the TPG broth in amounts of 1 μCi/100 ml. Unlabelled sodium acetate or butyric acid was added to a final conc of 2×10^{-3} M. The growth medium was inoculated with 10^6 cells of the MSp^+ or RSpoIIIa and incubated at 30 C. Poly-β-hydroxybutyrate granules were isolated and purified as described above. The purified polymer was dissolved in hot chloroform, evaporated to 0.2-0.3 ml.

Manometry

Carbon dioxide and hydrogen production rates were determined by standard manometric techniques (151). The reaction mixture consisted of 1 ml cell suspension in trypticase peptone broth without glucose (0.7 - 1.0 mg cells dry wt obtained from 10 h cultures), 0.5 ml of 0.1 M phosphate buffer pH 7.2, the reagents in the side arms and trypticase peptone broth to give a total volume of 3.0 ml. After equilibration at 30 C under oxygen-free nitrogen, 7.2 mg glucose was added from one side arm and the fermentation was allowed to proceed for a predetermined time period between 5 h and 35 h. At 5 h intervals, the reaction was stopped, CO_2 evolved was absorbed with 0.2 ml of 20% NaOH placed in the center cup and H_2 was measured. CO_2 was estimated by subtracting the hydrogen evolved from the total gas produced. Correction for the bound CO_2 was made by tipping in 0.1 ml of 40% phosphoric acid from one side arm. Flask constants were calculated. The residual liquid was centrifuged and analyzed for glucose, pyruvate, acetate, ethanol, and butyrate.

Gas chromatography of volatile fatty acids

Culture samples of 100 ml TPG with glucose as main carbon source were collected at 5 h intervals for 5 to 35 h, centrifuged and the supernatant solutions

adjusted to pH 8.0 with 1 N NaOH. The samples were concentrated to 10 ml by fanning at 4C and prior to steam distillation, an equal vol of 1 N H_2SO_4 was added bringing it to pH 2.0. Distillates were collected in 1 N NaOH, concentrated by freeze drying and acidified to pH 2.0 before analysis by gas chromatography.

The distillates were analyzed on a model 2100 Varian Aerograph gas chromatography unit, equipped with a flame ionization detector and a disc chart integrator. Pyrex U-shaped glass columns, 6 ft x 3 mm ID, were packed with Chromosorb 102, 100/120 mesh, (Chromatographic Specialists Ltd., Brockville, Ontario, Canada). The instrument was operated at these conditions: column temperature, 200 C; carrier gas, nitrogen, 24 ml/min/ hydrogen, 24 ml/min; and compressed air, 240 ml/min.

The standard mixture contained 1 mg/ml of each of the authentic fatty acids including both the n-types and the isotypes and mercaptoacetate. For separation, 5 µl were injected into the gas chromatograph and the peak areas and relative retention times were calculated from the disc chart integrator readings of the gas chromatograms. Supernatant solutions of actively growing cultures were monitored for residual glucose and volatile acid content during growth and sporulation.

Inhibition studies

To test the effect of inhibitors of sporulation on butyrate synthesis and acetate accumulation, β-phenethyl alcohol, 0.35% (109), fluoracetic acid, 5 mM and 2-picolinic acid, 5 mM (24, 50) were separately added to cultures at different intervals. Then growth, sporulation, acetate, and butyrate accumulation were estimated.

Assays of enzymes of butyric type of fermentation

Cells from cultures of sporogenic and asporogenic mutants were harvested at 4 h intervals between 0 to 24 h and MSp^+ spores and late stationary phase cells of RSpoIIIa were collected at 48 h. Cell-free extracts were prepared in 0.1 M Tris (hydroxymethyl) aminomethane-hydrochloride, 10 mM $MgCl_2$ and 0.001 M $MnSO_4$, pH 7.4, after disruption in Bronwill homogenizer for 90 sec. Acetate kinase was assayed at 30 C according to the method of Rose (113), by measuring hydroxamic acid formed from acetate in the presence of hydroxylamine and specific activity was expressed as μmoles acetyl phosphate formed per min per mg protein. Phosphotransacetylase was measured by the method described by Stadtman (138). Butyryl coenzyme A dehydrogenase was assayed by the method of Mahler (83) by both the Indophenol and the triphenyltetrazolium chloride pyocyanine (TTZ-pyocanine).

Uptake and utilization of radioactive glucose, pyruvate, acetate and butyrate

In these experiments [U-^{14}C] glucose (spec. activity 4.08 mCi/mM), [U-^{14}C] pyruvic acid, (sodium salt, spec. activity 120 mCi/mM), sodium [U-^{14}C] acetate (spec. activity 57 mCi/mM) and [1-^{14}C] butyric acid (spec. activity 16.5 mCi/mM) purchased from Amersham/ Searle Corp., Arlington Heights, Illinois 60005, U.S.A. were added separately to the growth medium in amounts of 1 μCi/100 ml medium. The organisms were harvested at 4 h intervals, between 0 to 24 h, by centrifugation at 15,000 g for 15 min and washed twice in 0.25 M NaCl containing 0.01 M $MgCl_2$ (114). Cell fragments were separated from the "soluble" fraction after osmotic lysis (109).

Extraction and fractionation of cell lipids

Lipids from cells maintained at three stages of the growth cycle were extracted with chloroform and methanol 2:1 by vol at 50 C for 30 min. Insoluble residue was removed by filtration through a No. 1 Whatman filter paper, previously extracted with chloroform and methanol. The solvent was evaporated by heating in a boiling water bath under a stream of

nitrogen gas and the lipid residue redissolved in 1 ml of chloroform. The lipid mixture so prepared was chromatographed on a 7 x 95 mm column (114) of activated silicic acid (100/300 mesh, reagent grade, Sigma Chemical Company, St. Louis, Mo., U.S.A.) which was prewashed with chloroform.

Neutral lipids were eluted from the column with 100 ml chloroform and polar lipids by 150 ml ethanol using positive pressure.

Other enzyme assays

The enzymes were prepared according to the flow sheet procedure outlined by Warren (156). Batch cultures, 500 ml, were harvested by centrifugation at 5000 g at 5 h intervals during growth and sporulation and the pellet was suspended in 10 ml of 0.05 M tris-HCl buffer, pH 7.1 and cooled on ice. The cell suspension was disrupted in a Biosonik sonicator (Bronwill Scientific, Rochester, N.Y.) set at maximum speed and centrifuged at 10,000 g for 15 mins. The supernatant was assayed for alkaline and acid phosphatase. In addition, an ammonium sulphate precipitate of the supernatant was analyzed for glucose dehydrogenase and DPNH oxidase activity.

Alkaline phosphatase

Sodium p-nitrophenyl phosphate, 3 µmoles in 1 M tris-HCl buffer, pH 8.0, in a total volume of 3 ml was allowed to equilibrate at 30 C in a quartz cuvette. The extinction at A_{410} (155) was recorded at 30 sec intervals after the addition of 0.1 ml cell extract.

Acid phosphatase

A modified method of Spahr et al (134) was followed using 3 ml acetate buffer pH 5.0, 1 ml 0-carboxyphenyl phosphate (Sigma) and 0.5 ml of extract. The reaction proceeded for 1 h at 30 C and was stopped by the addition of 3 ml of 2N NaOH and the absorbance was determined at A_{410}.

Glucose dehydrogenase

The reaction mixture contained 0.2 ml extract, 300 µmoles tris buffer, 100 µmoles glucose and 2 µmoles diphosphopyridine nucleotide in a total volume of 3 ml, and was adjusted to pH 7.6. Controls without glucose were also tested. The extinction at A_{340} (4) was recorded at 15 sec intervals for 1 to1.5 h.

DPNH Oxidase

The reaction was carried out with 0.2 ml extract added to 0.5 mmoles of DPNH in 3 ml 0.1 M

tris buffer, pH 7.6 and the extinction measured at 15 sec intervals for 1 to 1.5 h at A_{340}.

Effect of varying concentrations of glucose

Increasing amounts of glucose ranging from 22 mM to 0.27 M were added to trypticase peptone broth containing mercaptoacetate and adjusted to pH 7.2. Cells from 10 h cultures of both mutants were inoculated into the media and the percentage of refractile endospores, growth, glucose consumption and pH changes were measured after 12, 24, and 36 h incubation. The ultrastructure of the RSpoIIIa mutant grown for 24 h in broth containing 0.135 M glucose was examined by electron microscopy

Cyclic AMP assay

Cells grown in 45 ml TPG, 22 mM glucose, or TPGe, 0.27 M glucose, TPGe + adenosine 3',5' cyclic monophosphate (cAMP) (10^{-4}M) were harvested at intervals of 12, 24, 36 h after determining the absorbance and degree of sporulation. The cells were then washed twice with tris-EDTA buffer, 0.05 M pH 7.5 and disrupted with a Bronwill homogenizer in 4 cycles of 60 sec each. The supernatant fluid was deproteinized with an equal volume of 10% trichloroacetic acid.

Samples (50 µl) of the resulting fluids were assayed for cyclic AMP (cyclic AMP assay kit, Amersham/Searle).

Effect of cAMP or MB-cAMP on sporulation in TPG and TPGe

Varying concentrations of cAMP or monobutyrate adenosine 3',5'-cyclic monophosphate (MB-cAMP) were added into TPG and TPGe before cell inoculation. The percentage of endospores were estimated after incubation for 12, 24, 36 h.

Effect of adenine or guanine nucleotides on the sporulation of Clostridium spp. in TPGe.

To examine the specificity of cyclic AMP in reversing the repression of sporulation of glucose, the effect of some related substances on sporulation were tested. Concentrations of cAMP; MB-cAMP, adenosine 5'-triphosphate (5'-ATP), adenosine 5'-diphosphate (5'-ADP), adenosine, 5'-monophosphate (5', AMP); guanosine 3',5'-cyclic monophosphate (cGMP), guanosine 5'-triphosphate (5'-GTP), monobutyrate guanosine 3',5'-cyclic monophosphate (MB-cGMP), guanosine 5'-monophosphate (5'-GMP). (Sigma Chemical Co.), were added to the TPGe medium before inoculation. The percentage of endospores were estimated after incubation for 24 h.

Effect of 3',5'-cAMP or MB-3', 5-cAMP on growth, glucose utilization and pH.

TPG or TPGe medium with or without added cyclic AMP or MB-cyclic AMP was inoculated with the washed cells at a density of 10^6/ml. Samples were removed for analyses at intervals of 12, 24, 36 h. Growth was measured at A_{600}; glucose was determined by "Glucostat" reagent (Worthington Biochemical Corp., Freehold, N.J.) and the pH of the samples was determined.

Analytical methods

The protein content of whole cells and cell-free extracts was assayed by Lowry method (78). Glucose was estimated enzymatically with the glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). Pyruvic acid was determined by the method of Friedemann and Haugen (49) and ethanol by the enzymatic method described by Sigma, Bulletin No. 331-UV. 1971.

Radioactivity measurements

Samples (0.2 - 0.3 ml) of lysed organisms purified PHB and lipid extracts were transferred to scintillation vials. Then 10 ml of Bray's solution (21) was added and ^{14}C-counting done in a Packard scintillation counter for 10 min.

R E S U L T S

RESULTS

NTG-Induced mutants

Asporogenic mutants obtained by selective replicate sampling, by acriflavin or quinacrine treatment showed a high rate of reversion to the sporogenic state (on subsequent transfers). Stable asporogenic mutants were obtained only from the NTG-treated cells and the asporogenic state of the mutants was retained after more than 20 transfers.

RSpoIIIa and MSp^+ clones were clearly differentiated on liver veal agar. The RSpoIIIa clones were pinhead in size, whitish and transparent, while the MSp^+ clones were 5-10 mm in diam, pale-brown and opaque. Phase contrast microscopic examination showed that the RSpoIIIa cells were smaller in size and shorter in length than the MSp^+ cells. Satisfactory growth was obtained on LVA but not on other solid media tested. The colony description as well as some phenotypic characteristics of MSp^+, RSpoIIIa and the wild type are shown in Table 1. and photographs of the colonies of the mutants are shown in Fig. 14.

Cytoplasmic granules were observed in a few cells of both mutants at 12 h, in 80% of the cells by 16 h but were not seen in free spores.

Toxigenicity

Supernatants fluids from cultures of the wild type ATCC 9564 were lethal for mice within four hours whereas those from cultures of MSp^+ and RSpoIIIa proved innocuous.

Antigenicity

The asporogenic and sporogenic mutants showed precipitin lines of identity with the antiserum against the wild type E strain and a significant degree of fluorescence with a fluorescein-conjugate of the antiserum.

The sequence of the ultrastructural changes during growth of the RSpoIIIa mutant was as follows.

Stage I. The onset of sporulation was characterized by the aggregation of the nucleoplasm of the cell into a central axial filament at about 8 h after the transfer of cells from a 12 h culture. Sample photomicrographs are shown in Figs. 1 and 2.

Stage II. After 12 h a forespore septum, a double unit membrane, was formed by the infolding of the cytoplasmic membranes at one end of the cell pole (Figs. 3 and 4). Septation was not detected until 14 h and mesosomes, connected with the nuclear filaments were occasionally seen on both sides of the septum (Fig. 4). At this time a considerable number of translucent granules were visible in the cytoplasm of the cells and a few were trapped within the forespore.

Stage III. Figures 5 and 6 show the engulfment of the forespore. Chromatin material as wellaas electron-dense ribosome aggregates are clearly visible. Sporulation is blocked during this stage. Further incubation gives rise to aberrant changes in the surface layers including convolutions and severance of small segments of the inner surface of the cell wall (Fig. 7b). Highly disorganized cytoplasmic material was observed to disperse from the central region of the cell prior to lysis (Fig. 7a). Cell lysis was due to rupture of the cell wall and cytoplasmic membrane at multiple sites. The lytic activity was extensive and many wall membranes fragments were observed around each lysed cell (Figs. 8, 9, and 10).

The effect of increasing the amount of glucose, 0.135 M, on the asporogenic mutant was that 80% of the cells were maintained at stage II of septum formation for up to 36 h without lysis, and the forespore stage was not seen Figs. 11, 12, 13).

Changes in growth, sporulation and pH.

The kinetics of growth and sporulation and pH changes of the sporogenic and asporogenic strains grown in TPG are shown in Fig. 16. At 10 h, the OD of both cultures reached maximum, indicating the 'end of exponential growth'. The OD of the MSp^+ then decreased abruptly, reaching minimum at 15 h, 'early stationary phase' or 'prespore stage' and remained essentially unchanged afterwards, 'late stationary phase' or 'endospore stage', 15 h to 25 h. At the end of 'maturation stage', 25 h to 35 h, free spores were detected. At the end of exponential growth, the OD of the RSpoIIIa cultures remained constant for 10 h more and decreased to its lowest level at 30 h. Both cultures showed a rapid drop in pH during growth. The MSp^+ culture was pH 5.8 at the prespore stage, whereas, the asporogenic culture was pH 6.0 during late stationary phase. Refractile endospores were first detectable

in the MSp^+ strain at 15 h, then increased rapidly and reached maximum of 90% at 30 h.

Control cultures grown in trypticase peptone broth without glucose showed no significant changes in OD or pH, (Fig. 17) formed no detectable endospores and did not produce any intermediate compounds. Changes which occurred during growth and sporulation of the mutants are summarized in Table 2. At 16 h, dipicolinic acid, DPA (50 ng/mg cell dry wt) was detected and 12% endospores were observed in the MSp^+ culture. The DPA synthesis continued in parallel with the appearance of the refractile spores, for example by 30 h, 64 ng DPA/mg and 90% endospores have been formed (Fig. 16). Significant amounts of DPA were not detected in the RSpoIIIa strain or in vegetative cells of the MSp^+. Some of the cultures of the MSp^+ showed proteolytic and antibiotic activity during late logarithmic growth phase and early sporulation phase. The antibiotic activity was not a constant property and when present it was weak showing zones of inhibition of about 2 to 3 mm in diameter and disappeared completely during stationary phase.

Gases evolved

The isothermal gas chromatographic analysis yielded four peaks, which were identified as $HS\text{-}CH_2\text{-}$

CH_2-OH, CO_2, N_2O and NO. During sporulation of the MSp^+ the mercaptoethanol and CO_2 content decreased substantially, while at the same time the evolution of dinitromonoxide increased (Table 3 and Fig. 18). The RSpoIIIa culture showed no significant changes in the composition of the gases during growth and did not release N_2O.

The effect of carbon sources on growth and sporulation.

Various carbon sources were tested for their ability to promote growth and sporulation (Table 4 and 5). Glucose adapted cells of the sporogenic and asporogenic mutants fermented glucose, maltose, sucrose and fructose resulting in heavy growth, acid pH, intracellular granules and typical morphology. The MSp^+ cells sporulated with high frequency in these media but the RSpoIIIa cells did not form any detectable endospores. On the other hand, the strains showed only minimal growth in media with acetate, butyrate and succinate. Furthermore, an inoculum of MSp^+ cells maintained in the prespore stage sporulated well in these media, whereas the RSpoIIIa cells were lysed. The other carbon sources tested supported limited growth and no sporulation.

Increasing the glucose concentration (TPGe) reduced the amount and rate of growth and maximum absorbance was not reached until 24 h. No significant differences in growth rates were observed when cyclic AMP was added to either TPG or TPGe medium (Fig. 19).

Table 1. Segregation and designation of MSp^+ and RSpoIIIa clones.

Strain	Spores %	Toxigenicity	Colony type*
Type E, ATCC 9564 replicate sampling	>60	+	large, irregular, opaque
High sporulating (wild type) NTG treatment	>80	+	large, irregular, opaque
Clones			
Sporogenic MSp^+ **	>80	-	large, irregular, opaque
Asporogenic RSpoIIIa**	0	-	pinhead translucent

*After 48 h on liver veal agar (Fig. 14).
**designated according to the nomenclature of Young et al (163).

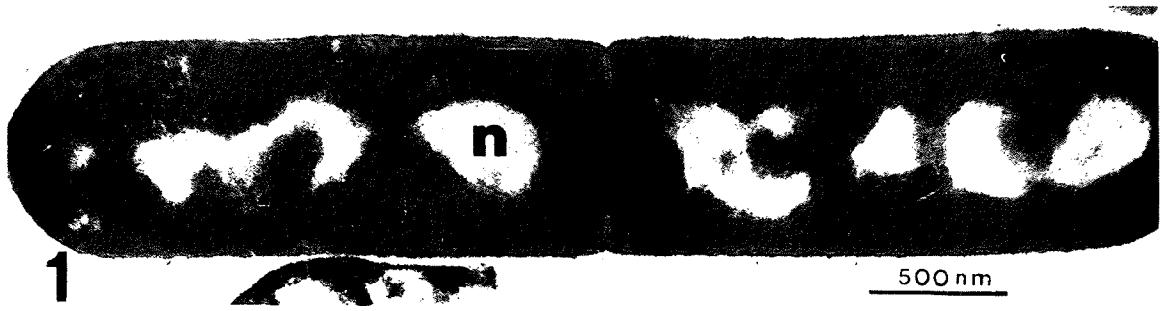
LIST OF FIGURES

Figure 7b. The area delineated in Figure 7a. has been enlarged to show the convolution and initial rupture of the cell wall.

