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Nutritional and Biochemical Studies on Spore Outgrowth and Spore Formation of Type E Strains of *Clostridium botulinum*

by

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"NUTRITIONAL AND BIOCHEMICAL STUDIES ON SPORE OUTGROWTH AND SPORE FORMATION OF TYPE E STRAINS OF CLOSTRIDIUM BOTULINUM"

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A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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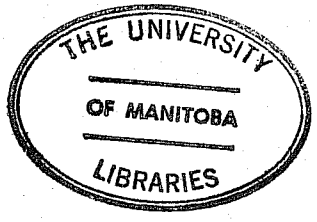

ABSTRACT

A chemically defined medium (CDM) was devised for the study of the nutritional requirements for germination, growth and sporulation of the sporogenic (MSp^+), asporogenic (RSpoIIIa) and temperature-sensitive (ts-25) mutants of Clostridium botulinum type E. The CDM consisted of a mixture of amino acids, vitamin factors, purines, pyrimidines, trace minerals, 0.4% glucose, 1.0% sodium acetate, 0.1% sodium bicarbonate and 0.2% sodium thioglycollate in phosphate buffer, pH7.0. The effect of single amino acid deletion studies showed that isoleucine, arginine, methionine and cysteine were essential for germination and outgrowth of MSp^+ heat-activated spores whereas valine, threonine and tryptophane were also essential for the ts-25 strain. No single amino acid was absolutely necessary for growth, however, for maximum growth, qualitative differences in amino acid requirements were observed. Sodium bicarbonate enhanced the rate and extent of spore germination and outgrowth. Glucose was required for growth and sporulation while sodium acetate increased the spore yield. The CDM supported germination, growth and sporulation of 8 other type E strains and also growth but not

sporulation of other serotypes of C. botulinum and C. sporogenes. Spore yield in CDM was usually lower when compared to complex medium, 65% in CDM and>90% in trypticase-peptone-glucose-yeast extract broth for MSp^+.

Experiments on incorporation of radioactive uracil, methionine and thymidine showed ordered syntheses of RNA, protein and DNA during spore outgrowth. Synthesis of DNA of the three test strains usually ceased by the end of log phase, T_0, while net RNA synthesis continued up to T_2 for both $MSp^+$and RSpoIIIa, but stopped at T_0 for ts-25. Net protein synthesis of the asporogenic strain stopped at T_3 while that of the sporogenic strains continued. Studies on rifampin treatment of cultures during stationary growth phase showed that synthesis of RNA continued during spore formation. The temperature shift experiment confirmed that the ts-25 strain was an early-blocked mutant which was expressed between T_0 and T_1 at the non-permissive temperature.

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INTRODUCTION

The growth cycle which occurs in the spore-forming bacteria belonging to the genus Bacillus or Clostridium consists of vegetative growth leading to the formation of an endospore, lysis of the mother cell to release the mature spore and germination of the spore followed by outgrowth into a new vegetative cell. Both sporulation and outgrowth of germinating spores have been employed as model systems for studying cellular differentiation (43, 61) since it involves a sequence of profound morphological alternations accompanied by biochemical changes and by the synthesis of qualitatively new proteins.

There is a remarkable similarity in the sporulation of species of Bacillus and Clostridium, both from the morphological and from the physiological points of view. The whole process involves continuous morphological changes which have been conventionally timed from the end of logarithmic growth (82, 101) designated as T_0 and the hourly periods after T_0 as T_1, T_2, ... T_n, etc. These cytological changes were first described in Bacillus cereus by Young and Fitz-James (135, 136, 137) and in Bacillus subtilis by Ryter (98). Similar changes occur in Clostridium histolyticum (12). Based on the

examination of thin sections of sporulating cells by electron microscopy, the process has been divided into seven stages (0 to VII). Stage 0 is the vegetative cell in early stationary phase, after growth and cell division have been completed. Formation of an axial nuclear filament, the first morphological sign of sporulation (Stage I), is followed by the development of a membrane diaphragm (forespore septum) and formation of the forespore in stages II and III. Stages IV to V is characterized by the development of spore cortex and coat. Maturation of the spore (Stages V and VI) consists of structural transformations of the spore envelopes which lead to the development of resistance and refractility. The final step (Stage VII) is the lysis of mother cell to release the mature spore. The time scale for the cytological events of the seven stages of sporulation which occurred in cultures of Bacillus subtilis has been established by Dawes et al (24). The designation of these developmental stages serves as a basis for following the chronology of genetic and biochemical events of sporulation.

Information concerning synthesis and turnover of macromolecules at the end of logarithmic growth and during sporulation (stationary phase) has come mainly from Bacillus spp as limited research has been done on Clostridium spp. Freese (43) has pointed out that one

of the criteria necessary for the initiation of normal sporulation is a decrease in the expansion of the cytoplasm, i.e. the reduction in the net synthesis of nucleic acids and protein, with continuous turnover of m-RNA and protein. The turnover of m-RNA and proteins is necessary for the synthesis of new enzymes and structural proteins and new classes of RNA for spore formation (26,134). The increase of DNA stops before or at the time of forespore septation and turnover of DNA does not occur.

The transformation of spores into vegetative cells includes three distinct and sequential processes ——— activation, germination and outgrowth. When activated spore is exposed to certain "germinants" which are usually species-specific, it is transformed into a metabolically active cell which is still quite different from the vegetative one. Germination is metabolism-mediated (126) and is not dependent on RNA or protein synthesis (110). During germination 30% (61) of the dry weight of a spore (including DPA, calcium and cortical material) is expelled into the medium and the spore becomes non-refractile and stainable. Both activation and germination are processes responsible for the termination of cryptobiotic state. The germinating spore, when exposed to nutrients,will start to swell, elongate and produce new kinds of proteins and cell wall and emerge as a typical

vegetative cell. This process is known as outgrowth and can be suppressed by inhibitors of macromolecular synthesis. In an outgrowing spore, RNA synthesis starts shortly after germination while protein synthesis lags behind and begins 2-4 minutes after the initiation of RNA synthesis (54, 64, 119). DNA synthesis commences about 200-300 minutes after germination when the amount of RNA has almost doubled (131).