Figure 8, 9, and 10. Lysis of the mutant cells. The extensive lytic activity has severed the cell wall and the plasma membrane into many fragments. Note the highly disorganized cytoplasmic material in the lysed cells and the granules (i).

Figure 11, 12 and 13. Asporogenic mutant cells stabilized with glucose. Sporulation is equilibrated at the end of stage II and lysis was not seen.

Figure 14. A composite photo of clones of MSp^{+} and RSpoIIIa on liver veal agar after 24 h growth; actual size by transmitted light.



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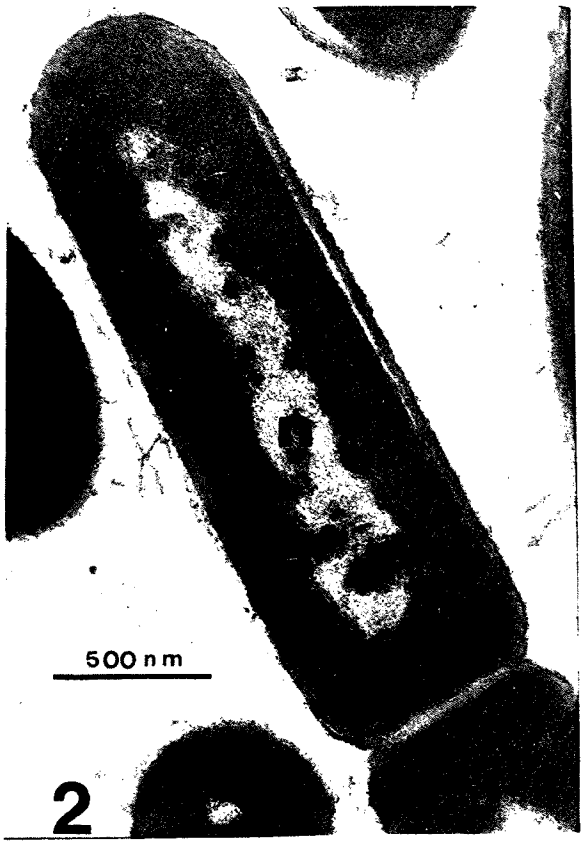

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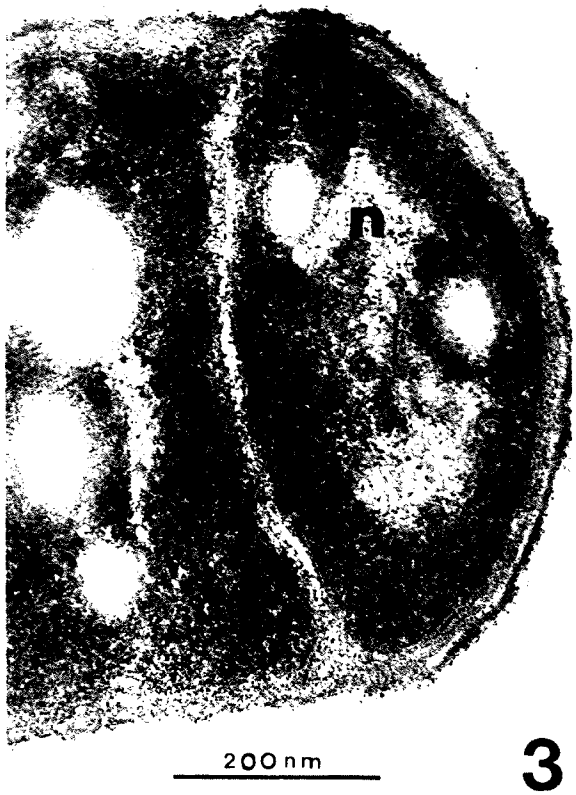

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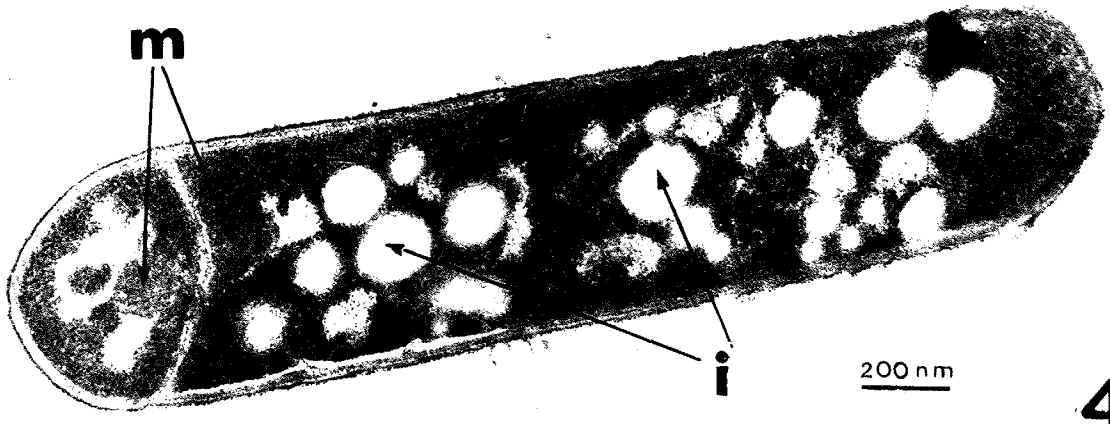

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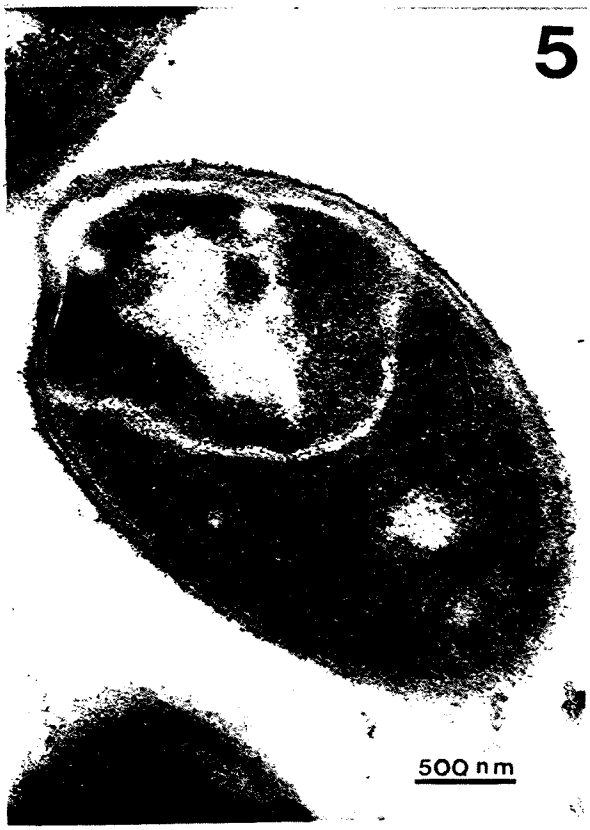
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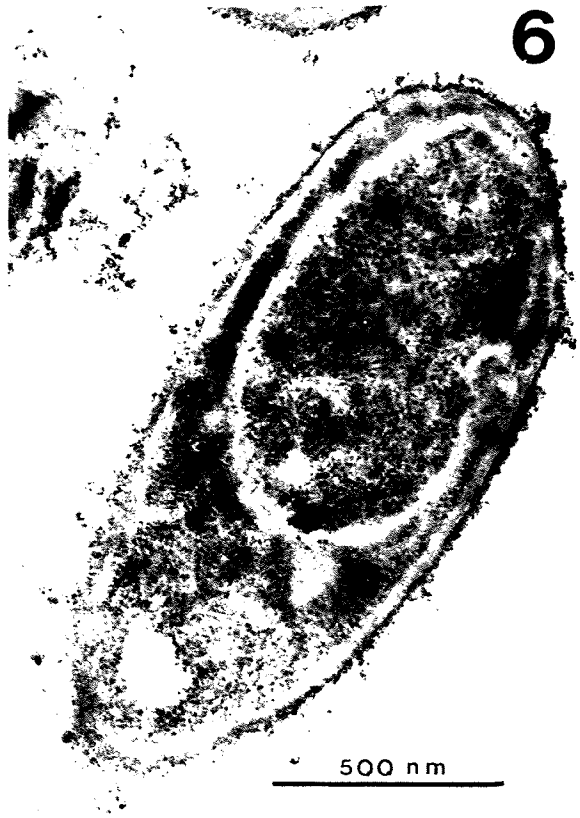
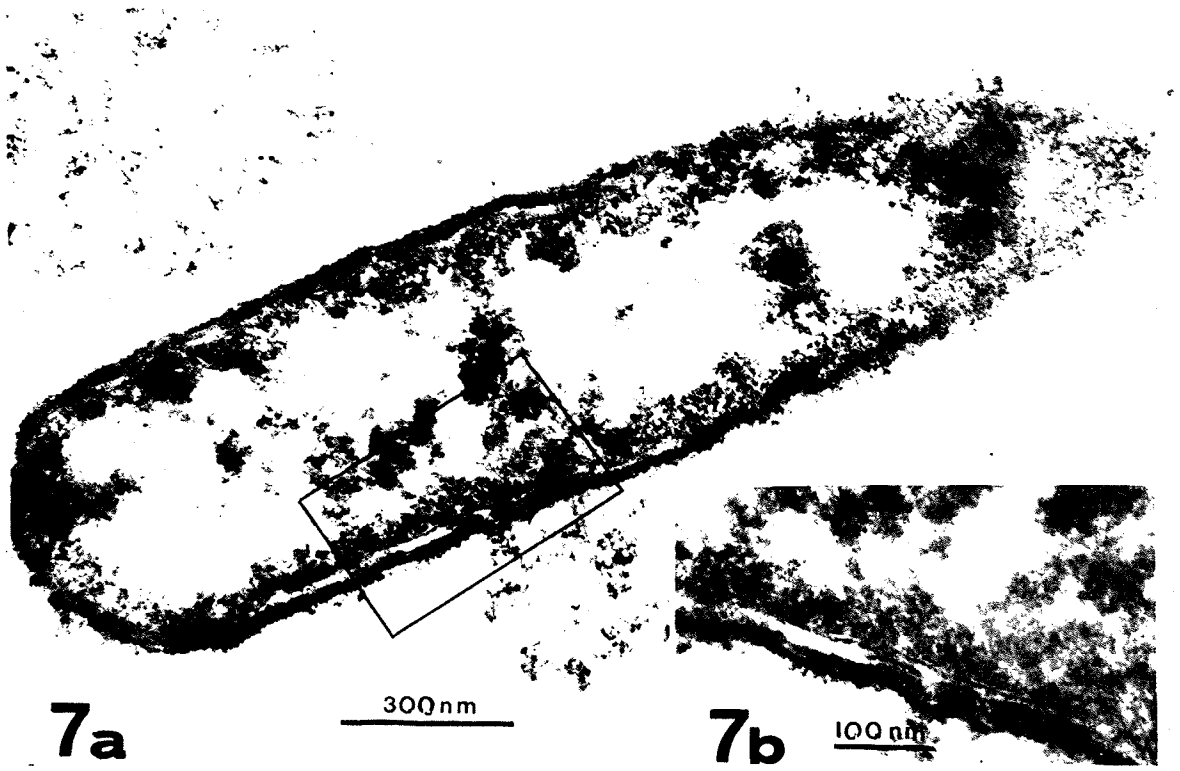
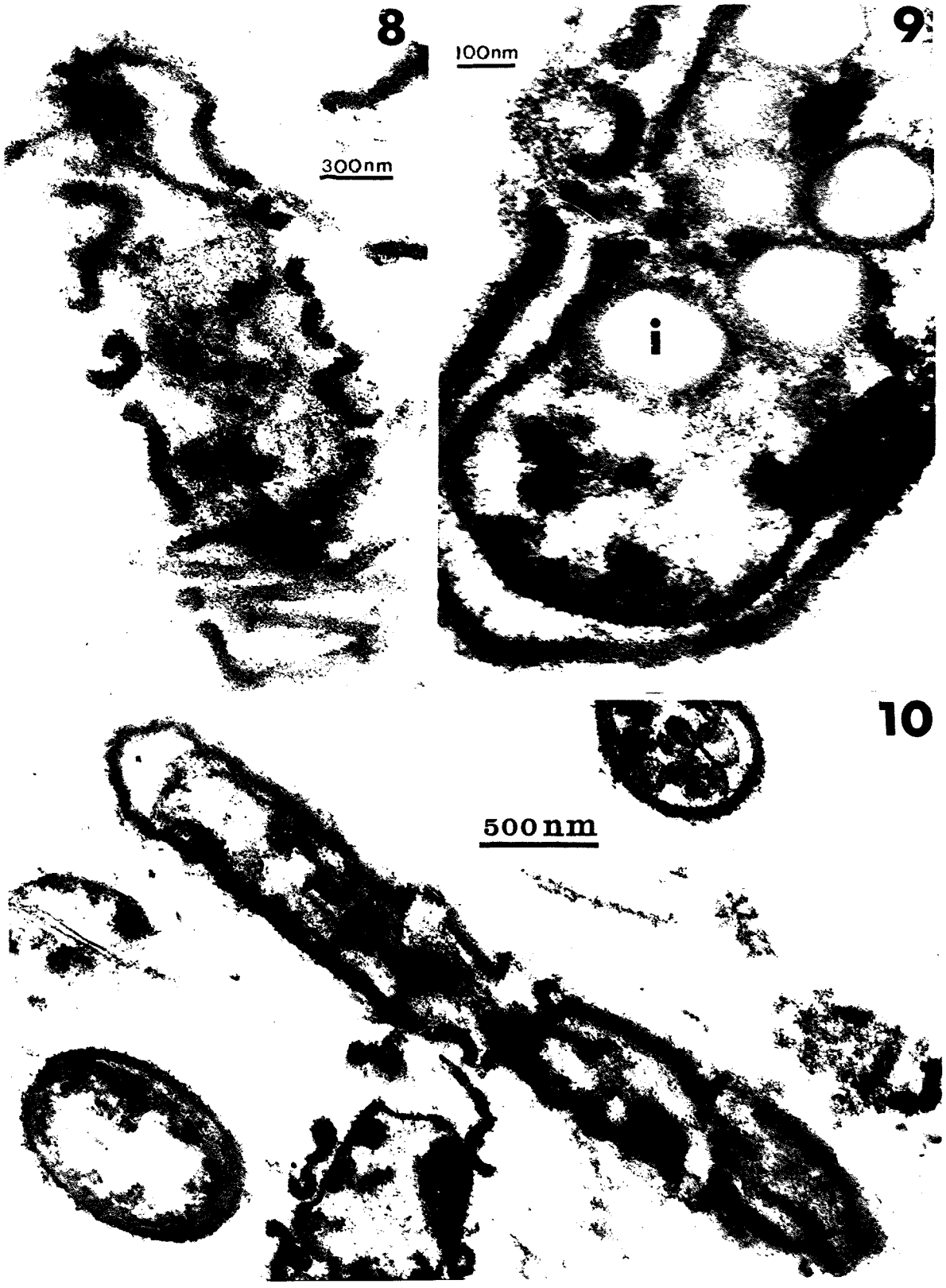
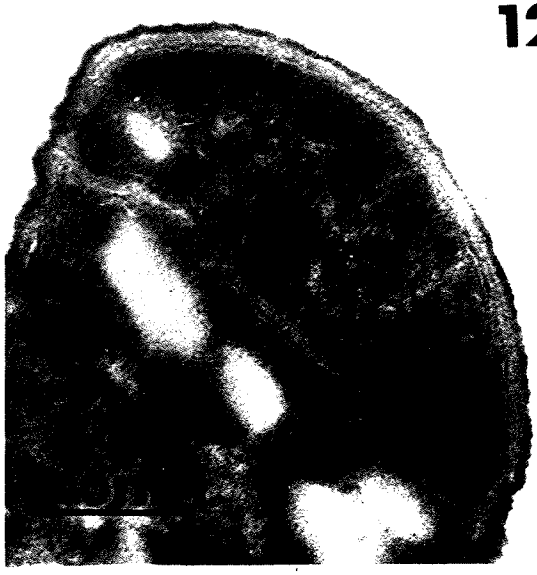

6
500 nm


7a
300nm
7b
100 nm


8
100nm
9
300nm
i
10
500nm

11

12



13

14

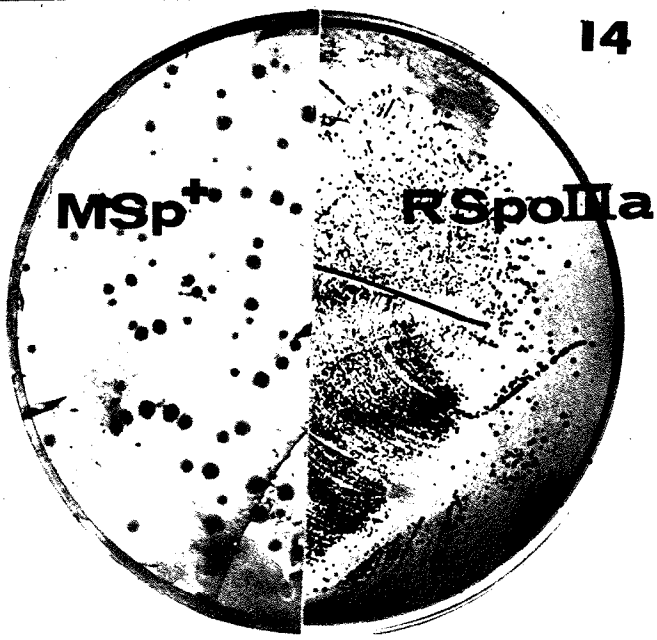


Figure 16. Kinetics of growth and sporulation. The sporogenic, MSp^+, and the asporogenic RSpoIIIa strains were cultivated in TPG. Samples taken at intervals were assayed for growth, sporulation, pH, and DPA synthesis. The arrow indicates the time that protease and antibiotics were first detected.

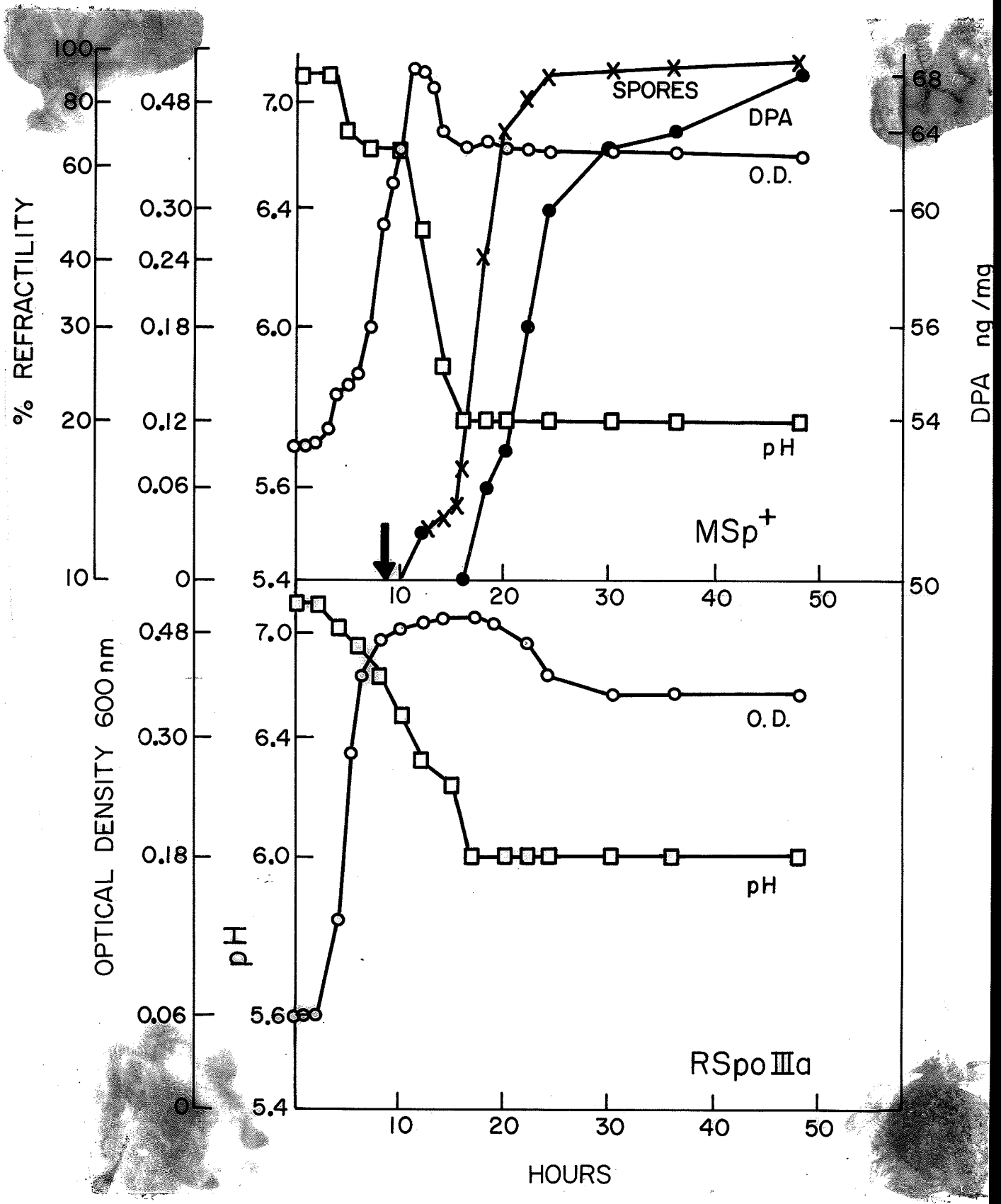

% REFRACTILITY
OPTICAL DENSITY 600 nm
DPA ng/mg
pH
HOURS
SPORES
DPA
O.D.
pH
MSp+
O.D.
pH
RSpo III a

Figure 17. Growth and pH changes with and without glucose.

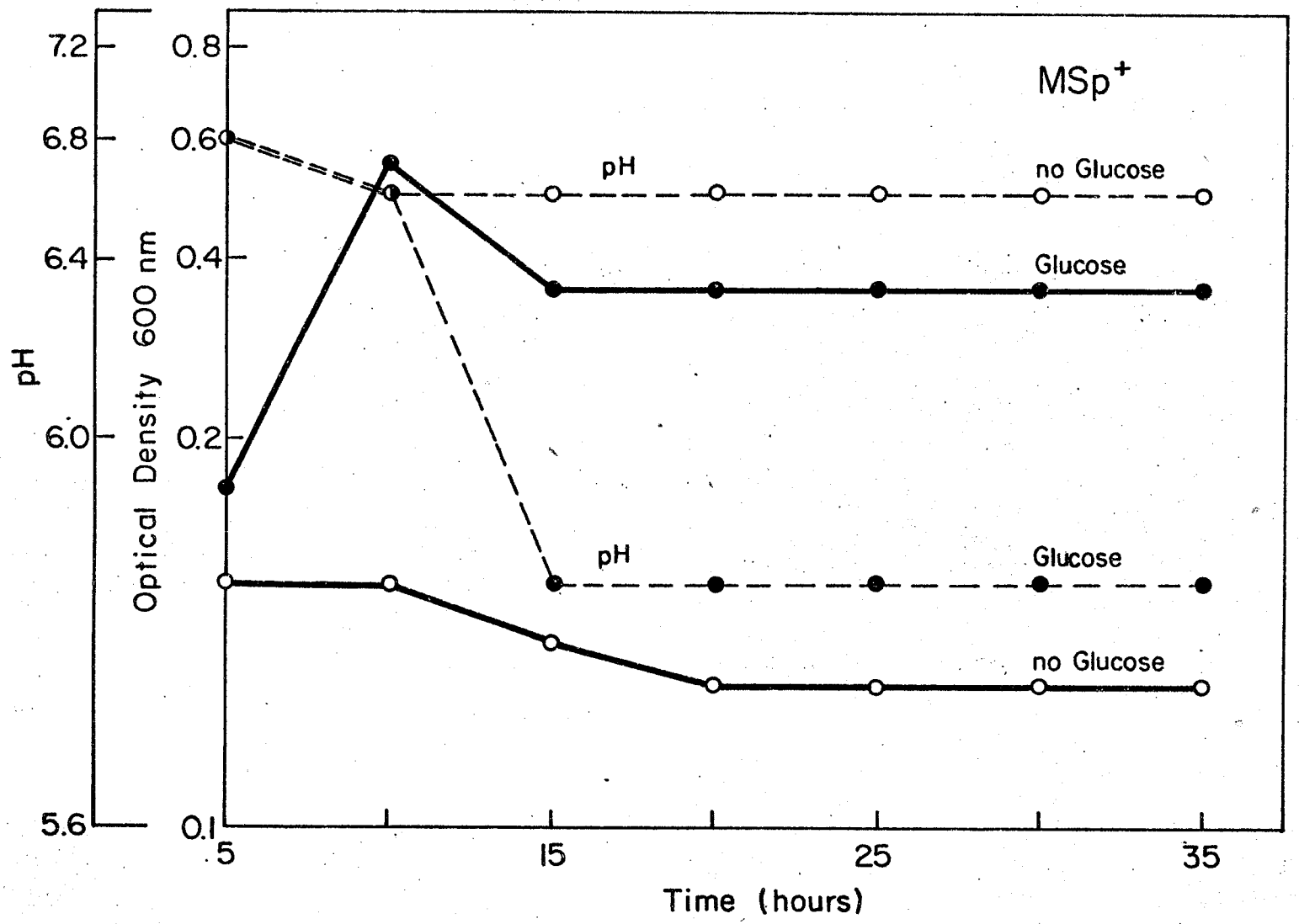

MSp+
pH
no Glucose
Glucose
pH
Glucose
no Glucose
pH
Optical Density 600 nm
7.2
6.8
6.4
6.0
5.6
0.8
0.6
0.4
0.2
0.1
5
15
25
35
Time (hours)

Table 2. Comparison of changes during sporulation

Time hr	Refractile bodies %	O.D. 600 nm		pH		Morphology
	MSp^+	MSp^+	RSpoIIIa	MSp^+	RSpoIIIa	RSpoIIIa
0*	0	0.50	0.48	6.5	6.5	nuclear filaments
2	0	0.42	0.48	6.2	6.2	
4	0	0.42	0.48	5.7	6.0	septation, rare forespore
8	12	0.42	0.47	5.7	6.0	
10	20	0.40	0.46	5.7	6.0	a few lysed cells
12	75	0.40	0.45	5.7	6.0	
22	90	0.40	0.40	5.7	6.0	90% cells lysed

*Eight hours after the end of logarithmic growth

Figure 18. The amount of each gas evolved from 100 ml of TPG during growth and sporulation was determined from the per cent of total area of the peaks. The gases were identified by comparing their relative retentions with known standards.

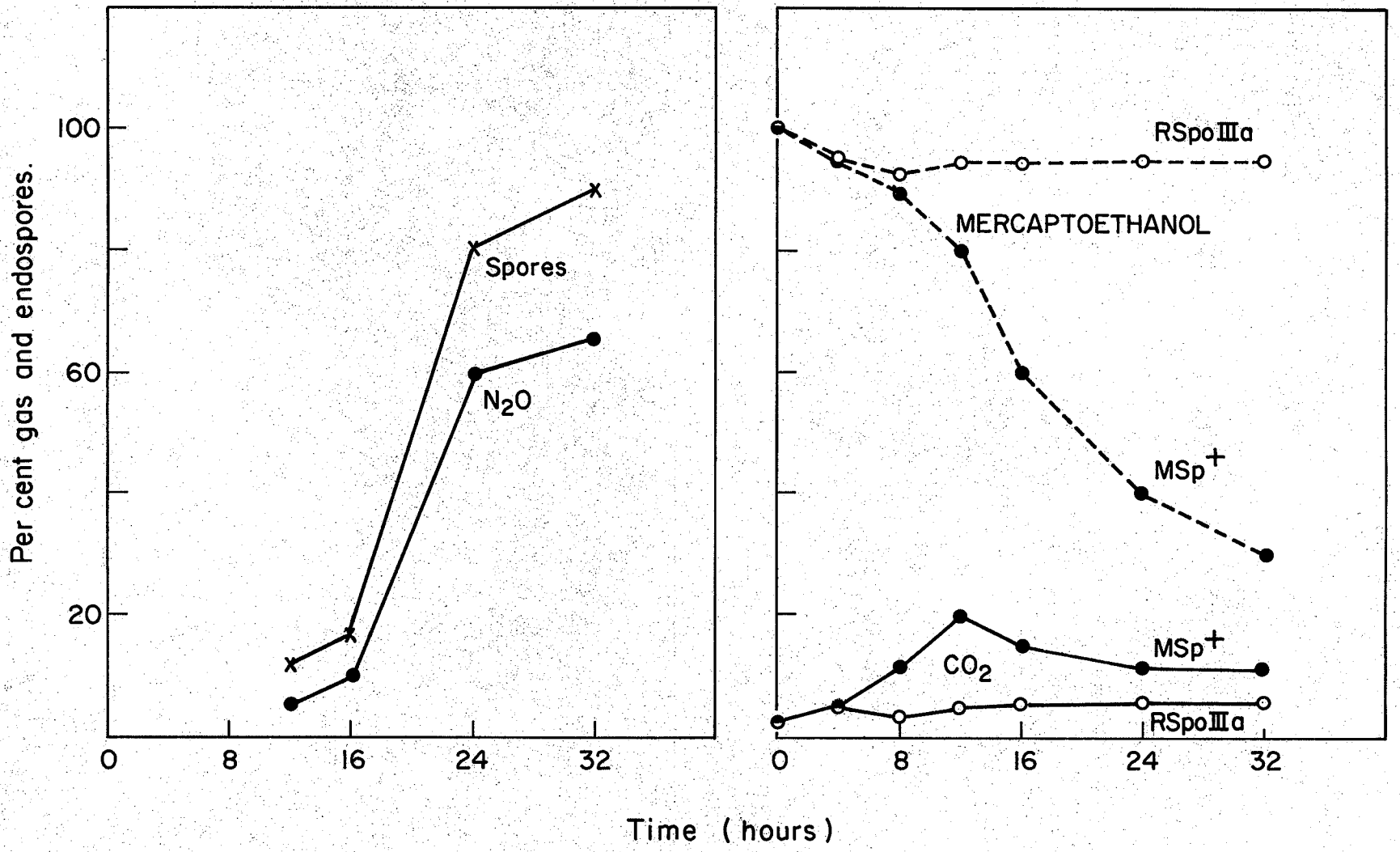

Per cent gas and endospores.
100
60
20
0
8
16
24
32
Spores
N_2O
RSpoIIIa
MERCAPTOETHANOL
MSp^+
CO_2
MSp^+
RSpoIIIa
Time (hours)

Table 3. Composition of gases during growth and sporulation.

Gases	Strain	Per Cent Gas Evolved Time (Hrs)							Dist. in cm	Relative Retention
		0	4	8	12	18	24	30		
Gas 1	MSp^+	99	98.6	95	74	64	34	30	9.9	0.870
	RSpoIIIa	99	97.0	97	96	95	95	95		
	Blank	99	95.0	95	95	95	95	95		
$HS-CH_2-CH_2-OH$									9.9	0.870
Gas 2	MSp^+	1.0	1.4	5	18	9	7	8	14.1	1.27
	RSpoIIIa	1.0	1.2	0.7	3.2	4.6	4.6	5		
	Blank	1.0	1.0	1.0	1.0	1.3	1.2	1.0		
CO_2									13.9	1.24
Gas 3	MSp^+	0	0	0	8	27	59	62	26.5	2.32
	RSpoIIIa	0	0	0	0	0	0	0		
	Blank	0	0	0	0	0	0	0		
N_2O									26.5	2.35
Gas 4	MSp^+	0	0	0	0	0	0	0	1.2	0.11
	RSpoIIIa	0	1.8	2.3	0.8	0.4	0.4	0		
	Blank	0	4.0	4.0	4.0	3.7	3.8	4.0		
NO									1.3	0.12

Table 4. The effect of carbon sources on growth and sporulation of C. botulinum type E, MSp+ in Trypticase peptone broth.

Carbon[a] source	Growth[b] %A_{600}	Final pH	Cytoplasmic Granules	Sporulation[c] %	Morphology
Glucose	100	5.8	+	80	Typical
Maltose	140	5.5	+	60	Typical
Sucrose	100	5.8	+	60	Typical
Lactose	20	6.6	-	0	Small
Fructose	70	5.8	+	80	Elongated
Galactose	0	6.6	-	0	Short and Cubical
Xylose	10	6.6	-	0	Short and Swollen
Pyruvate	20	6.1	-	0	Short and Swollen
Acetate	30	6.2	+	20	Typical
Butyrate	40	6.0	+	30	Typical
Lactate	20	6.7	-	0	Elongated
Succinate	20	6.7	±	20	Oval
Glutarate	20	6.8	-	0	Short and Swollen
Control	10	6.9	-	0	Short and Swollen

[a]All carbon sources were in total concentrations of 0.4% (w/v), except butyrate which was

Table 4 Continued.

0.2% (v/v).

[b]Percent optical density, after 10 h growth was calculated by designating the absorbance obtained in the normal sporulating medium (TPG) as 100%.

[c]Sporulation was determined after 48 h incubation.

Table 5. The effect of carbon sources on growth and sporulation[c] of C. botulinum type E, RSpoIIIa in Trypticase peptone broth.

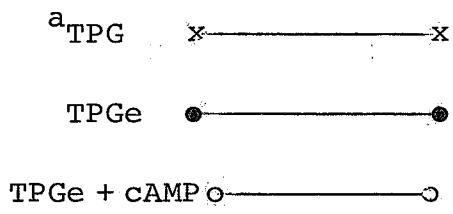
Carbon[a] source	Growth[b] (% A_{600})	Final pH	Granules	Morphology of Cells
Glucose	100	6.0	+	Typical
Maltose	140	6.0	+	Typical
Sucrose	100	6.0	+	Typical
Lactose	10	6.6	-	Lysed
Fructose	80	6.0	+	Typical
Galactose	10	7.0	-	Short
Xylose	10	7.0	-	Lysed
Pyruvate	10	7.0	-	Lysed
Acetate	10	7.0	-	Lysed
Butyrate	10	7.0	-	Lysed
Lactate	10	7.0	-	Non-viable
Succinate	10	7.0	-	Non-viable
Glutarate	10	7.0	-	Short and swollen
Control	10	7.0	-	Non-viable

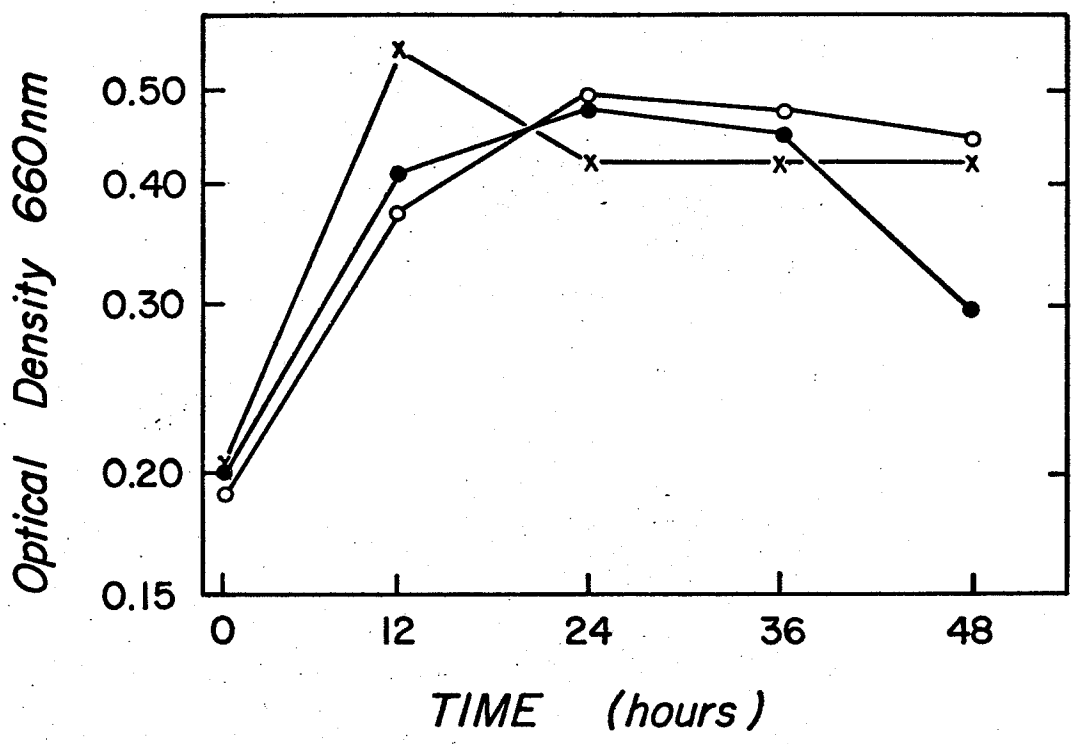
[a]All carbon sources were in total concentrations of 0.4% (w/v), except butyrate which was 0.2% (v/v).

[b]Percent optical density was calculated by designating the maximum OD obtained in the normal sporulating medium (TPG) as 100%.