A synthetic or chemically defined medium offers the most direct route to the elucidation of nutritional requirements, yet the literature is relatively sparse in this respect for different strains of Clostridium botulinum, particularly type E. Inukai and Haga first used a semi-synthetic medium containing vitamin-free casaminoacids to study the vitamin requirements of the Iwanai strain of type E (58). Germination and growth of spores of strain D8 in a synthetic medium was reported by Ward and Carroll (128) in 1966. In 1967, Gullmar and Molin (50) perfected their chemically defined medium for sporulation of type E strain 1537/62 by increasing the choline chloride concentration to 5 mg/100 ml medium. Later, Strasdine and Melville (111) also noted the sporulation of Minnesota strain in a defined medium. None of the workers reported growth of type E strains other than the test strains and also that the media could support the complete growth cycle of

germination, growth and sporulation.

The purpose of this work was to develop a pre-reduced chemically defined medium which would support germination, growth and sporulation of Clostridium botulinum type E strains and to determine the amino acids requirements of three type E mutants —— sporogenic, asporogenic and temperature-sensitive —— previously isolated in our laboratory (29, 67). In addition a preliminary study of the synthesis of macromolecules —— DNA, RNA, protein —— in these mutants during growth and sporulation, utilizing radiochemicals and inhibitors of macromolecular synthesis, was carried out.

H I S T O R I C A L

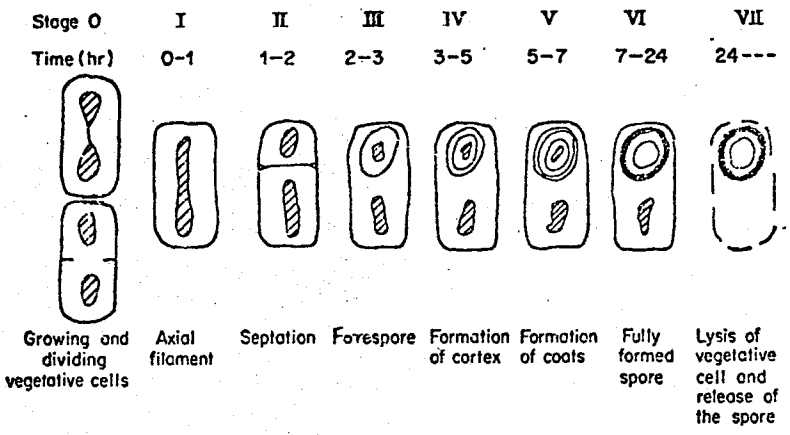
HISTORICAL

Extensive nutritional, cytological, physiological, biochemical and genetical studies of bacterial sporogensis have been carried out on the aerobic Bacillus species which have proved to be easy to grow and work with. Most of our knowledge about sporulation has come from studies on Bacillus subtilis and Bacillus cereus. Although the anaerobic spore-formers are important to the food industry and public health, the clostridia have not been studied so extensively because of the stringent requirements for growth and strict anaerobiosis. With the improvement in anaerobic techniques, the nutritional as well as increasing number of cytological, physiological and biochemical studies are being done on Clostridium species.

The cytological changes which occur during sporulation have been defined on a temporal sequence. Seven morphological steps have been described. The process is essentially the same in Bacillus and in Clostridium species (12,38, 59, 82). The end of the logarithmic growth phase (Stage 0) is followed by the formation of an axially disposed filament of condensed chromatin (Stage I). Stage I is completed when a

septum begins to form near the pole of the cell. The completion of the forespore septum during stage II results in the segregation of the nuclear material into two compartments referred to as the mother cell and forespore cytoplasmic units. During stage III, the forespore protoplast is engulfed as a result of unidirectional growth of the cytoplasmic membrane of the sporangium. As a consequence of engulfment, the two membrane surfaces that normally face the exterior of the cell now face each other at the surface of the forespore cytoplasm. Peptidoglycan precursors are transported to the space between the two membranes while the cortical peptidoglycan and germ cell wall are being synthesized during stages III and IV. Coat and exosporium formation occur during stages IV and V. The forespore becomes partially refractile during stages IV and early stage V. The end of stage V is characterized by the presence of a complete coat structure. During stage VI, a maturation process occurs. The refractility of the endospore increases, heat resistance develops, and changes in the fine structure of the cytoplasm of the spore protoplast occur that cause it to appear more homogeneous and electron-dense. Stage VII consists of the liberation of the mature free spore via autolysis of the mother cell. Subsequent to maturation of the endospore a lytic enzyme is synthesized or activated which brings about its

release from the sporangium. The following diagram shows the morphological stages in the sporulation of Bacillus subtilis Marburg (Adapted from Schaeffer et al., 1969)



Growth and spore formation of Clostridium botulinum usually require complex media, such as meat and vegetative infusions (120, 90, 17), and strict anaerobic conditions. Cooked-meat medium supports spore formation of many clostridial species (94) but the presence of tissue particles renders the harvesting of the spore crop inefficient and cleaning of the spores is even more laborious. Aparticulate liquid media prepared from commercially available dehydrated peptones, usually supplemented with other constituents, generally support the production of stable, clean spores (87). Perkins(87) obtained superior sporulation of C. parabotulinum types A and B and PA3679 in casein supplemented with proteins of animal origin.