[c]Spores were not detected after 48 h incubation.

Figure 19. Growth in TPG or TPGe with and without 10^{-4}M cAMP.




Optical Density 660nm
0.50
0.40
0.30
0.20
0.15
0
12
24
36
48
TIME (hours)

The rate of glucose consumption was rapid in TPG, moderate in TPGe with added cyclic AMP and slow in TPGe without cAMP (Fig. 20A). The profile of the pH curve of each medium paralleled glucose consumption (Fig. 20B). The degree of sporulation decreased as the glucose concentration increased (Table 6). A medium containing 0.27 M glucose repressed sporulation to < 30% and maximum sporulation was obtained at 36 h irrespective of the concentration of glucose.

Uptake and utilization of glucose-^{14}C, pyruvate-^{14}C and butyrate-^{14}C.

During log phase, the mutants rapidly incorporated glucose, butyrate and some acetate as shown in Fig. 21 and 22. The uptake of glucose, butyrate and acetate continued in sporulating cells but decreased in aging cells of the asporogenic mutant.

Poly β-hydroxybutyrate biosynthesis

The cytoplasmic granules were identified as poly β-hydroxybutyrate (PHB) and chemical analysis before and after purification showed 70% and 96% content respectively on the basis of total dry weight.

The biosynthesis of (PHB) occurred during growth of the sporogenic and asporogenic mutants of C. botulinum (Fig. 23). The polymer was first detected after 8 h of growth and continued to accumulate, reaching 9% and 13% dry weight of sporogenic and asporogenic cells respectively at the early stages of stationary phase. In the sporogenic strain, the PHB content of the cells then decreased. At the free spore stage, only about 2% dry weight was detected. In the asporogenic mutant, the amount of PHB in aging cells remained essentially unchanged.

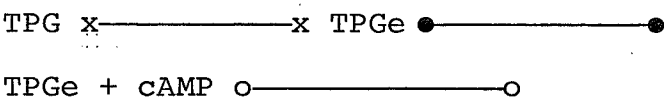
^{14}C-acetate or ^{14}C-butyric acid was incorporated into PHB of growing cells in "active culture" (Fig. 24). In the sporogenic and asporogenic mutants, acetate incorporation paralleled the accumulation and catabolism of the polymer. The specific activity of ^{14}C-acetate rapidly reached 1.2×10^3 cpm/mg PHB and did not change significantly. Butyric acid incorporation into PHB was low, with a specific activity of 0.2×10^3 cpm/mg PHB.

Cytoplasmic distribution of PHB

Before the end of the log phase, granules were observed in the cytoplasm of sporogenic and asporogenic cells by phase contrast microscopy. Stained cells showed that the granules were sudanophilic whereas volutin granules were not observed and the iodine-stained cells gave variable results.

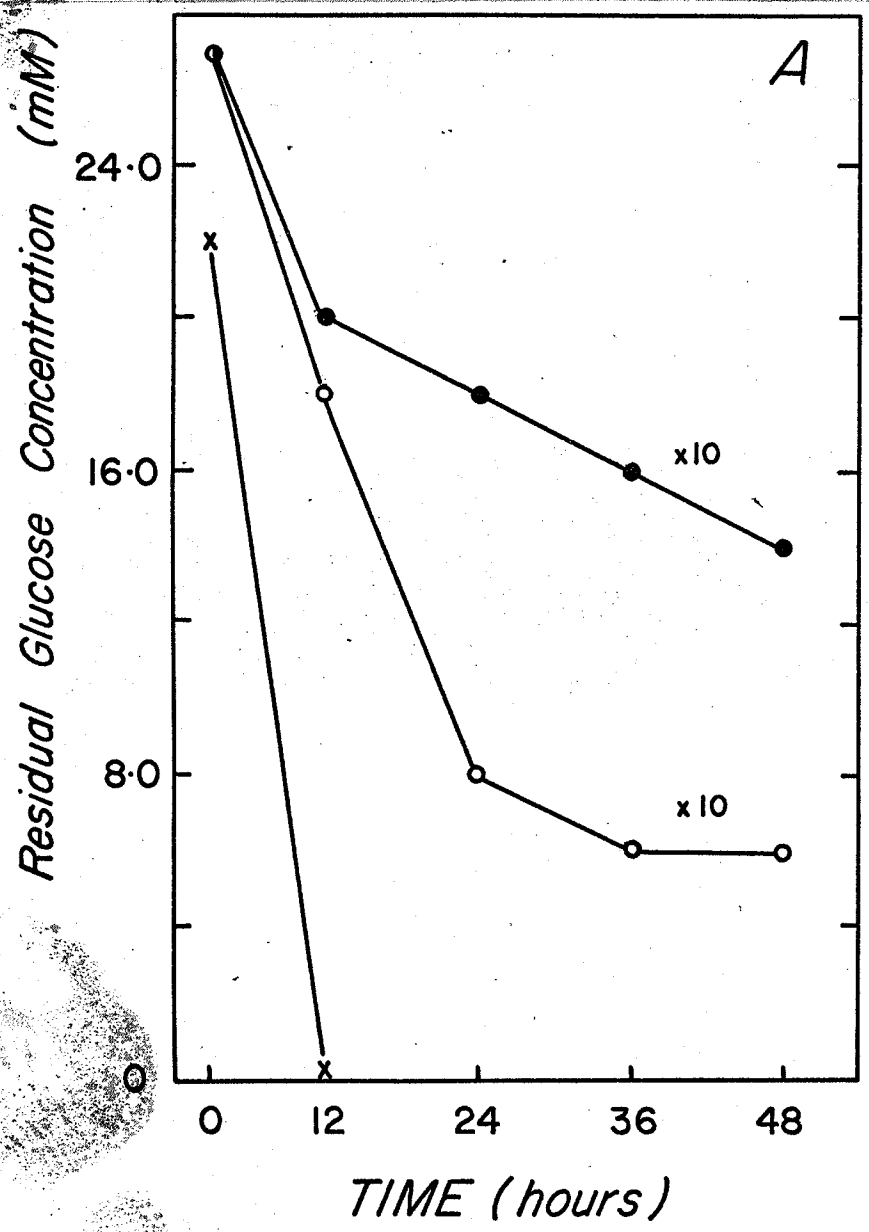
The electron micrographs of thin sections showed that granules in sporulating cells were dispersed throughout the cytoplasm and a few were trapped within the forespore (Fig. 25 and 26). The electron micrograph of purified PHB granules which had been shadow casted with carbon are shown in Fig. 27. PHB content of both mutants at various stages of growth cycle is compared with a schematic representation of ultrastructural changes during sporulation (Fig. 28).

Figure 20. Glucose metabolism during growth and sporulation.



A. glucose consumption; B. pH changes

x10 Indicates residual glucose conc. multiplied by a factor of 10.


A
Residual Glucose Concentration (mM)
24·0
16·0
8·0
0
x10
x10
0
12
24
36
48
TIME (hours)

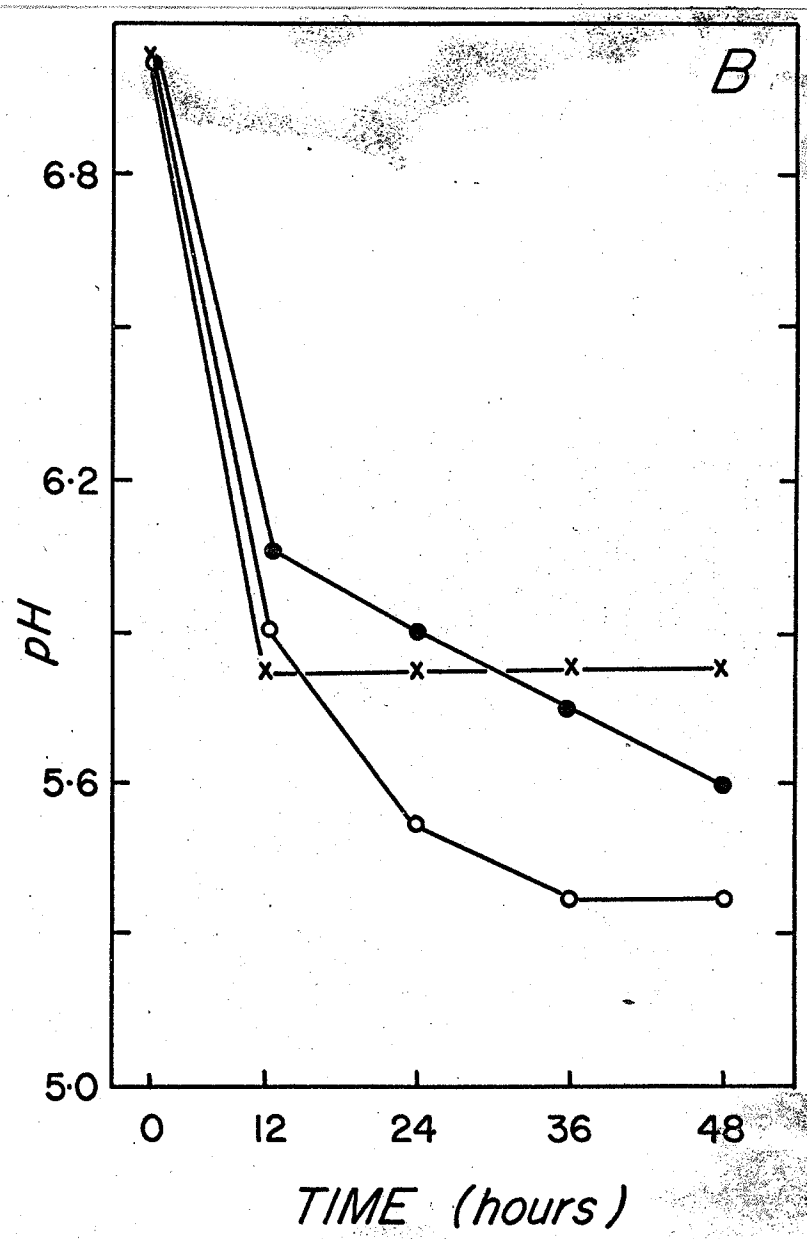

B
pH
6·8
6·2
5·6
5·0
0
12
24
36
48
TIME (hours)

Table 6. Effect of glucose on sporulation of MSp^+.

Time (h)	Glucose Concentration (M)				
	0.02	0.04	0.07	0.13	0.27
12	12.0[a]	10.0	8.0	5.0	5.0
24	80.0	70.0	65.0	60.0	20.0
36	90.0	80.0	75.0	70.0	30.0

[a] % refractile endospores per 10^3 cells by phase contrast microscopy.

Figure 21. Uptake and utilization of glucose and pyruvate by growing and sporulating cells of C. botulinum. Glucose ———— pyruvate -------.

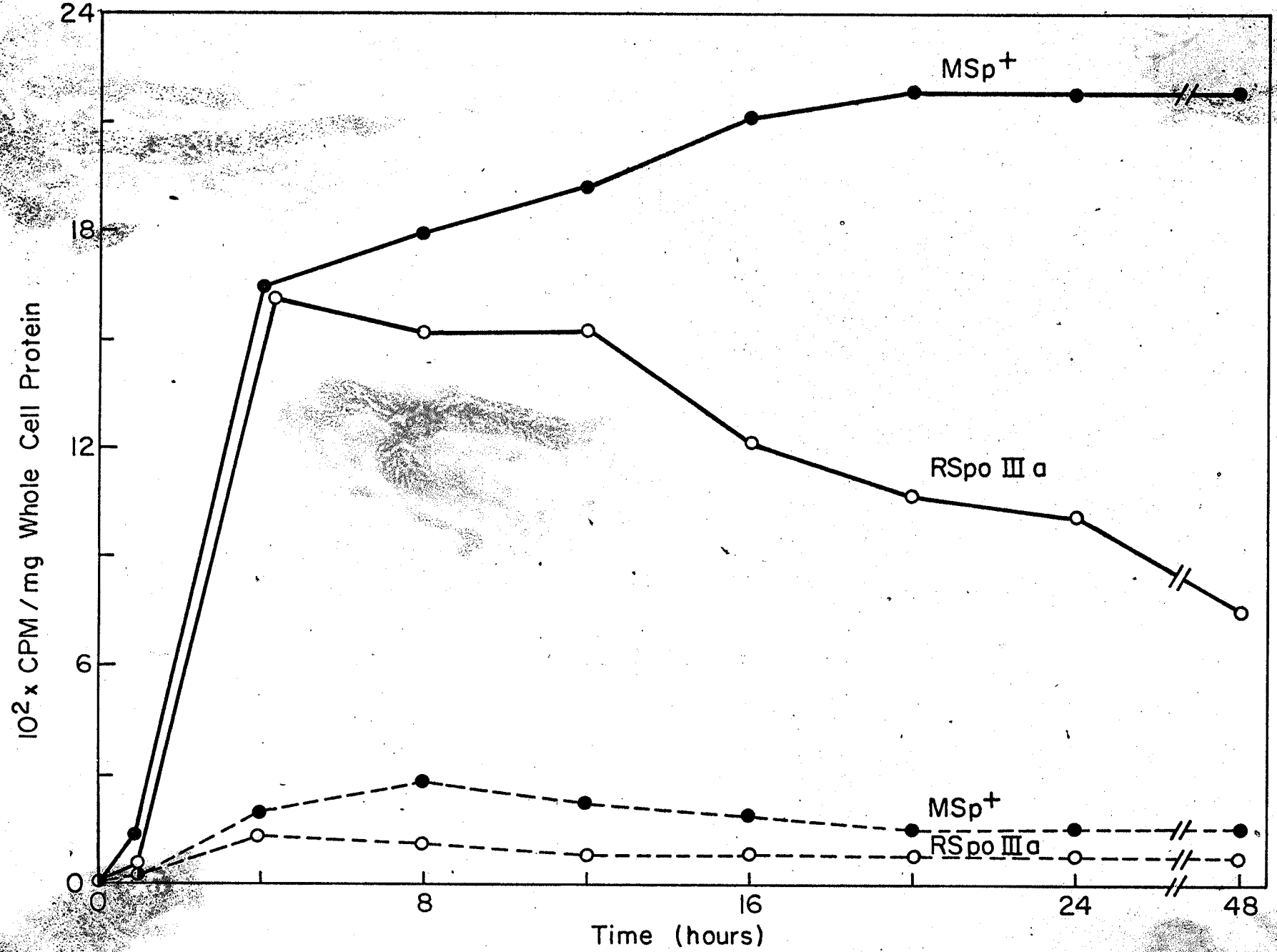

24
18
12
6
0
10^2 x CPM / mg Whole Cell Protein
MSp+
RSpo III a
MSp+
RSpo III a
0
8
16
24
48
Time (hours)

Figure 22. Uptake and utilization of acetate and butyrate by growing and sporulating cells of C. botulinum. Butyrate -----, Acetate ——————.

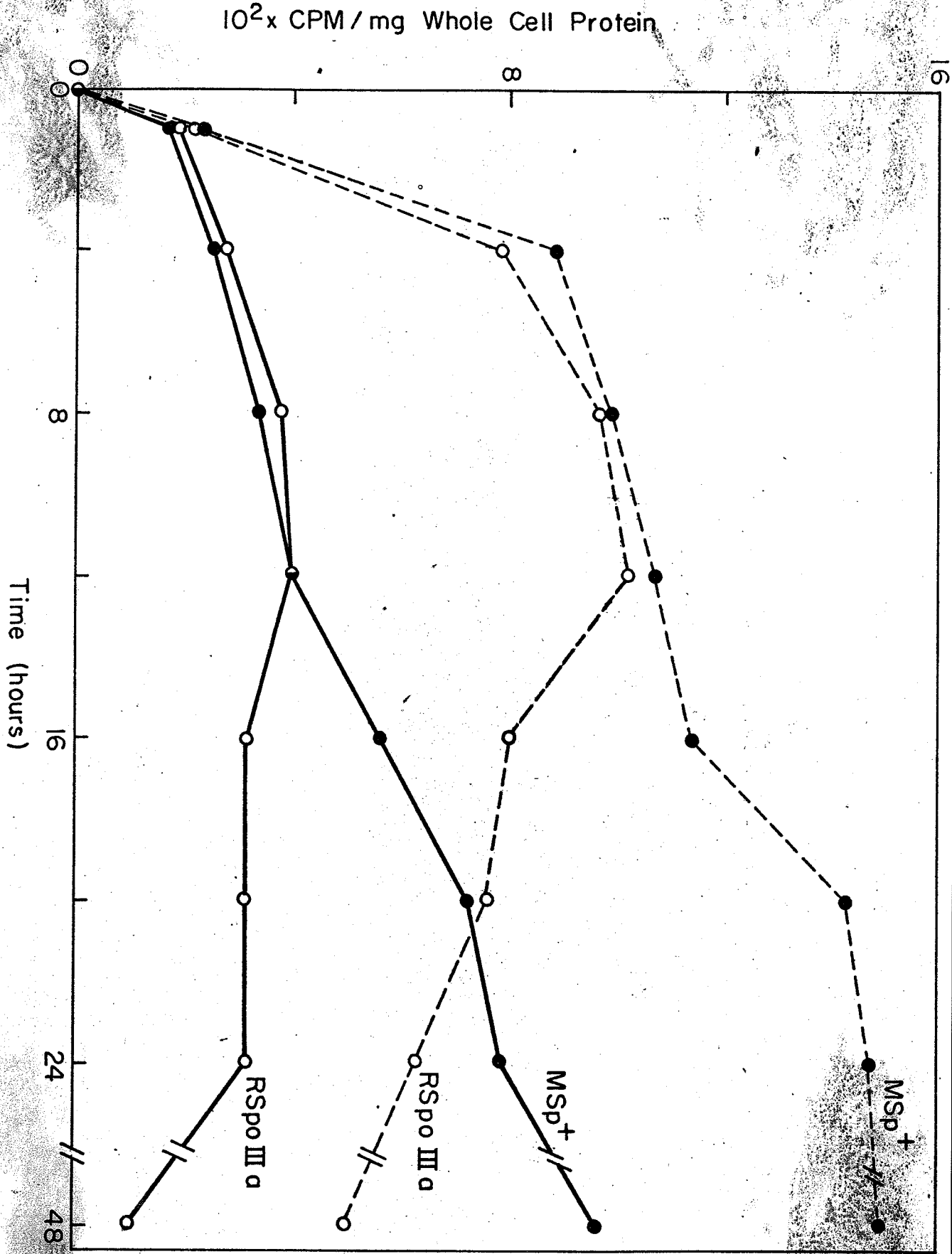

10^2x CPM / mg Whole Cell Protein
0
8
16
Time (hours)
0
8
16
24
48
RSpo III a
RSpo III a
MSp+
MSp+

Figure 23. Poly-β-hydroxybutyrate accumulation and degradation during the growth cycle of the MSp^+ and RSpoIIIa mutants. Cells were harvested at intervals and granules were isolated, purified and assayed for PHB spectrophometrically. Refractility is expressed as percentage of refractile spores in total cells counted as determined by phase-contrast microscope.

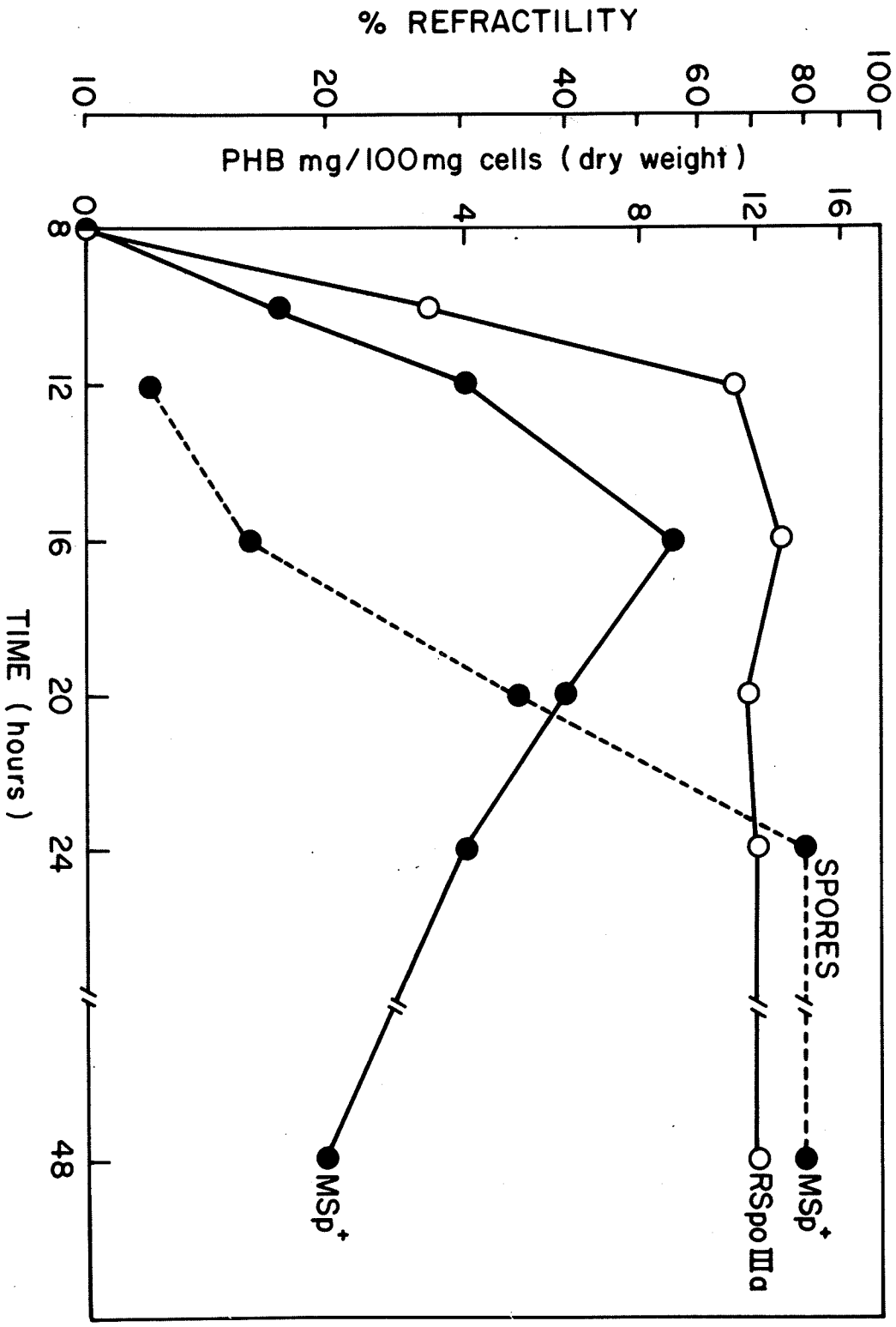

% REFRACTILITY
10
20
40
60
80
100
PHB mg/100mg cells (dry weight)
0
4
8
12
16
TIME (hours)
8
12
16
20
24
48
SPORES
MSp+
RSpo III a
MSp+
MSp+

Figure 24. Incorporation of ^{14}C-labelled acetate or butyrate into Poly-β-hydroxybutyrate during the growth cycle of MSp^{+} and RSpoIIIa mutants of C. botulinum. PHB granules were isolated and purified from cells at various stages after growth in a medium containing 1 μCi/100 ml acetate-^{14}C or butyrate-1-^{14}C and assayed for ^{14}C radioactivity. The total counts/min at each interval were obtained by multiplying the specific activity (1.2×10^{3} cpm/mg PHB) by total mg PHB/100 mg cell dry weight.

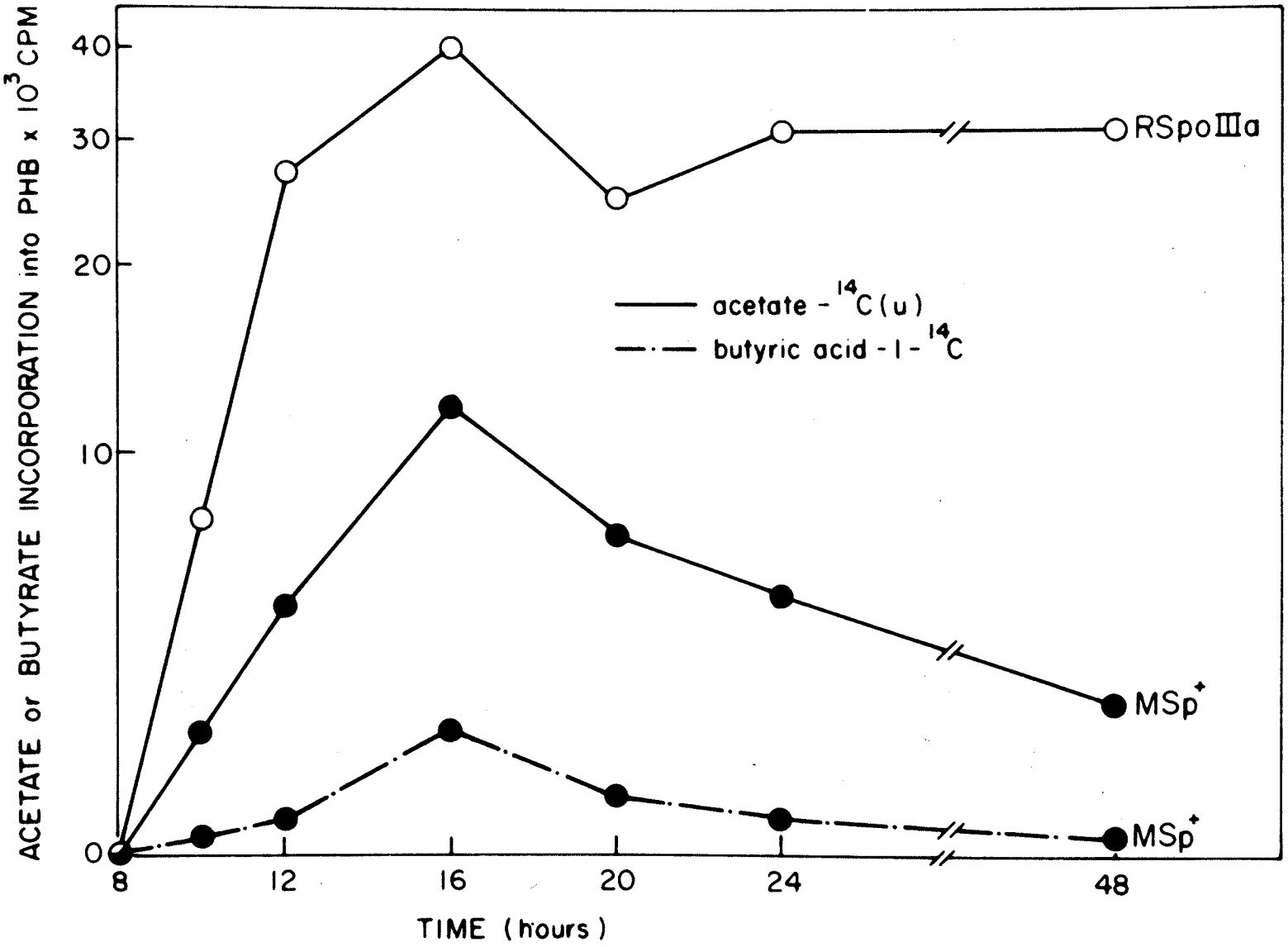
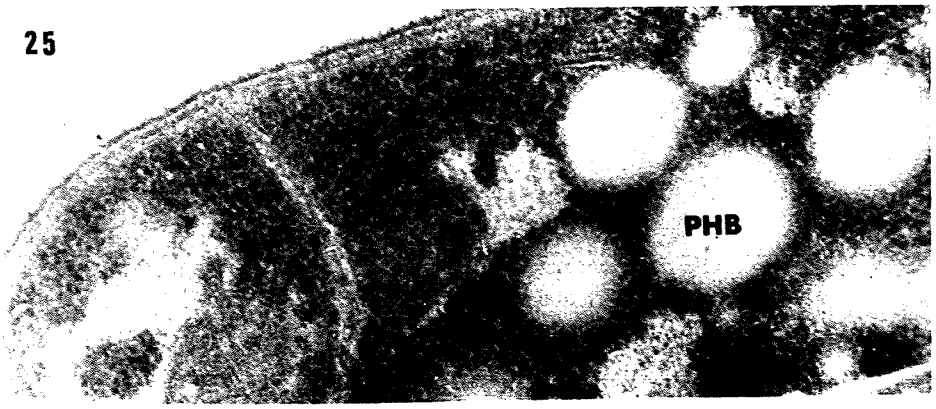

ACETATE or BUTYRATE INCORPORATION into PHB x 10^3 CPM
40
30
20
10
0
8
12
16
20
24
48
TIME (hours)
RSpoIIIa
acetate - ^{14}C(u)
butyric acid - 1 - ^{14}C
MSp$^+$
MSp$^+$

Figure 25. Longitudinal-section of MSp^+ showing PHB inclusions.

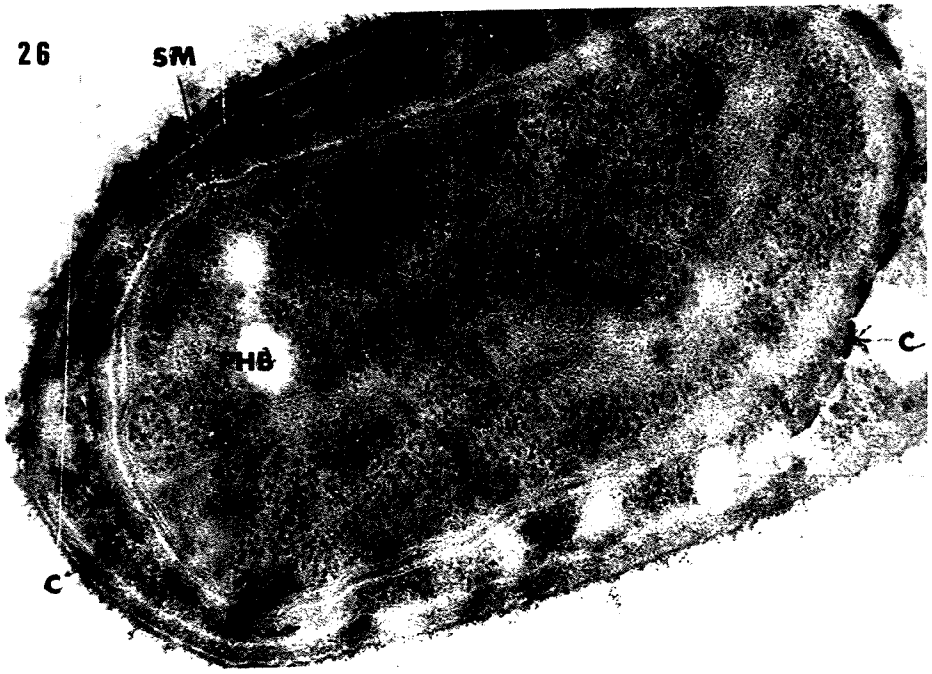
Figure 26. Longitudinal-section of forespore showing PHB inclusions. SM refers to spore membrane; C to spore coat segments.

Figure 27. Poly-β-hydroxybutyrate granules isolated from MSp^+ shadowed at 20^o with carbon x 12,800.

25



26



27

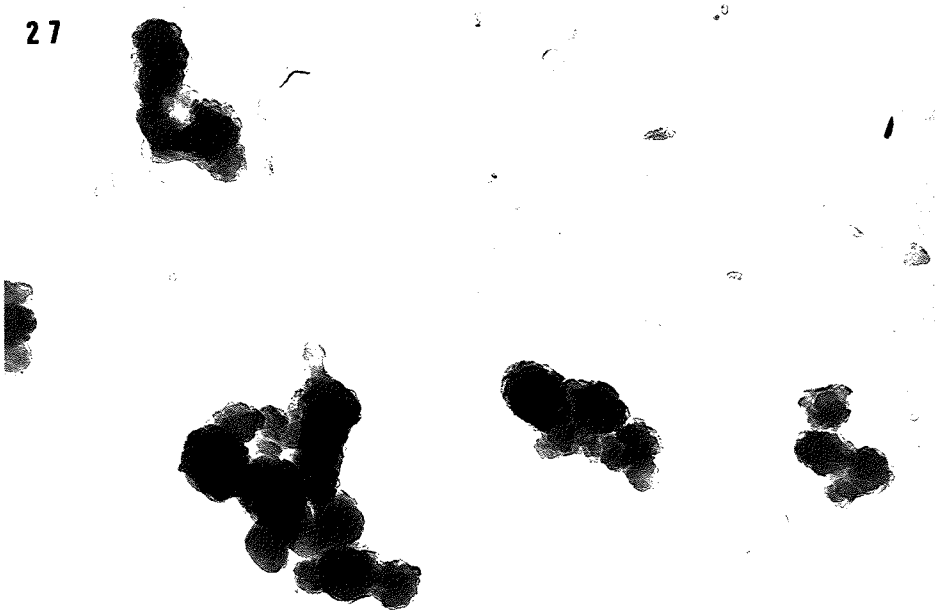
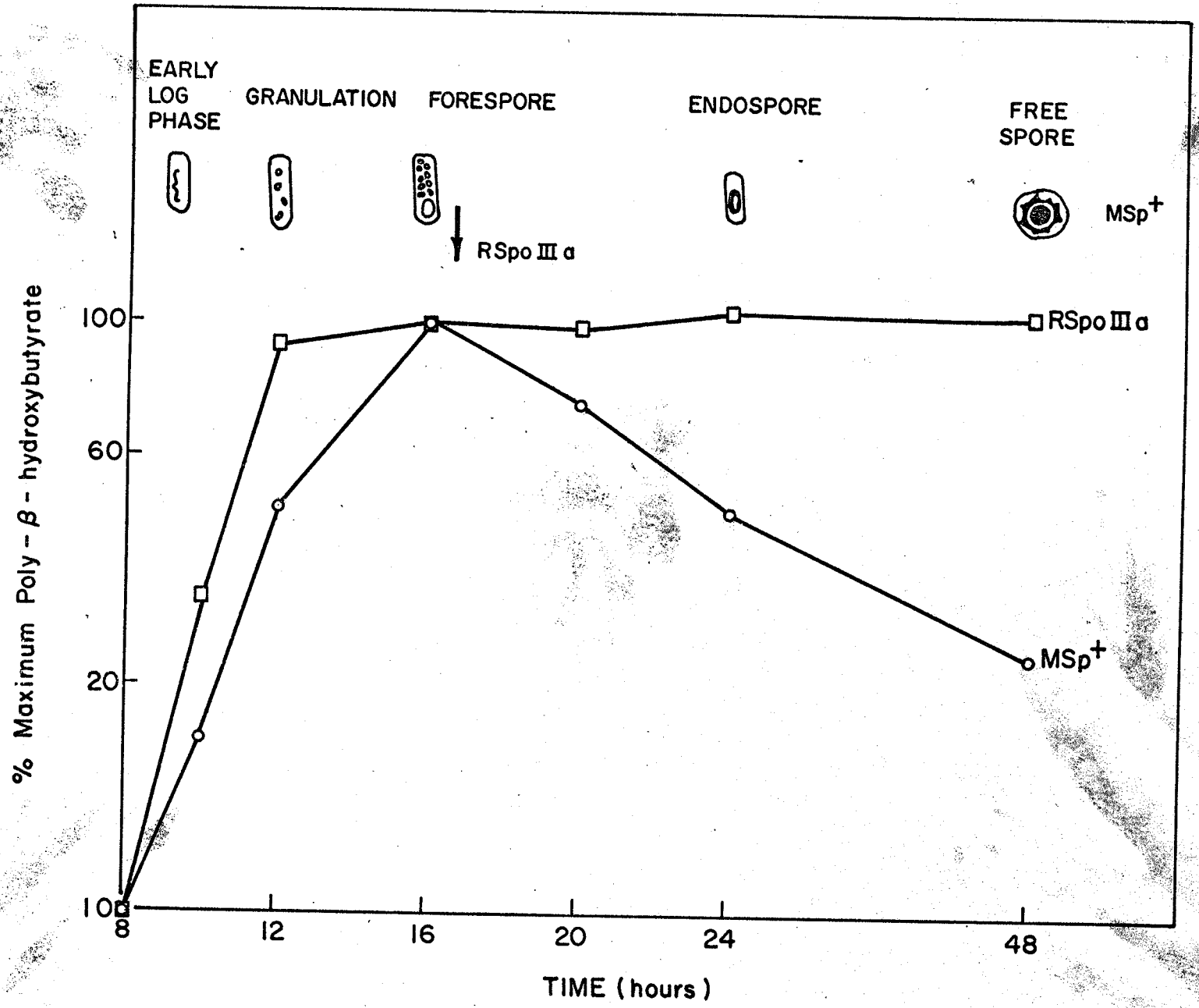


Figure 28. Poly-β-hydroxybutyrate levels in sporogenic (o———o) and asporogenic cells (□———□), expressed in arbitrary units using 100% as maximum accumulation. The sequential morphological stages of sporogenesis are indicated diagrammatically. The arrow (↓) indicates the developmental stage of blockage of the asporogenic mutant.


EARLY LOG PHASE
GRANULATION
FORESPORE
ENDOSPORE
FREE SPORE
RSpo III a
MSp+
RSpo III a
MSp+
% Maximum Poly - β - hydroxybutyrate
100
60
20
10
8
12
16
20
24
48
TIME (hours)

Intermediates of glucose metabolism

Fatty acids

The acids identified in the supernatant fluids of the MSp^+ and RSpoIIIa cultures by gas chromatography were acetate and butyrate (Fig. 29., Table 7). The changes in levels of residual glucose, acetate, and butyrate during the growth cycles of the sporogenic and asporogenic mutants are shown in Fig. 30. During exponential growth, glucose was rapidly dissimilated by both strains and the accumulation of acetate in MSp^+ cultures reached a maximum level at the prespore stage and then decreased during endospore formation. The acetate levels in the cultures of the asporogenic mutant showed a continuous increase during the entire growth cycle and were always lower than MSp^+ except during the late stationary phase when lysis occurred.