Leifson (69) reported 90% sporulation for C. parabotulinum with 1.0% peptone and 0.5% beef extract. Sugiyama (112) obtained spores of C. botulinum in a medium containing 5.0% casitone and 0.5% peptone. Clostridium botulinum strain 62A produced over 90% spores in 5% solutions of phytone, thiotone, basamin, yeast extract, trypticase or polypeptone (122). TPG medium (5% trypticase, 0.5% peptone and 0.4% glucose) had been used successfully for the production of spore crops of type E strains of C. botulinum (102, 93, 111,29); over 80% spores were obtained for MSp^+ (29) and about 60% for five other strains (93). Robert showed that over 80% spores of C. botulinum type 7272A were formed in TPG medium supplemented with 0.1% yeast extract and 1% ammonium sulfate (TPAY-GT). C. botulinum type C also produced satisfactory spore crops in TPAY-GT medium (94). Day and Costilow (23) obtained 80-90% sporulation for C. botulinum 62A in a medium containing 4% trypticase and 1 ppm of thiamine. Emodi and Lechowick (34) used TPSY medium (5% trypticase, 0.5% peptone, 0.5% sucrose and 1.0% yeast extract) to produce spore crops of C. botulinum type E strains. The most important advantage of these "semi-defined" peptone media is that their amino acid concentration can, within the limits of the ratios of the constituent hydrolysed proteins, be closely controlled.

Synthetic or chemically defined media offer the most direct route to the elucidation of nutritional requirements. The early literature concerning this aspect was mainly restricted to types A and B which produce lethal toxins. Burrows (16) was the first to report growth of types A and B in an inorganic medium supplemented with alanine, leucine, lysine, proline, cystine, glycine and glucose and stated that cystine, proline and leucine were essential for C. botulinum. Clifton (20, 21) later verified that glycine and proline act as hydrogen acceptors, while alanine and leucine act as hydrogen donors in the so-called "Stickland reaction". Knight and Fildes (63) also determined that the "sporogenous" vitamins and tryptophane were essential for the growth of C. botulinum. The amino acid requirements of C. parabotulinum A, now designated C. botulinum type A, were studied by Elberg and Meyer (28) and later by Mager et al (76). They found that tryptophane, threonine, valine, leucine, isoleucine, methionine, arginine, phenylalanine and tyrosine were essential for growth. Roessler and Brewer (96) obtained similar results and in addition, found that threonine was essential for type A and histidine for type B.

The vitamin requirements of C. botulinum type A were studied by Lamanna and Lewis (66) who observed that

the requirement for pantothenate could be substituted by either thiamine or choline. Roessler and Brewer (96) also noted that biotin was the only essential vitamin for types A and B; however, better growth occurred if riboflavin, para-aminobenzoic acid and niacinamide were included. The vitamin requirements of clostridial species and serotypes of C. botulinum were studied by Kindler and Mager (62).

Sporulation of C. botulinum in synthetic medium was first reported by Williams and Blair in 1950 (130). Perkins and Tsuji (88), however, could not duplicate their findings with strain 62A as limited growth and no sporulation was observed. By modifying the composition of the medium of William and Blair and increasing the arginine content sevenfold, Perkins et al succeeded in obtaining 70% sporulation after 7 days at 30°C. Replacement sporulation of 62A was reported by Day and Costilow (23) using solutions containing L-alanine and L-proline, L-isoleucine and L-proline, or L-alanine and L-arginine. Germination of heat-activated C. botulinum 62A spores was reported to occur in a defined medium containing L-cysteine, sodium bicarbonate and sodium thioglycollate in TES buffer, pH7.0, by Rowley and Feeherry in 1970 (97).

A semi-synthetic medium containing vitamin-free casaminoacids was first used by Inukai and Haga (58) to study the vitamin requirements of a type E strain of

C. botulinum. Germination and growth of spores of a type E strain occurred in a synthetic medium, described by Ward and Carroll (128), which contained 15 aminoacids, 9 vitamin factors, 0.4% glucose, 0.06% sodium thioglycollate and 3 salts in phosphate buffer, pH7.4; however, spores were not produced. The addition of purines and pyrimidines did not greatly influence growth. Sporulation of C. botulinum type E strain 1537/62 was observed by Gullmar and Molin (50) in a medium containing 20 amino acids, 7 vitamin factors, 0.243% acetate, 0.4% glucose, 0.06% sodium thioglycollate and 5 salts in phosphate buffer, pH7.0. Similarly, spore production and growth of type E, strain Minnesota was reported by Strasdine and Melville (111) in a defined medium containing 16 amino acids, 10 vitamin factors 0.5% glucose and 5 salts in phosphate buffer, pH7.0. After 24 hours, virtually all the endospores had become refractile. These workers also studied the effects of various carbon sources on total growth of the organism in their medium; slow germination and no sporulation was observed when acetate was used in place of glucose. Strasdine et al also confirmed that choline was an essential factor in promoting cell division.

Asporogenic, sporogenic and conditional sporogenic mutants (e.g. temperature-sensitive) blocked at different stages of spore development have proved very useful for

elucidating the biochemical events and regulation during bacterial sporogensis. Mutants of Bacillus subtilis blocked in each of the stages of sporulation (101,138) have been mapped by transformation and transduction. Mutations were located at numerous loci scattered over the B. subtilis genome, although some were clustered. Most of the asporogenic mutants can be divided into two classes —— the early-and late-blocked mutants. The early-block mutants, i.e. those affected in the first morphological stages of sporulation, exhibit unidirectional pleiotropic effects, preventing the expression of all the late functions. Mutants of Bacillus belonging to this class had been subjected to extensive genetic (11, 56, 101,117,118) and biochemical (71, 116) studies. The morphological changes have been correlated to the biochemical events that occur in the wild type and in asporogenic mutants (10, 42, 85 86, 127). Electron microscopy studies showed that some of the temperature-sensitive mutants of B. cereus (74, 75)and B. subtilis (71, 116) were blocked at stage 0 of spore development at the non-permissive temperature (116).

Asporogenic mutants of Clostridium histolyticum and Clostridium perfringens had been reported (18, 103, 104). In our laboratory, three types of mutants have been isolated from NTG-treated Clostridium botulinum type E

strains (29, 67). MSp^+ and RSpoIIIa were high frequency sporogenic and asporogenic mutants of ATCC 9564. Ultrastructure and some physiological and biochemical events occurring during the developmental stages have previously been compared (29,30, 31, 32, 33, 53). The temperature-sensitive mutant, ts-25, dervied from the type E Beluga strain, sporulated at 28°C but not 37°C. Electron micrograph of ts-25 grown at the non-permissive temperature (67) indicated that blockage was at stage I of the sporulating process.

The timing of DNA synthesis and the state of replication of the bacterial chromosome could have important implications in the initiation of sporogensis. According to Dawes, Kay and Mandelstam (25) and Mandelstam, Sterlini and Kay (78), the time at which the B. subtilis cell was sensitive to initiation of sporulation was limited to the period just after completion of DNA replication. This suggested that all DNA synthesis stopped before sporulation had commenced. Szulmajster and Canfield (115), using ^{32}P-suicide technique, reported that DNA synthesis ended at $T_{1.5}$ in B. subtilis. In B. cereus, DNA synthesis did not occur during stage I (135, 136), thus Young and Fitz-James suggested that the axial chromatin thread was formed from two discrete chromatin bodies present in the cell at the end of logarithmic growth and that the spore inherited

an amount of DNA equal to half of that present in the vegetative cell (36). However, electron microscopic observations did not support the hypothesis of nuclear fusion. Aubert et al (6) found that in B. subtilis DNA synthesis continued during the first three hours after initiation of sporogenesis and the bacteria contained twice as much DNA at T_3 as at T_0. They therefore suggested that the axial chromatin thread represented a replicating state of genetic material in this species so that one chromosome could enter the prespore cell while another one remained in the mother cell. Another interesting result of their work was that by T_5 40% of the DNA present at T_0 , was excreted into the medium and degraded. As a result of new synthesis and loss of DNA, only 30% of the spore DNA orignated from the vegetative cells, while 70% was formed between T_0 and T_3. The contradiction between the results of Aubert et al and the others might be due to the arbitrary definition of T_0 and differences in the degree of synchrony in the different cultures.

Previous observations have dealt with the DNA content of a heterogenous population, only part of which underwent sporulation at a given time. Ryter and Aubert (99), using technique of autoradiography combined with electron microscopy, followed DNA synthesis in individual sporulating cells of the Marburg

168 strain of B. subtilis and found that DNA synthesis had almost completely stopped in the sporulating cells when they had reached stage I; moreover the excretion of preformed DNA seemed to be restricted to non-sporulating cells (blocked before Stage II). These observations suggest a close relationship between the arrest of DNA synthesis and commitment to sporulation.

During spore formation of Bacillus subtilis, the total amount of RNA remained constant, until lysis occurred. However, although net synthesis stopped at T_0, the incorporation of radioactive precursors was indicated by a rapid turnover of the RNA. The rate of this turnover was constant during spore formation and reached 20%/h , which was about five times higher than in non-sporulating stationary phase cells. Sucrose gradient centrifugation and other techniques showed that all three types of RNA (messenger, ribosomal and transfer RNA) were synthesized during sporulation (8, 11, 40, 81, 114). Freese and his co-workers (39 ,44) observed that RNA synthesis and the maintenance of normal pool levels of ATP after the end of logarithmic growth in B. subtilis depended on the presence of a functional tricarboxylic acid cycle which, together with cytochrome-linked electron transport system, provided the necessary energy (ATP, electron transport) for continuous synthesis of cytoplasmic macromolecules during sporulation.

Recently, Hutchison reported that a decrease in the energy level (expressed as ATP conc. or adenylate energy charge) of B. subtilis cells had occurred at the end of logarithmic growth (57). Some controversy exists as to the stability of sporulation-specific m-RNA. Sporulating cells of Bacillus subtilis (7, 9, 113, 115) and B. cereus (37, 64) which were treated with actinomycin D, an inhibitor of RNA synthesis, synthesized unstable m-RNA species. Leighton and Doi (70) confirmed these findings by adding rifampin, an inhibitor of DNA-dependent RNA polymerase, to B. subtilis cells at different times of the sporulation cycle. However, pulse-labelling and hybridization techniques used on B. cereus by Aronson (4, 5) showed that after initiation of sporulation, in addition to the unstable m-RNA species, a persistently stable fraction of membrane-bound spore m-RNA was formed which was conserved even in the presence of actinomycin D. Existence of stable m-RNA implied translational, in addition to transcriptional control in sporulating system (43).

Rapid and extensive protein turnover occurred during sporulation and, in general terms, protein synthesis followed closely the synthesis of RNA. When total protein was measured during sporulation of B. subtilis it was found that net synthesis stopped at T_0, but the proteins were subject to rapid turnover. This

MATERIALS & METHODS

turnover rate was estimated to about 20%/h and remained constant throughout sporulation (9, 108, 109). The proteins of the spore and those present in the mother cell at stage VI, including ribosomal protein, were, for the most part, newly synthesized (52) and 75-90% of the soluble proteins of B. subtilis spores were synthesized during sporulation (65). Protein turnover which required protein synthesis after completion of growth has not been observed in many asporogenic mutants blocked early in sporulation (101). However, Mandelstam and Waites (77) have observed normal protein synthesis during the post-logarithmic phase of asporogenic mutants which did not exhibit rapid protein turnover. The disappearance, alternations or new production of sporulation-specific proteins has been reviewed by Halvorson (51), Kornberg et al (65), Murrell (82), Slepecky (106) and Hanson et al (52).