Butyrate accumulation in MSp^+ cultures increased significantly during spore formation and spore maturation, whereas the levels in cultures of the asporogenic mutant remained low and did not increase significantly. Early log phase (5 h) cultures contained small amounts of butyrate likely carried over with the inocula.

Production of pyruvate, ethanol, CO_2 and H_2.

Results of chemical analyses for pyruvate and ethanol are shown in Fig. 30. Ethanol levels in cultures of both mutants were identical during growth and endospore formation but continued to increase in RSpoIIIa during late stationary phase. During spore maturation of the MSp^+, the level of ethanol dropped.

CO_2 and H_2 levels in cultures of MSp^+ and RSpoIIIa determined by manometric assays are shown in Fig. 30. By early stationary phase, 15 h , the sporulating cells showed approximately a twofold increase in the amount of H_2 and CO_2 evolved as compared with the asporogenic cells. Subsequently the MSp^+ culture showed a marked decrease in levels of H_2 and CO_2 which were then maintained at approximately the same levels as in the asporogenic mutant throughout the late stationary growth phase.

Effect of inhibitors

β-Phenethyl alcohol, fluoroacetic acid and 2-picolinic acid inhibited acetate and butyrate synthesis by 50 to 62% and 87 to 97% respectively. The addition of inhibitors to cells in the log phase of growth reduced but did not prevent growth (Table 8). Fluoro-

acetate reduced sporulation whereas sporulation was completely inhibited by β-phenethyl alcohol and 2-picolinic acid.

Incorporation of ^{14}C substrate into lipids

The amount of acetate, pyruvate or butyrate assimilated into lipids of the cells which was expressed as per cent of total radioactivity, was 22 to 32% in the MSp^+ and 38 to 53% in the asporogenic mutant at the end of the exponential growth (Table 9). During the stationary phase, the ^{14}C from the pyruvate dropped from 28 to 18%, whereas that of butyrate increased from 22 to 28% in the MSp^+. Acetate assimilation into lipids was reduced to 16% in the sporulating cells. Due to cell lysis, the lipid content of the asporogenic mutant was not analyzed during the stationary phase. Most of the ^{14}C in the lipids was recovered in the polar and neutral fractions.

Fermentation balance

The data and fermentation balances are shown in Table 10. The carbon recovery and O/R balance were higher in the MSp^+ than in the asporogenic mutant.

Enzymes of butyric fermentation

The maximum specific activities of acetokinase, phosphotransacetylase and butyryl-CoA dehydrogenase during growth cycles of the MSp^+ and RSpoIIIa in TPG are shown in Fig. 31. In the sporogenic strain, the enzyme levels increased rapidly during spore formation and then decreased abruptly. In the RSpoIIIa only the acetokinase showed significantly activity but was lower than that observed in the sporulating cells.

Other enzymes

The specific activities of alkaline and acid phosphatase in the MSp^+ and the RSpoIIIa mutants during growth and sporulation are shown in Fig. 32. During the exponential growth phase both of the enzyme levels in the mutants remained relatively unchanged. As endospores are formed by the MSp^+ the alkaline phosphatase increased to a maximum whereas the acid phosphatase declined abruptly. However, during stationary growth phase of the RSpoIIIa mutant the activity of both of the enzymes increased simultaneously.

Glucose dehydrogenase was detected only in the sporogenic strain and showed maximum activity during endospore production. β-DPNH oxidase increased to a maximum at the end of exponential growth phase of the RSpoIIIa mutant but during spore maturation in the MSp^+ (Fig. 33).

Catabolite derepression by cAMP or MB-cAMP

Varying concentrations of 3'-5'-cAMP or MB-3',5'-cAMP were tested for their ability to reverse the catabolite repression of glucose on sporulation. As expected, these cyclic nucleotides had no effect on the sporulation pattern in TPG. However, in medium containing 0.27 M glucose, both compounds showed maximum ability to reverse the repression of sporulation by glucose between 10^{-5} and 10^{-4} M, with MB - cAMP being slightly more effective than cAMP (Table 11).

In order to examine the specificity of cAMP on catabolite repression, the effect of various adenine and guanine nucleotides on sporulation was tested (Table 12). MB-cAMP, ATP and ADP, but not AMP, reversed the repression of sporulation by glucose at 10^{-4} and 10^{-3} M concentrations. However, of all the

guanine nucleotides tested, only GTP reversed catabolite repression.

The time sequence at which catabolite derepression of sporulation by cAMP or MB-cAMP occurred, showed that both nucleotides could reverse the repression before 24 h (Fig. 34A and B). Because of the influence of cyclic AMP on the *in vivo* sporulation process, the cyclic AMP content in the cells during growth was determined and the results are shown in Table 13. In TPG, the level of cyclic AMP, assayed by protein binding, increased before the end of log phase reaching maximum during endospore formation but decreased at maturation. Glucose-repressed cells contained low amounts of cyclic AMP at all stages.

Figure 29. Representative gas chromatogram of the volatile acids from distillates of acidified cultures.

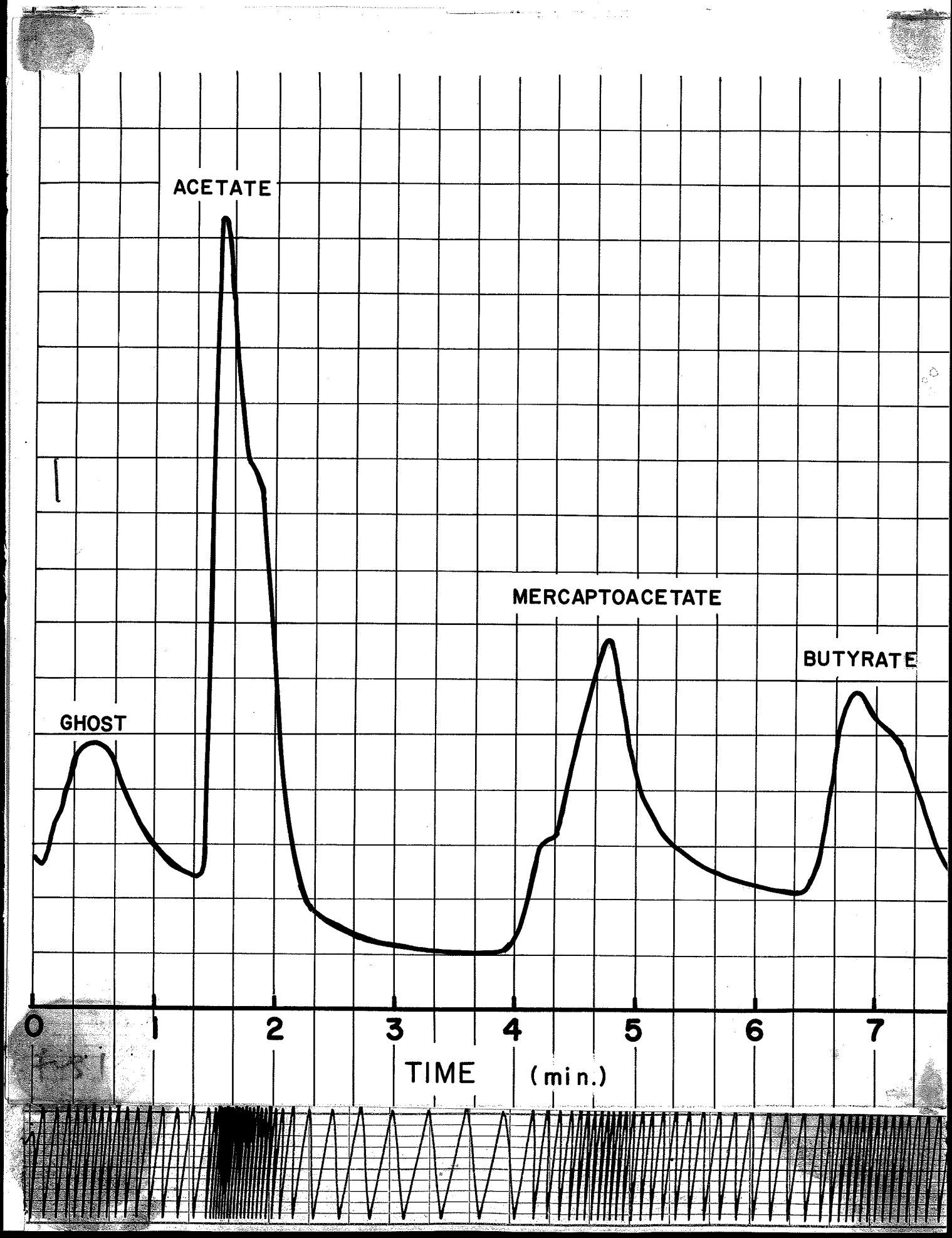

ACETATE
MERCAPTOACETATE
BUTYRATE
GHOST
0
1
2
3
4
5
6
7
TIME (min.)

Table 7. Relative retention times of authentic free fatty acids on chromosorb 102

Peak number	Fatty acid	Relative retention time
1	Ghost	0.56
2	Acetic acid	1.00[a]
3	Propionic acid	1.84
4	Mercaptoacetic acid	2.50
5	Isobutyric acid	3.20
6	n-butyric acid	3.60

[a]Relative retention time of 1 = 1.5 min.

Figure 30. Changes in concentrations of butyrate, acetate determined by gas chromatograph; CO_2, H_2 by manometric techniques; ethanol and glucose by enzymic analyses and pyruvate by chemical analysis.

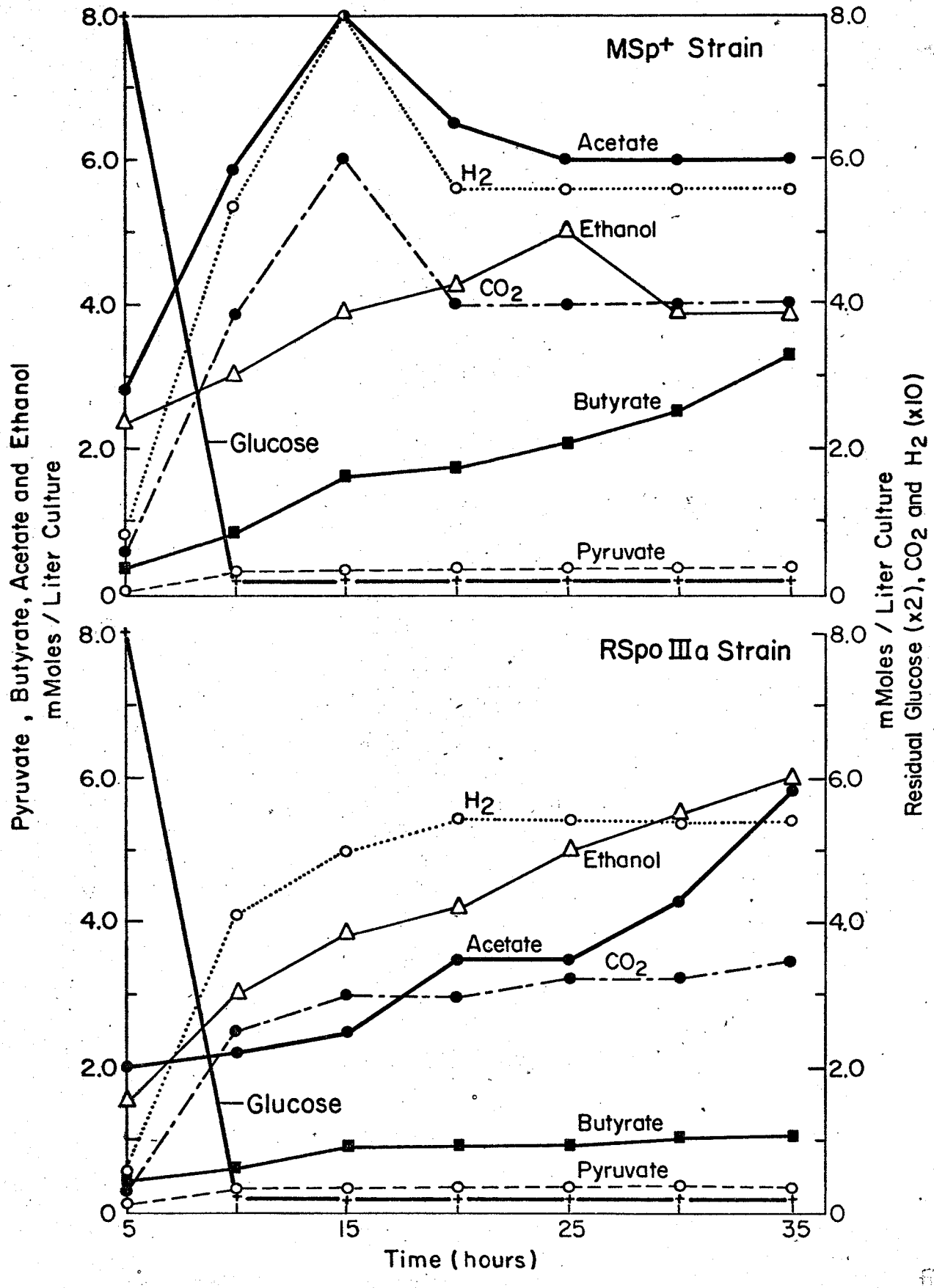

MSp+ Strain
RSpo IIIa Strain
Acetate
H2
Ethanol
CO2
Butyrate
Glucose
Pyruvate
Pyruvate , Butyrate, Acetate and Ethanol
mMoles / Liter Culture
Residual Glucose (x2), CO2 and H2 (x10)
mMoles / Liter Culture
Time (hours)
0
2.0
4.0
6.0
8.0
5
15
25
35

Fig. 2

Table 8. Effect of inhibitors on acetate and butyrate accumulation as well as growth and sporulation of MSp^+.

Experimental condition	Growth OD 600 %	Sporulation %	Inhibition Acetate %	Inhibition Butyrate %
Control[a]	100	80	0	0
Control + 0.35% β-phenethyl alcohol	43	0	56	97
Control + 5 mM fluoroacetic acid	67	20	50	90
Control + 5 mM 2-picolinic acid	24	0	62	87

[a]Trypticase peptone glucose broth (TPG)

Table 9. Incorporation of glucose, pyruvate, acetate and butyrate by growing and sporulating organisms into lipid fractions.

TIME (hours)	STRAIN	SUBSTRATE	RADIOACTIVITY (cpm)				INCORPORATION (%)
			WHOLE[a] ORGANISMS	TOTAL LIPIDS	NEUTRAL FRACTION	POLAR FRACTION	
12	RSpoIIIa	Glucose	4.0×10^4	2.0×10^3	670	1270	5
		Pyruvate	1.5×10^3	8.0×10^2	410	390	53
		Acetate	1.0×10^4	3.8×10^3	1680	2160	38
		Butyrate	2.6×10^4	1.3×10^4	5800	7400	50
	MSp^+	Glucose	4.8×10^4	3.0×10^3	850	2160	5
		Pyruvate	5.7×10^3	1.6×10^3	860	780	28
		Acetate	1.0×10^4	3.2×10^3	1430	1740	32
		Butyrate	2.7×10^4	5.9×10^3	2760	3130	22
20	MSp^+	Pyruvate	3.9×10^3	6.9×10^2	500	190	18
		Acetate	1.8×10^4	2.9×10^3	1600	270	16
		Butyrate	3.6×10^4	9.9×10^3	5230	4620	28
24	MSp^+	Pyruvate	3.9×10^3	8.2×10^2	704	114	21
		Acetate	2.0×10^4	3.4×10^3	1680	1700	17
		Butyrate	3.7×10^4	1.0×10^4	6800	3230	27

[a]All determinations were made on 25.0 mg whole cell protein.

Table 10. Metabolic activities of MSp^+ and RSpoIIIa cells.
mmoles / 100 mmoles glucose fermented

End Products	MSp^+	RSpoIIIa
Pyruvate	0.09	0.09
Butyrate	15.0	5.0
Acetate	37.0	11.6
CO_2	277.0	185.0
H_2	380.0	300.0
Ethanol	23.0	25.0
% C recovered	76	46
O/R balance	1.21	1.03
% C_1 recovered	25	20

Figure 31. Changes in the levels of some enzymes of butyric acid fermentation present in cell extracts of the sporogenic and asporogenic strains during growth and sporulation. Enzyme activities are expressed as arbitrary units by designating the maximum specific activity in the MSp^{+} mutant as 100%.