MATERIALS AND METHODS

Organisms

MSp$^+$, a non-toxigenic sporogenic mutant of *Clostridium botulinum* type E, ATCC 9564, was derived by treatment with N-methyl-N'-nitrosoguanidine (NTG) (29). It produced over 80% spores in trypticase-peptone-glucose (TPG) medium. RSpoIIIa, an asporogenic mutant, isolated from the same parent strain, was blocked at stage III of spore development (53).

Ts-25, a temperature-sensitive mutant of NTG-treated *C. botulinum* type E strain Beluga, grew equally well at 28 and at 37°C. Mature spores were formed at 28°C, whereas at 37°C only aggregation of nucleoplasm into an axial filament, a characteristic feature of stage I spore development, was observed by electron microscopy (67). Other strains of *Clostridium spp.* used in this study are listed in Table 7.

Media

(1) Complex

Trypticase-peptone-glucose-yease extract(TPGY) medium containing 5% trypticase (BBL), 0.5% proteose peptone (Difco), 0.4% glucose, 0.4% yeast extract (Difco) and 0.2% sodium thioglycollate (Difco) was used for

maintenance of cultures and for spore production. TPGY agar plates, containing 3% agar, were used from time to time to check the purity of the cultures.

(2) Defined

Empirical attempts were used to develop the chemically defined medium (CDM) which is described in Table 1. Stock solutions of reagent grade chemicals, L-amino acids, vitamin factors, purines and pyrimidines (Nutritional Biochemical Corporation) were prepared with deionized distilled water and added together either individually or as blocks (Table 2). The medium was adjusted to pH7.0 by .1 N HCl, diluted to the required volume, sterilized by membrane filtration (Nalge disposable filter unit, 0.45 micron), and dispensed aseptically into sterile screw-cap test-tubes (15x125 mm), test-tubes (15x125 mm) or 250 ml. flasks. The test-tubes and flasks were fitted with Suba-seal stoppers. In order to maintain the medium in its pre-reduced state, screw-cap tubes containing the medium were stored in Gas-Pak anaerobic jars at room temperature whereas sealed tubes and flasks were flushed with a mixture of sterile gas (95% N_2 and 5% CO_2). Since Na-thioglycollate gradually became slightly toxic to microorganisms, all media were freshly prepared and used within three days.

Table 1. Chemically Defined Medium (CDM) for germination, growth and sporulation of *C. botulinum* type E

Component	mg/100ml	Component	mg/100ml
L-alanine	40	Biotin	0.002
L-arginine	27	Calcium pantothenate	0.2
L-aspartate	65	Choline chloride	5.0
L-cysteine HCl H_2O	44	Folic acid	0.2
L-glutamate	37	Inositol (meso)	0.2
Glycine	23	Niacin	0.1
L-histidine	24	Niacinamide	0.1
L-isoleucine	62	p-aminobenzoic acid	0.005
L-leucine	164	Pyridoxal HCl	0.1
L-lysine HCl	141	Pyridoxine HCl	0.1
L-methionine	10	Riboflavin	0.2
L-ornithine HCl	70	Thiamine HCl	0.2
L-phenylalanine	108		
L-proline	50	K_2HPO_4	50.0
L-serine	100	KH_2PO_4	50.0
L-threonine	114	NaCl	0.2
L-tryptophane	5	$CaCl_2$	0.2
L-tyrosine	27	$MgSO_4$ $7H_2O$	4.0
L-valine	114	$MnSO_4$ $4H_2O$	1.0
		$FeSO_4$ $7H_2O$	0.2
Adenine	1.0	$ZnSO_4$ $7H_2O$	0.2
Cytosine H_2O	1.0	$CuSO_4$ $5H_2O$	0.1
Guanine	0.5		
Thymine	0.5	$NaHCO_3$	100
Uracil	1.0		
Xanthine	1.0	Na-acetate	1000
Glucose	400	Na-thioglycollate	200

pH adjusted to 7.0 with 0.1 N HCl

Table 2. Stock solutions[a] used to prepare CDM

Ingredients	ml/100ml medium
19 individual amino acids[b] (25X)	76
Block vitamin factors[c] in H_2O (100X)	1
Riboflavin in 0.02 N acetic acid (100X)	1
Folic acid in 0.01 N NaOH (100X)	1
Block adenine, cytosine and guanine in 0.1 N HCl (100X)	1
Block thymine, uracil and xanthine in 0.1 M NH_4OH (100X)	1
Block trace minerals in phosphate buffer[d] (100X)	1
$NaHCO_3$ in H_2O (50X)	2
Na-acetate in H_2O (20X)	5
Sodium thioglycollate in H_2O (50X)	2
Glucose in H_2O (50X)	2

[a] All stock solutions were filter-sterilized and kept in tightly capped milk dilution bottles at 0^{o} C.

[b] Aspartate, glutamate and tyrosine were dissolved in 0.1 N NaOH; tryptophane in 0.1 N HCl and the others in H_2O.

[c] With the exception of folic acid and riboflavin.

[d] K_2HPO_4 & KH_2PO_4 dissolved in 50 ml H_2O were mixed with the trace minerals which had been dissolved in 40 ml H_2O, the precipitate formed in the combined solution was dissolved by adding 10 ml 0.1 M EDTA.

Preparation of stock spore suspension

A spore suspension (1 ml) from stock MSp^+ culture, which was heat-activated at 65°C for 15 minutes, was added to 9 ml. TPGY medium in screw-cap tubes and incubated at 28°C until most of the spores had germinated into young vegetative cells (approx. 10 to 12 h). A 10% inoculum of the young culutre was added to fresh TPGY medium (9 ml) and incubated for 2 to 4 h before transferred to 90 ml of fresh TPGY. After 24 to 36 h of incubation, cultures showing greater than 90% spore were centrifuged for 15 minutes at 4080 g, 5°C. The pellet was washed three times with sterile deionized distilled water and treated with a solution containing 100 μg/ml of trypsin and 200 μg/ml of lysozyme in 0.02 M phosphate buffer (pH7.0) for 2 to 4 h at 37°C with continuous agitation. The cellular debris was removed by differential centrifugation at 1,000 g for 20 minutes, 4,000 g for 10 minutes and 10,000 g for 5 minutes. The clean spores were suspended in 0.02 M phosphate buffer (pH7.0) to an optical density (O.D.) of about 0.6 at 600 nm, containing approximately 10^9 spores per ml. This stock spore suspension was dispensed into screw-cap tubes (5 ml/tube), stored at 0 to 2°C and later used as inocula.

Stock spore suspensions of other strains of Clostridium spp. were prepared in a similar manner except vegetative cells of RSpoIIIa were maintained in TPGY medium at 0° to 2°C.

Growth and sporulation

Spores from stock spore suspension were suspended in test media and heat-activated at 65°C for 15 minutes. A 10% inoculum was added to test media in stoppered test-tubes and incubated at 28°C unless stated otherwise. Germination and vegetative growth was monitored spectrophotometrically (600nm, Coleman Junior IIA, model 6/20A). Samples for phase contrast microscopic examination were withdrawn with a hypodermic syringe to maintain anaerobic conditions. The percentage of sporulation was computed from the ratio of the number of refractile endospores in relation to the total number of cells (300) examined.

Amino acid requirements for germination and growth

Batches of chemically defined media prepared with each of the amino acids deleted were dispensed in duplicate in test tubes (9 ml) and were tested for supporting germination and growth of MSp^+. Requirements for germination were determined by inoculating media with heat-activated spores while requirements for growth were established through 2 successive transfers of vegetative cells in defined media after the spores had germinated into young culture in TPGY medium.

Effect of $NaHCO_3$ in TPGY medium

TPGY medium supplemented with 0.1% $NaHCO_3$ was inoculated with heat-activated MSp^+ spores to study the effect of $NaHCO_3$ on germination, growth and sporulation.

Effect of glucose, Tween 80 and ethanol in CDM

Defined media with varying glucose concentrations (0 - 1.5%) or supplemented with varying concentrations of Tween 80 (0.01 - 0.1%) or ethanol (0.1 - 1.0%) were inoculated with heat-activated MSp^+ spores.

Effect of rifampin on growth and spore formation

Heat-activated spores were inoculated into 200 ml TPGY medium. After incubation for 10 h at 28°C, samples (5 ml) were withdrawn from the culture at one hour intervals for 12 h and were transferred to vacutainers (15x100 mm) containing rifampin solution (final conc. 1.5 µg/ml). Incubation was continued for 24 h.

Assay of macromolecular synthesis

Incorporation of thymidine-6-^3H (specific activity 5 Ci/m mol), uracil-2-^{14}C (specific activity 61 mCi/m mol) and L-[methyl-^{14}C] methionine (specific activity 56 mCi/m mol) by cells was used as a measure of DNA, RNA and protein syntheses respectively. The labeled compounds were added to cultures of heat-activated spores

of MSp^+ at time of inoculation or at the end of log phase, with or without inhibitors (rifampin or chloramphenicol) of macromolecular synthesis. For RSpoIIIa, the labeled compounds were added to stationary phase cells at time of inoculation or 11 h after while for ts-25, they were added to early log phase cells. The culture (0.1ml) was sampled at appropriate time intervals and immediately mixed with 0.2 ml cold 10% trichloroacetic acid (TCA) solution. After standing at 0^oC for one hour, the precipitate formed was placed on presoaked membrane filter (0.45 micron, 25 mm) by filtration and washed three times with approximately 10 ml 5% TCA solution and then twice with 95% cold ethanol. The dried membrane filter was then placed in a counting vial with 10 ml of Aquasol. Radioactivity (cpm) was counted with a Beckman liquid scintillation counter, model SC-230. Five-minute counts were taken for all samples.

Temperature sensitive period for temperature-sensitive mutant

A series of 10-ml cultures of the temperature-sensitive mutant, ts-25, was prepared in TPGY medium. In the "shift up" experiment, cultures were grown at 28^oC for varying periods of time and then shifted to 37^oC for a total incubation period of 32 h. Similarly, a second series of cultures were grown at 37^oC and then shifted "down" to 28^oC.

R E S U L T S

RESULTS

PART I Nutritional studies in chemically defined medium

Effect of $NaHCO_3$ in TPGY medium and CDM

The growth curves of MSp^+ in TPGY medium and in bicarbonate supplemented TPGY medium (0.1% final concentration) are shown in Fig. 1. In the bicarbonate supplemented medium, most of the spores turned phase-dark within 4 h after inoculation and vegetative cells were seen at 6 h., whereas in TPGY medium without bicarbonate, vegetative cells were not observed until 10 h after inoculation. Outgrowth occurred about 4 h earlier in the bicarbonate medium and sporulation began at 16 h, about 6 h earlier compared with TPGY medium. Over 90% of the cells formed spores in both media.

The rate and extent of germination and outgrowth of MSp^+ was reduced inCDM without $NaHCO_3$. (Fig. 2)

Fig. 1 The effect of $NaHCO_3$ on germination and growth of MSp^+ spores in TPGY medium.