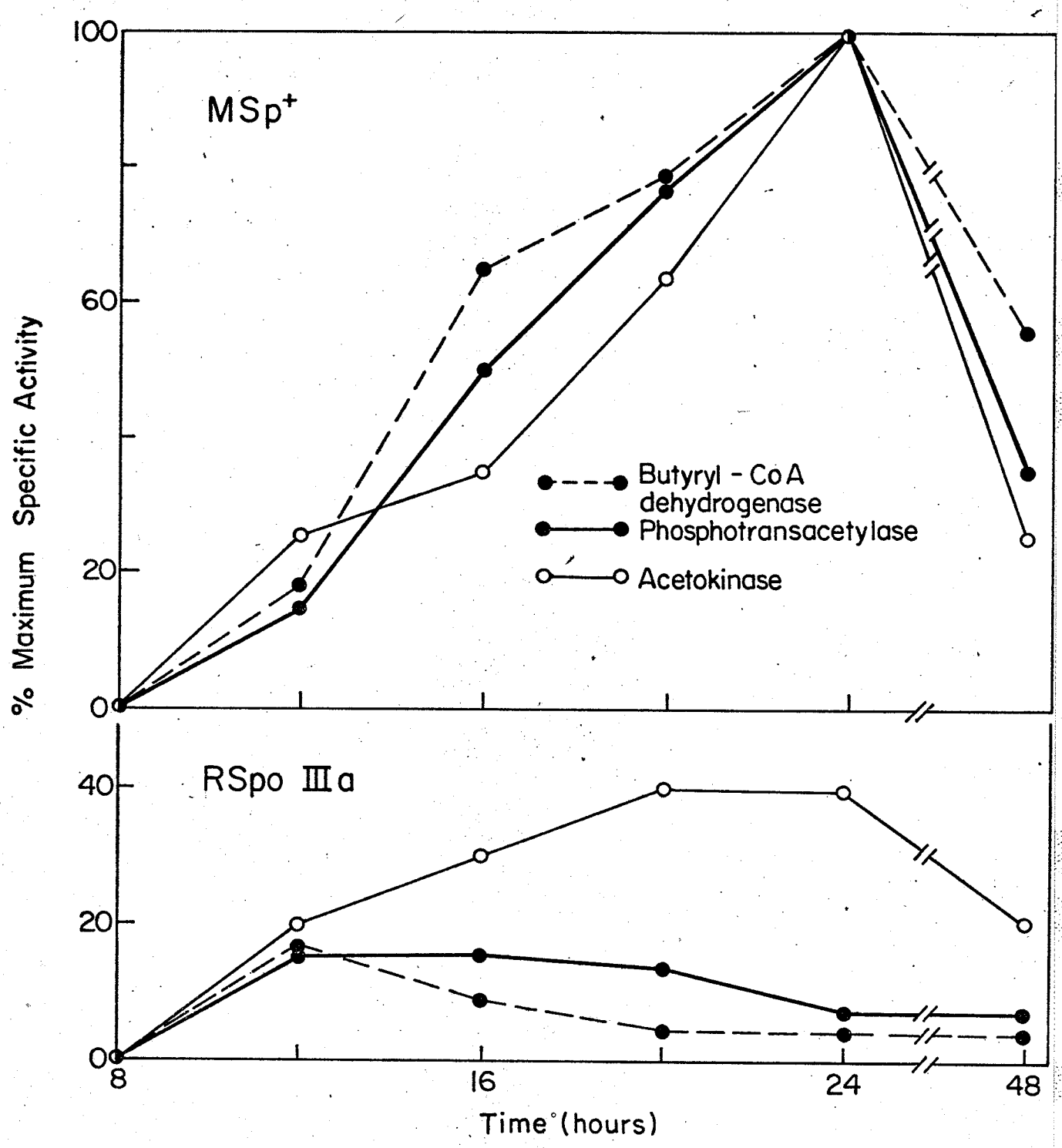

MSp+
RSpo III a
% Maximum Specific Activity
100
60
20
0
40
20
0
8
16
24
48
Time (hours)
Butyryl - CoA dehydrogenase
Phosphotransacetylase
Acetokinase

Figure 32. Alkaline and acid phosphatase levels in cell extracts during growth and sporulation. One unit of enzyme is defined as the amount of enzyme that catalyzes the oxidation of 1 μ mole of p-nitrophenol phosphate or O-carboxy phenyl phosphate per min at 410 nm.

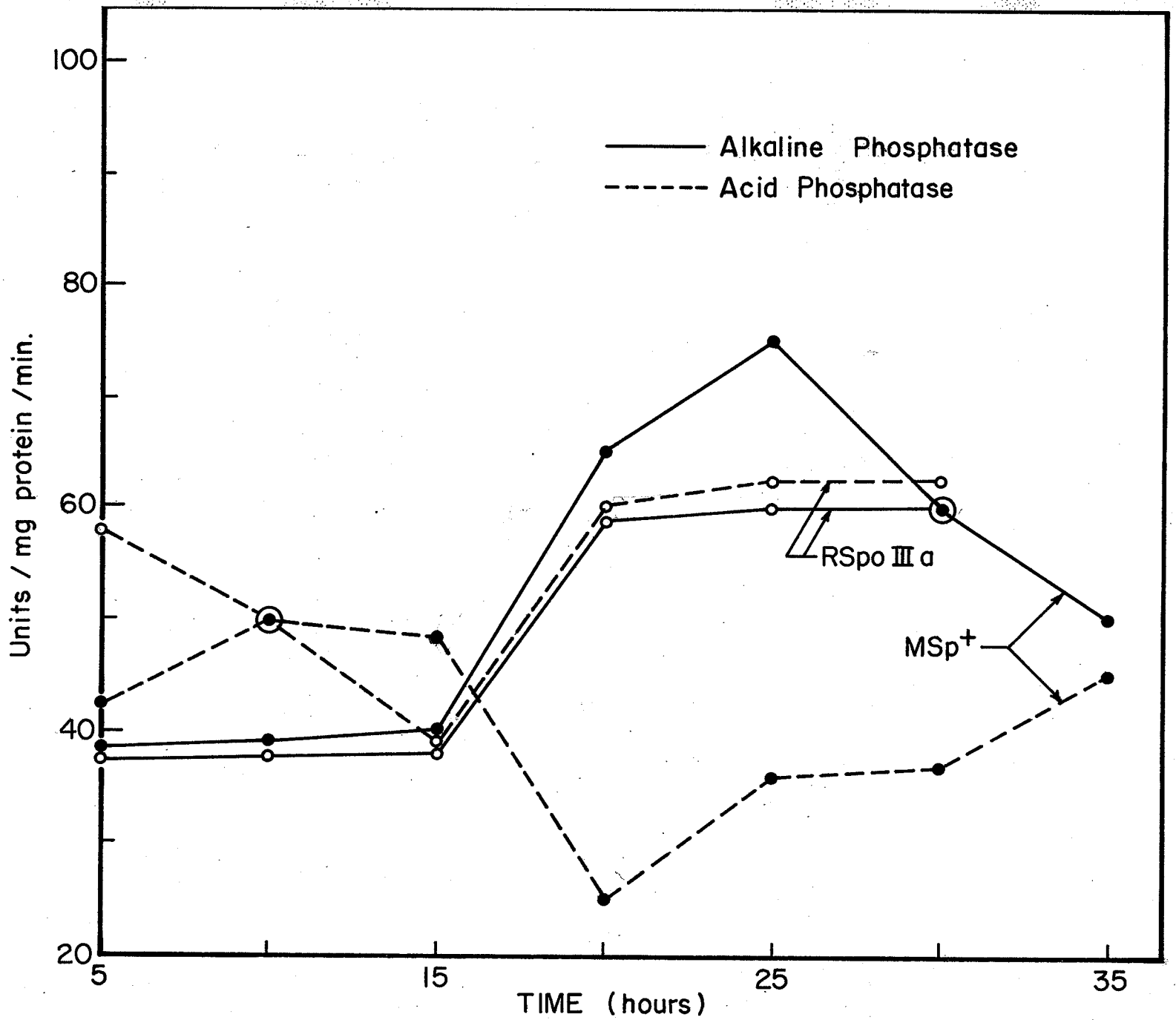

Alkaline Phosphatase
Acid Phosphatase
RSpo III a
MSp+
Units / mg protein /min.
100
80
60
40
20
5
15
25
35
TIME (hours)

Figure 33. Glucose dehydrogenase and DPNH oxidase levels in cells during growth and sporulation. One unit of enzyme catalyzes the reduction or oxidation of 1 micromole of DPN or DPNH respectively per min at 340 nm.

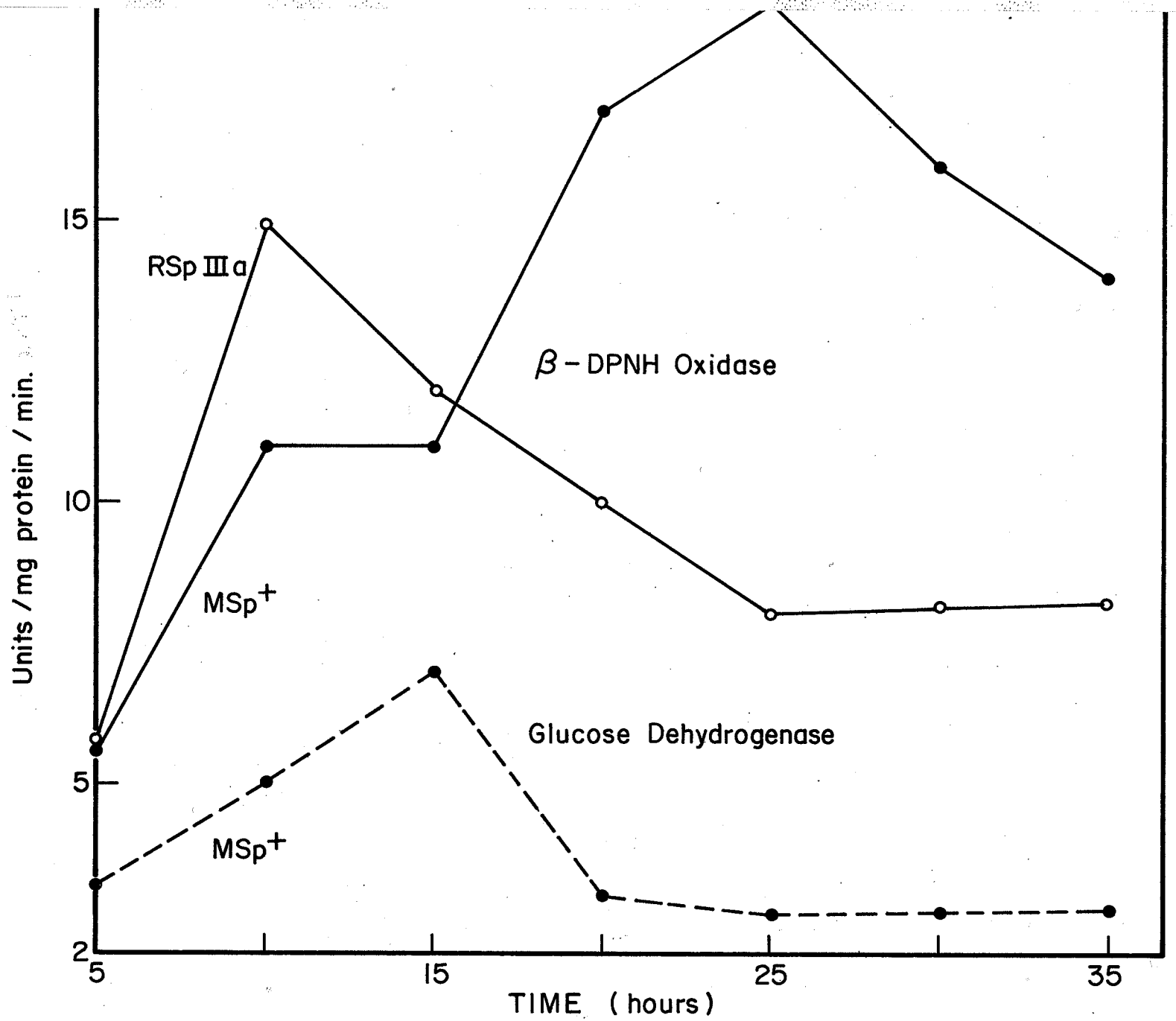

15
10
5
2
Units / mg protein / min.
RSp III a
MSp+
β – DPNH Oxidase
Glucose Dehydrogenase
MSp+
5
15
25
35
TIME (hours)

Fig 33

Table 11. Effect of cAMP or MB-cAMP on glucose repressed sporulation.

Nucleotide Conc. (M)	Incubation hours 24	36
0	30[a]	30
10^{-6} cAMP	30	30
MB-cAMP	40	60
10^{-5} cAMP	50	70
MB-cAMP	60	90
10^{-4} cAMP	80	80
MB-cAMP	80	90
10^{-3} cAMP	60	60
MB-cAMP	50	70

[a] % refractile endospores per 10^3 cells; at 12 h samples showed 5% endospores.

Table 12. Effect of nucleotides on sporulation of Clostridium spp. in 0.27 M glucose.

Nucleotides	Conc. (M) 10^{-3}	10^{-4}
3', 5'-cAMP	[a]60	70
MB 3', 5'-cAMP	50	70
-ATP	60	60
-ADP	60	60
-AMP	40	40
-GTP	60	60
3', 5'-cGMP	30	30
MB 3', 5'-cGMP	30	30
-GMP	30	30

[a] % refractile endospores in 10^3 cells were estimated after 24 h incubation. Control samples without added nucleotide contained 30% endospores.

Figure 34. The effect of the addition of 10^{-4} and 10^{-5} M cAMP or MB-cAMP on sporulation in TPG x ------ x and TPGe ———

A. cAMP; B. MB-cAMP

10 h cells were inoculated into TPG or TPGe with or without cAMP or MB-cAMP. After 12, 24, and 36 h of incubation samples were removed and percentage of cells containing endospores out of 10^3 cells was estimated by phase contrast microscopy.

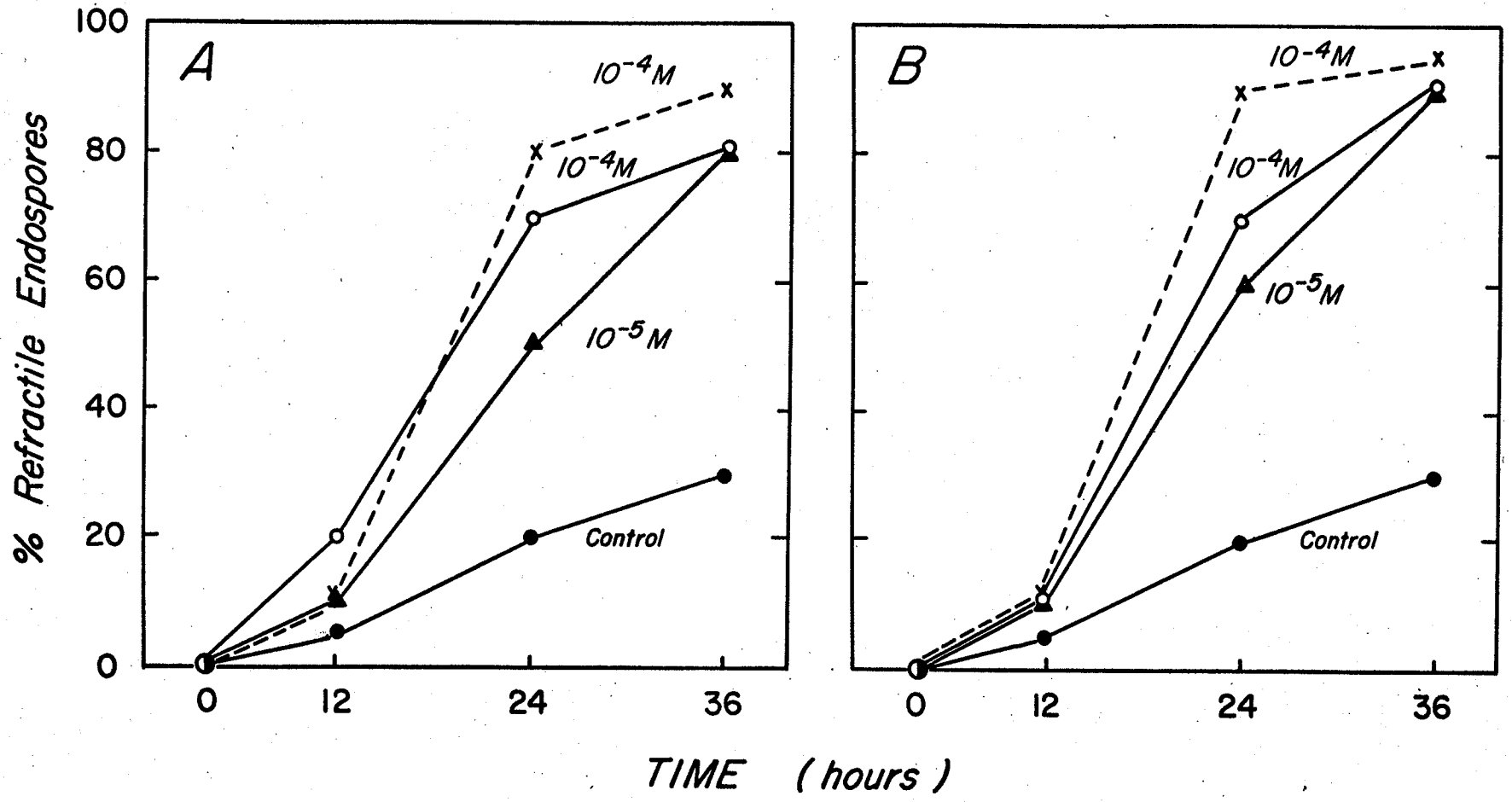

A
B
$10^{-4}M$
$10^{-4}M$
$10^{-5}M$
Control
$10^{-4}M$
$10^{-4}M$
$10^{-5}M$
Control
% Refractile Endospores
100
80
60
40
20
0
0
12
24
36
0
12
24
36
TIME (hours)

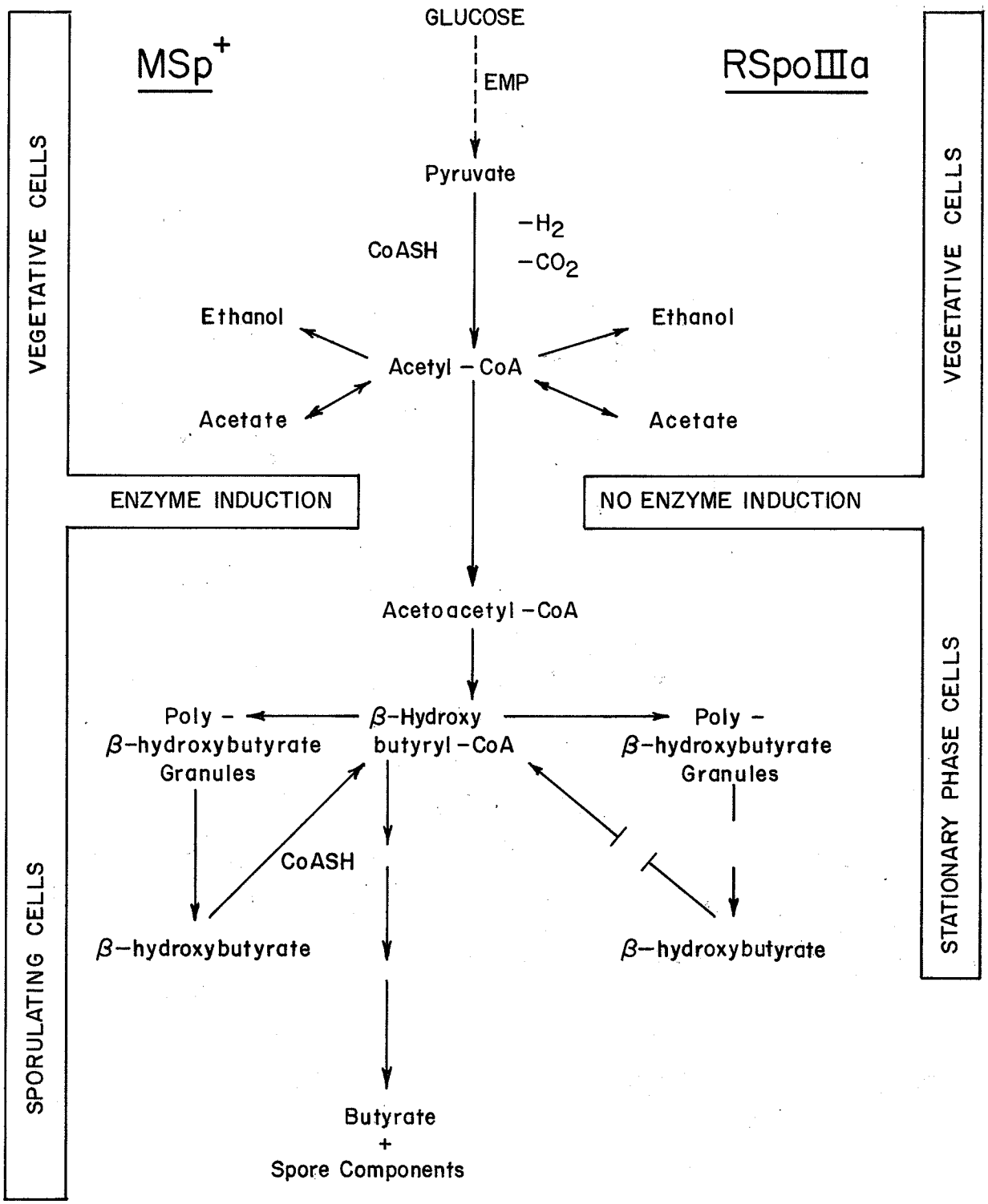
Table 13. The effect of glucose on cyclic AMP in cells during growth and sporulation.