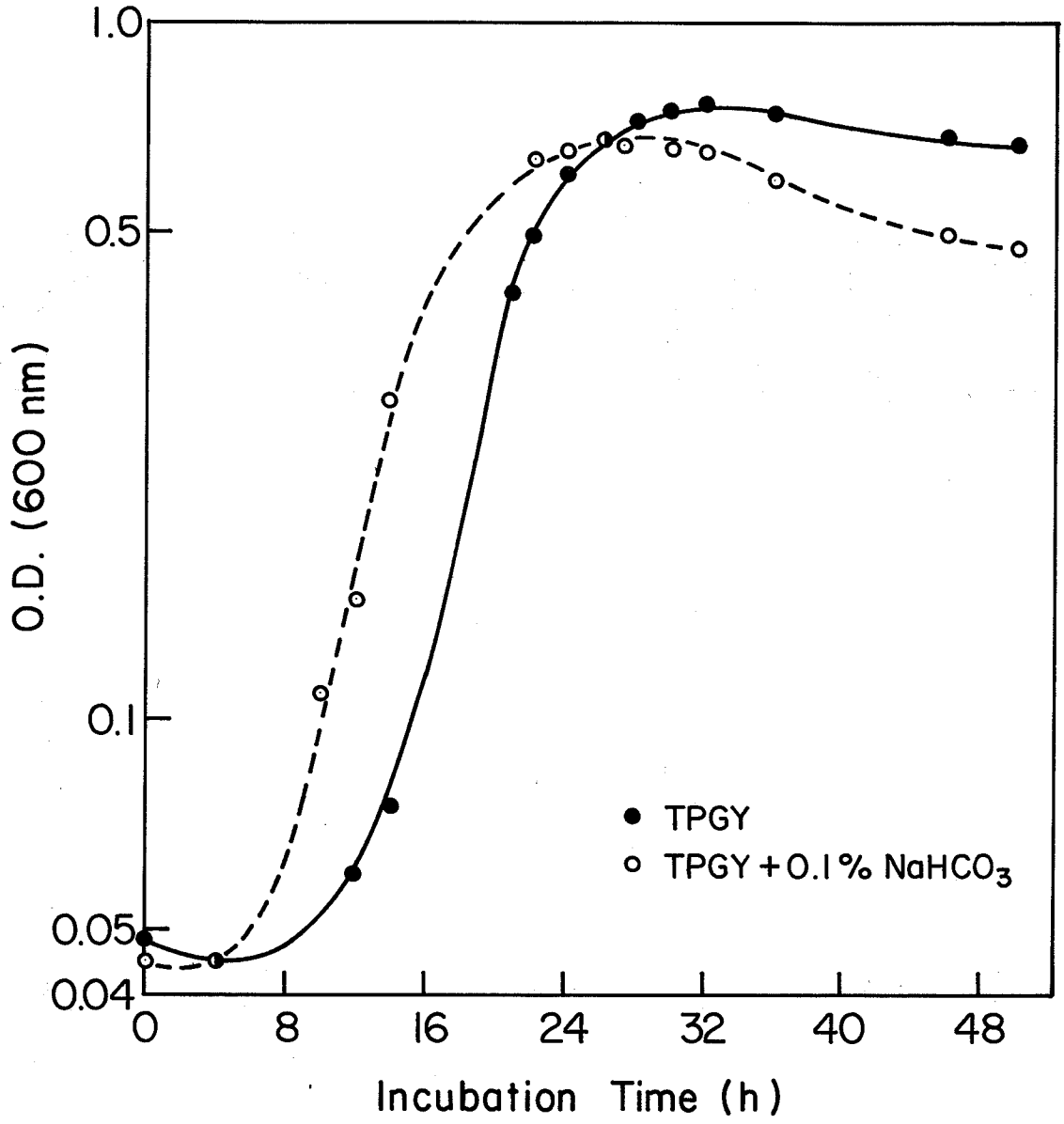
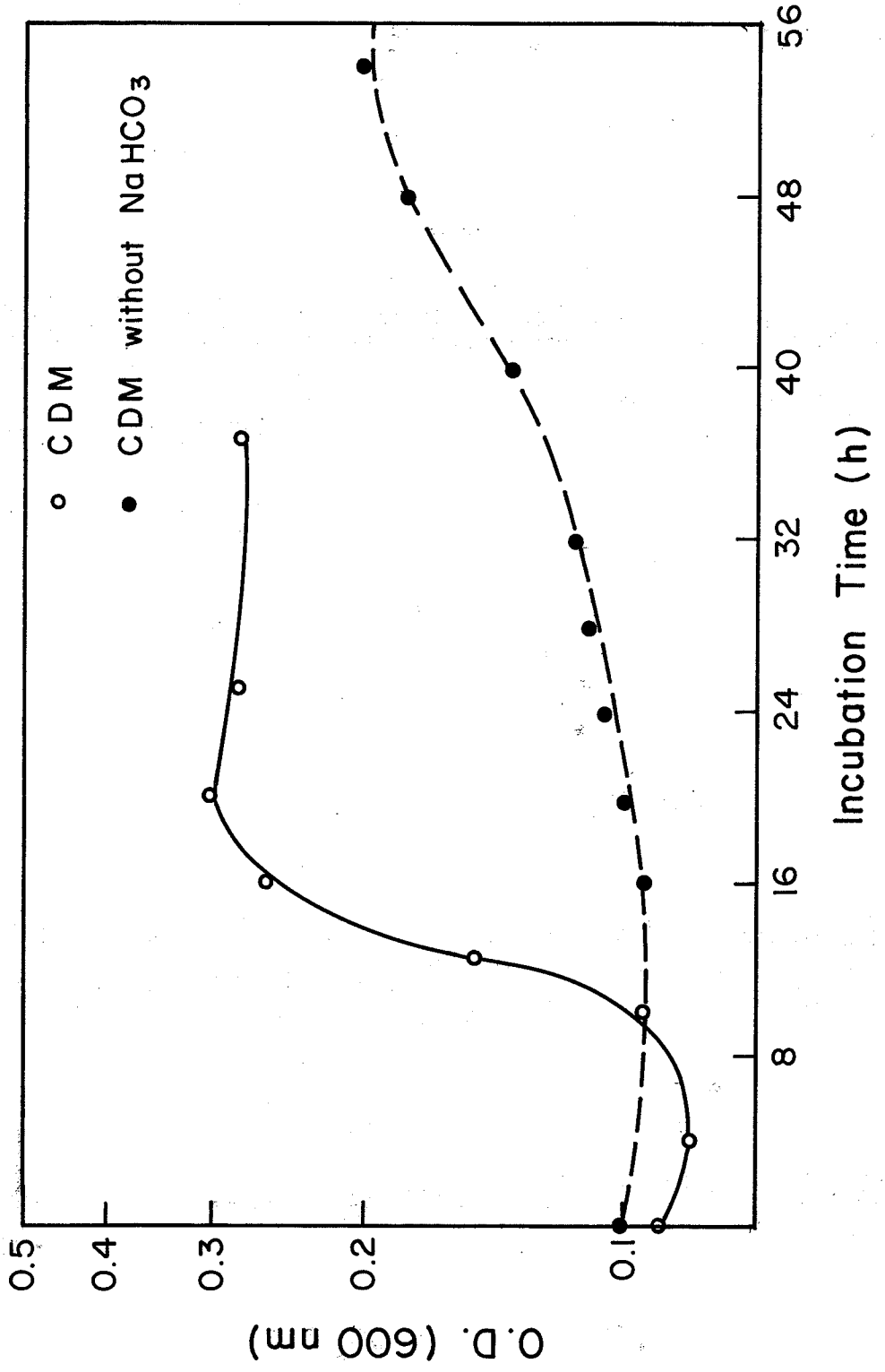