Time (hours)	Cyclic AMP (pmols/mg cells, dry wt) TPG*	TPGe**	TPGe + 10^{-4} M cAMP
12	15.6	4.0	10.0
24	20.5	5.0	11.0
36	7.0	5.0	6.0

*TPG 22.0 mM glucose

** TPGe 0.27 M glucose

Figure 35. A schematic representation of butyric type of fermentation occurring in sporogenic and asporogenic mutants of C. botulinum. The site of blockage of the RSpoIIIa mutant is shown with ———/ /———→.


GLUCOSE
EMP
MSp+
RSpoIIIa
Pyruvate
CoASH
−H2
−CO2
Ethanol
Ethanol
Acetyl − CoA
Acetate
Acetate
VEGETATIVE CELLS
VEGETATIVE CELLS
ENZYME INDUCTION
NO ENZYME INDUCTION
Acetoacetyl − CoA
Poly − β−hydroxybutyrate Granules
β−Hydroxy butyryl − CoA
Poly − β−hydroxybutyrate Granules
CoASH
β−hydroxybutyrate
β−hydroxybutyrate
SPORULATING CELLS
STATIONARY PHASE CELLS
Butyrate + Spore Components

D I S C U S S I O N

DISCUSSION

The stable mutants, sporogenic MSp^+ and asporogenic, RSpoIIIa isolated from cells of Clostridium botulinum after treatment with N-methyl-N-nitrosoguanidine (NTG) were useful tools for comparing the events which occur during growth and sporulation. In general, only a few stable mutants have been isolated from Clostridium species (25, 37, 128).

In accord with Adelberg (1), the addition of NTG to actively growing cells of the wild type gave rise to a high death-rate, thus suggesting that cells should not be allowed to grow and metabolize in the presence of the mutagen. The standard procedure adopted consisted of NTG treatment of washed cells suspended in buffer which yielded > 40% mutants. The mutant clones were differentiated on liver veal agar containing Mn^{++} ions which also supported good growth and thus provided a convenient method for the recovery of mutants. The pale-brown color of the complex formed between the by-products

of sporulation and the Mn^{++} ions constituted the basic distinction between sporulating and non-sporulating clones. The concentration of $MnSO_4$ used, was sufficient to ensure increased sporulation as well (39).

The mutants derived by acriflavin or quinacrine treatments (112) and selective replicate sampling were unstable, suggesting that an episomic factor for sporulation may be lacking in C. botulinum. However, it is more likely that the effect of NTG, a chromosomal mutagen was upon one or more mutable sites, indicating that the genetic determinants of anaerobic sporulation are similar to those reported for Bacillus spp (134).

The cells derived from the sporogenic clone (MSp^+) differed markedly from those of the asporogenic (RSpoIIIa) in cultural and metabolic properties, but not from the wild type strain except for toxigenicity (Table 1 and 3). Immunodiffusion tests confirmed the identity of the sporogenic and asporogenic mutants as type E strains of C. botulinum. Previously, Sebald and Schaeffer (127) and Duncan (167) had indicated that there was direct relationship between toxigenicity and the ability to form spores of Clostridium species,

however, our results showed that cells of the sporulating clone, as well as, the asporogenic mutant were non-toxigenic. It has recently been reported that phage conversion of toxigenicity occurs in C. botulinum type C (40), thus the loss by the sporogenic strain may be due to a one site mutation, a view supported by the similarities in cultural, antigenic and metabolic properties with the wild type strain. The asporogenic strain lost both the ability to produce toxin and to sporulate because of the effect of the NTG on two sites of the genome.

The ultrastructure of the mutants showed a thin layer between the double membrane of the forespore septum of the sporogenic strain, 'germ cell wall', which appeared prior to the development of either spore coat or cortex and remained unchanged throughout all stages of sporulation. Since the 'germ cell wall' was not observed in the asporogenic mutant (Fig. 6) blockage of the mutant was defined as occurring early in stage III at the time of forespore formation just prior to the synthesis of 'germ cell wall'. Fig. 6 shows a nearly completed forespore in which the nucleoplasm has been replaced by ribosomes and a double mem-

brane with defects at two or three locations.

The designation of the sporogenic mutant as MSp^+ and the asporogenic as RSpoIIIa follows the nomenclature proposed by Young and Wilson (164) based on colony type, ability to sporulate, the precise stage of blockage and the antibiotic activity.

Most of the data on spore formation and sequential differentiation have been obtained from batch cultures, in which the composition of the media is being changed during growth cycle due to the accumulation of metabolic end products. The accuracy of the batch culture method in the study of growth and sporulation is dependent on the use of a synchronized culture. Nearly synchronous cultures were achieved by serial transfer of preselected cultures of both of the strains as shown by the short lag and the identical log growth phases (Fig. 16).

The kinetics of growth of the strains showed significant differences at the end of the log phase denoting the region of blockage and the inability of the mutant to progress to stage III in the sporulation process. The abrupt drop in OD of the

MSp^+ strain is probably due to protein turnover initiated by exoprotease (14), thus providing subunits for the formation of spore specific substances. On the other hand, the gradual decrease of the optical density of the RSpoIIIa strain was primarily due to autolysis. The lysis of the asporogenic strain, occurring 24 h after growth may be a consequence of cells which are committed but unable to sporulate since the forespores are without 'germ cell walls' and in addition, the cells are incapable of reverting to the vegetative state. There is some evidence that this strain formed fragments of spore envelopes but was unable to assemble them (Fig. 9). During stationary growth phase, the decrease in pH was due to the rapid oxidation of glucose to pyruvate and acetate (97) and other acids, which accumulated in the medium. Thus, the slight pH difference between the two strains suggests that only a small portion of the acids was utilized for sporulation. However, it is clear from the results that MSp^+ strain grows and sporulates well in an acid medium.

The extracellular proteolytic activity associated with sporulation has recently been defined in terms of excretion of S-proteinase and esterase (93),

however, the exact nature of the relationship is still to be clarified. The weak inhibitory action of supernatant fluids of cultures of MSp^+ against Staphylococcus aureus was interpreted as an antibiotic activity, a finding which could not be confirmed later. This suggests either that the antibacterial substance detected is not actually an antibiotic (83) or that only a minute quantity of the antibiotics is produced and used up during sporogenesis. In any event, further work needs to be done in this area. The RSpoIIIa mutant, which did not synthesize DPA, showed neither proteolytic nor antibiotic activity probably because, at the site of mutation, the biochemical sequence of events controlling their synthesis was arrested. Since mutants of Bacillus spp. blocked at a later stage produced protease and antibiotics without synthesizing DPA, it appears that aerobic as well as anaerobic sporogenesis follows the same ordered metabolic sequence.

The stable asporogenic mutant blocked during early forespore state afforded a comparative means of studying the metabolic activities of C. botulinum during growth and sporulation. Since the mutants are

saccharolytic, utilizing glucose, fructose, maltose and sucrose, it is assumed that catabolism proceeds via Embden-Meyerhof pathway to pyruvate during vegetative growth (8). The data presented here have led us to make the following deductions about the pathways of glucose fermentation as shown schematically in Fig. 35. Since neither the intermediate compounds nor endospores were detected when glucose was omitted from the growth medium, it appears that the amino acid metabolism of these mutants may not contribute significantly to the pathway suggested here.

In trypticase peptone glucose broth, carbon and energy for growth is derived from the catabolism of glucose (35, 50, 55) and sporulation is suppressed until the monosaccharide is depleted. Acetate accumulates in the cultures resulting in a pH drop (Fig. 30).

Some of the acetate is used for the biosynthesis of poly-β-hydroxybutyrate (PHB) via coenzyme A esters (129) and only a portion is converted to lipid (Table 9) with some directed to ethanol production. The production of acetate and its utilization during sporulation of the sporogenic mutant (Figs. 30 and 32) is similar to that reported for the aerobic spore formers

(35, 55) and by Day for C. botulinum type A except that granules were not detected in the latter (32). The effect of acetate accumulation may also be to induce enzymes associated with acetate metabolism and with butyric acid type of fermentation during the transition from vegetative growth to sporulation. As growth ceases, the cells of the sporogenic strain begin to form endospores accompanied by induction of aceto-kinase, phosphotransacetylase and butyryl-CoA dehydro-genase. These events increased the metabolic rate via the butyric type of fermentation and the energy available for sporogenesis.

The various developmental stages of C. botulinum examined for poly-β-hydroxybutyrate (PHB) included vegetative, forespore, endospore and free spore (Fig. 28) as determined by phase contrast and electron microscopy. The asporogenic mutant blocked at the forespore stages also accumulated many intracellular granules and hence provided a basis for comparison with the sporogenic mutant.

The results showed that the granules which accumulated by the start of the stationary phase were PHB and were seen throughout the cytoplasm with some in the matrix of the forespore (Fig. 25 and 26). The

asporogenic mutant synthesized a larger amount of PHB even in aging cells undergoing lysis. Since ^{14}C-acetate was readily incorporated into the PHB granules (Fig. 24) it would appear, as suggested for Bacillus spp. (36, 69, 94, 131) that one of the important roles of acetate was to supply carbon precursors via PHB for the synthesis of spore materials. As observed by Doudoroff and Stanier (36) when ^{14}C-butyric acid was used as substrate, a low yield of PHB and reduced rate of breakdown resulted, suggesting that butyric acid is an unlikely precursor.

The polymer was not utilized to any significant degree by the asporogenic mutant, suggesting an impaired mechanism for degradation (131). Furthermore, the failure of the asporogenic mutant to catabolize PHB could be due to a feedback or mass effect due to the developmental block at stage III. On the other hand, most of the PHB was catabolized by the sporogenic strain. Only 2% PHB dry weight remained in the spores which is in accord with the findings of Akashi (2) in spores of Bacillus subtilis. Since the polymer was catabolized between 16 and 48 h of the growth cycle, corresponding to stages III to VI of sporulation (Fig. 28), it would appear that PHB granules are utilized as carbon and energy sources for spore maturation.

The data indicate that most of the butyric type of fermentation occurs concurrently with sporogenesis in the MSp^+ and it is suggested that when the PHB granules are catabolized, the β-hydroxybutyrate which is formed undergoes dehydration and reduction to produce butyrate. The PHB metabolism may also serve as a "reduction sink" for energy yielding reactions and the butyric acid pathway generates energy and materials for spore formation.

Under similar conditions when glucose is exhausted and growth ceases, cells of the asporogenic mutant enter into an extended stationary phase (Fig. 16) without enzyme induction. Because of the low enzyme levels and the impaired mechanism of PHB catabolism the cells did not synthesize significant amounts of butyrate. Although the amount of cells in the sporogenic and asporogenic cultures was the same (Fig. 16), considerably lower levels of metabolic products were accumulated by the RSpoIIIa cells, presumably because of slower metabolic rate.

The isotopic tracer experiments indicated that log phase cells of both mutants preferentially utilized external substrates supplied in the following order: glucose > butyrate > acetate > pyruvate (Fig. 21 and 22).

The sporulating cells continued to assimilate additional butyrate and acetate which is in keeping with the suggested precursor role of butyrate and acetate during sporogenesis. As indicated by the substantial amounts of ^{14}C-butyrate incorporated into spore lipids and the sporangia of the MSp^{+} strain, butyrate may also serve as a carbon precursor for the biosynthesis of spore lipids and other spore specific materials. The failure of the pyruvate medium to support sporulation, may be because of the inability of the compound to be readily transported into the sporulating cells as shown by radioactive studies (Fig. 21).

Gas-solid chromatography (10) was used to identify gases based on their relative retention, relative to methane, in order to obtain some insight in the type of metabolic processes occurring during growth and sporulation. For comparison of the amount of gases evolved with time, gas compositions (Table 2) were calculated from gas chromatographic peak areas in per cent of the total area. The amount of oxygen or hydrogen evolved was not determined because it was difficult to separate the two gases on Porapak Q. The mercaptoethanol was probably released during autoclaving from the mercaptoacetate added to the TPG

for the purpose of maintaining anaerobic conditions. The decrease of the alcohol during sporulation but not in the course of growth may reflect the extent of anaerobiosis or the actual utilization of the gas. The evolution of N_2O concurrently with sporulation may indicate that it is a metabolic waste product. The significance of these findings remains to be determined.

The fermentation balance sheet (Table 10) indicates that the mutants produce butyrate, ethanol and acetate as the major end products of glucose catabolism together with carbon dioxide and hydrogen. The excess C_1 observed is presumed to be due to the loss of carbon into cellular materials and not because of the fermentation of other substances since control experiments consistently gave negative results. The low carbon recovery and the normal O/R balance of the asporogenic mutant may be accounted for by the intracellular PHB that was not catabolized. The high O/R balance together with the relatively high lipid content of sporulating cells seems to suggest that the unrecovered carbon of the MSp^+ strain, may be in the form of spore lipids which are more reduced than the granules.

Inhibition studies indicated that β-phenethyl alcohol, fluoroacetic acid and picolinic acid almost completely inhibited butyrate biosynthesis and endospore formation. Although the site of inhibitory action was not determined, the findings seem to indicate a direct relationship between the butyric type of fermentation and anaerobic sporulation.

Other enzymes which showed increased activity during anaerobic sporulation of the MSp^+ were alkaline and acid phosphatase, glucose dehydrogenase and β-DPNH oxidase. In both mutants, alkaline and acid phosphatase activities remained relatively constant during the growth phase, but as sporulation proceeded, the alkaline phosphatase increased to maximum suggesting that it may play a role in spore formation. The abrupt decline of acid phosphatase at the onset of sporulation is difficult to explain. The function of alkaline phosphatase in sporulation is not known, but Waites et al (155) have suggested that it may include the hydrolysis of phosphate esters which generates inorganic phosphate required for the synthesis of phospholipids to be used in the formation of forespore membrane. The enzyme may also take part in the regulation of DNA synthesis since its activity is affected by the concentrations of ATP and Mg^{++}.

During the stationary growth phase of the RSpoIIIa, both alkaline and acid phosphatase showed increased activity and may participate in the autolysis of the cells.

Glucose dehydrogenase was found only in extracts of sporogenic cells which is in accord with the findings of Bach and Sadoff (134). However, further studies were not carried out and more data are needed before an appropriate interpretation can be made. The low activity of glucose dehydrogenase (133) may be due to the increase of DPNH oxidase during spore maturation (Fig. 33).

In the standard growth medium, trypticase peptone broth containing glucose, 22 mM (TPG), cells of the asporogenic mutant undergo autolysis after 24 h of growth but when the amount of glucose was increased > 0.135 M, the cells were stabilized at stage II of sporulation (Fig. 11-13) suggesting that glucose represses spore formation.

The sporogenic mutant which forms > 80% endospores in TPG (22 mM glucose) produced only about 30% spores in trypticase peptone broth when the glucose was increased to 0.27 M, indicating that sporulation in Clostridium as in yeast and in Bacillus (48, 122, 150, 152) is under catabolite repression.

Cyclic AMP has been shown to be a mediator in the reversal of glucose repressed sporulation of yeast (152) of inducible enzymes in Escherichia coli (99, 110, 166) and the data presented here shows that cyclic AMP displaced the catabolite repression of sporulating MSp^+ cells and increased their glucose consumption, suggesting that the reversal may be via glucose metabolism and thus functions as an indirect mediator. Since we have reported the presence of enzymes of butyric fermentation during sporogenesis of the MSp^+, it is likely that these enzymes are stimulated by cyclic AMP. It appears that the exogenous cyclic AMP increases the glycolysis in the derepressed cells in order to produce high levels of metabolites which are capable of inducing spore enzymes with an increase in "sporulation metabolism" and therefore the degree of sporulation. The reversal of the catabolite repression seems specific to cyclic AMP because only those nucleotides which could be converted to it mimicked its effect.

In accord with Van Wijk and Konijn (152), intracellular concentration of cyclic AMP in glucose repressed cells is lower than in derepressed cells. Moreover, since a higher level exists in sporulating cells than in vegetative cells, so it would appear

that repression of sporulation of the anaerobe is controlled by the fluctuations in cyclic AMP levels. The results of the intracellular analyses of cyclic AMP must be interpreted with caution since the validity of the binding protein method is questionable, and cAMP has not been detected in extracts of Bacillus (17, 130) so that the assay needs to be confirmed by another method.

CONCLUSION AND PROSPECTS

CONCLUSION AND PROSPECTS

It is concluded that glucose adapted cells of the sporogenic MSp^+ strains of Clostridium botulinum catabolizes glucose via the EMP pathway to yield acetate which serves as a precursor for poly-β-hydroxybutyrate granules. The enzymes of acetate metabolism as well as those of butyric acid fermentation may be partly susceptible to induction by acetate. As the glucose is exhausted, the cells shift to the butyric acid type of fermentation whereby the PHB granules are degraded to β-hydroxybutyrate, which undergoes dehydration and reduction to form butyrate yielding energy for sporulation and spore components.

Anaerobic sporulation is catabolite repressible and cyclic AMP appears to mediate indirectly in the derepression by enhancing the metabolic activities.

It would be of interest to correlate the biochemical and genetic studies of anaerobic sporulation. For example, although the dipicolinic acid (DPA) found in spores of both Bacillus and Clostridium has similar chemical properties, the biosynthetic pathway is obviously different for each genus and indeed the DPA may arise from different precursors in each case.

Permeability studies of metabolites such as acetate, butyrate and some amino acids during the development of the forespore may assist in understanding the synthesis and function of the spore layers and components. Only very limited studies have been reported on the germination of clostridial spores and it will be useful to investigate further, since toxins are generally produced at this time. Furthermore, a chemically defined medium containing only nutrients essential for growth and sporulation of Clostridium species is needed to elucidate the nutritional requirements, mechanism of endospore, toxin and enzyme production. It should be possible to select the nutrients so as to derive one medium for growth only - presporulation medium and another medium for spore formation. Such media would permit a study of the effect of individual nutritional components on the development of structures and properties that are specific to spores.

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