1.0
0.5
0.1
0.05
0.04
O.D. (600 nm)
0
8
16
24
32
40
48
Incubation Time (h)
TPGY
TPGY + 0.1% NaHCO3

Fig. 2 The effect of $NaHCO_3$ on germination and growth of MSp^+ spores in CDM.


O.D. (600 nm)
0.5
0.4
0.3
0.2
0.1
8
16
24
32
40
48
56
Incubation Time (h)
○ CDM
● CDM without $NaHCO_3$

Development of chemically defined medium (CDM)

Heat-activated spores of MSp^+ did not germinate in the basal medium, medium of Strasdine and Melville (111) supplemented with $NaHCO_3$. Most of the spores remained refractile after incubation for 16 h. In the medium which was supplemented with 0.1% casaminoacids, a few vegetative cells were observed, however, when 0.4% yeast extract was used as a supplement, many swollen vegetative cells were seen by 16 h and about 20% of the cells formed endospores. In the medium supplemented with both casaminoacids and yeast extract, normal slender shaped cells were observed by 16h and about 50% of the cells formed endospores by 22 h.

The quantity of the constituent amino acids, vitamin factors and inorganic components present in yeast extract and casaminoacids was estimated according to the analytical reports supplied by DIFCO and BBL (Table 3). An initial chemically defined medium which contained the components of the basal medium and constituents which simulated the composition of yeast extract and casaminoacids was developed. In this medium, most of the spores germinated into vegetative cells after incubation for 16 h and 35% of the cells sporulated by 28 h while cell autolysis began at 40 h. Growth was increased when purines and pyrimidines were added to the initial chemically defined medium and spore yield was higher when 1% sodium acetate was also included.

The chemically defined medium (CDM), comprising all the above mentioned components, supported germination, growth and sporulation of strain MSp^+. About 65% of the cells formed endospores in CDM as compared to over 90% in TPGY medium. A comparison of the growth and sporulation achieved by MSp^+ in CDM and TPGY medium is shown in Fig. 3.

Effect of glucose in CDM

When glucose was omitted from the CDM, only a few spores could germinate into vegetative cells. The rate and amount of growth was increased when the glucose concentration was raised from 0.4% to 1.0% (Fig. 4). However, spore production with glucose concentration at 1.0 or 1.5% was only 35-40% as compared to 65% in CDM with 0.4% glucose.

Table 3. Typical analyses of yeast extract & casaminoacids

	yeast extract (BACTO)	casaminoacids (BBL)
Ash (%)	10.0	-
Total nitrogen	9.5	8.0
Amino nitrogen	7.0	6.4
Amino Acids (%)		
Arginine	1.0	1.4
Aspartate	5.0	3.7
Cystine	-	0.3
Glycine	2.5	1.0
Glutamate	6.5	14.2
Histidine	1.0	0.7
Isoleucine	3.0	2.7
Leucine	3.5	3.5
Lysine	4.0	3.7
Methionine	1.0	1.7
Phenylalanine	2.0	0.7
Proline	-	4.0
Threonine	3.5	2.5
Tyrosine	0.5	3.1
Valine	3.5	4.1
Vitamin Factors (mcg/g)		
Biotin	1	0.018
Cyanocobalamin	-	0.00006
Folic acid	-	0.0057
Niacin	280	0.1
Pantothenate	-	0.26
Pyridoxine	20	0.024

Table 3 continued.

Riboflavin	20	0.10
Thiamine	3	0.105
Inorganic Components		
NaCl (in %)	0.51	37.2
Ca	0.0406	0.05
Fe	0.028	0.0045
K	0.042	0.4
Mg	0.03	0.003
P	0.89	0.32
S	-	0.066
SiO_2	0.052	-
Mn (in ppm)	7.8	-
Pb	16.0	-
Cu	19.0	-
Zn	80.0	-
Arsenic	0.11	-

Fig. 3 Growth and sporulation of MSp^{+}.

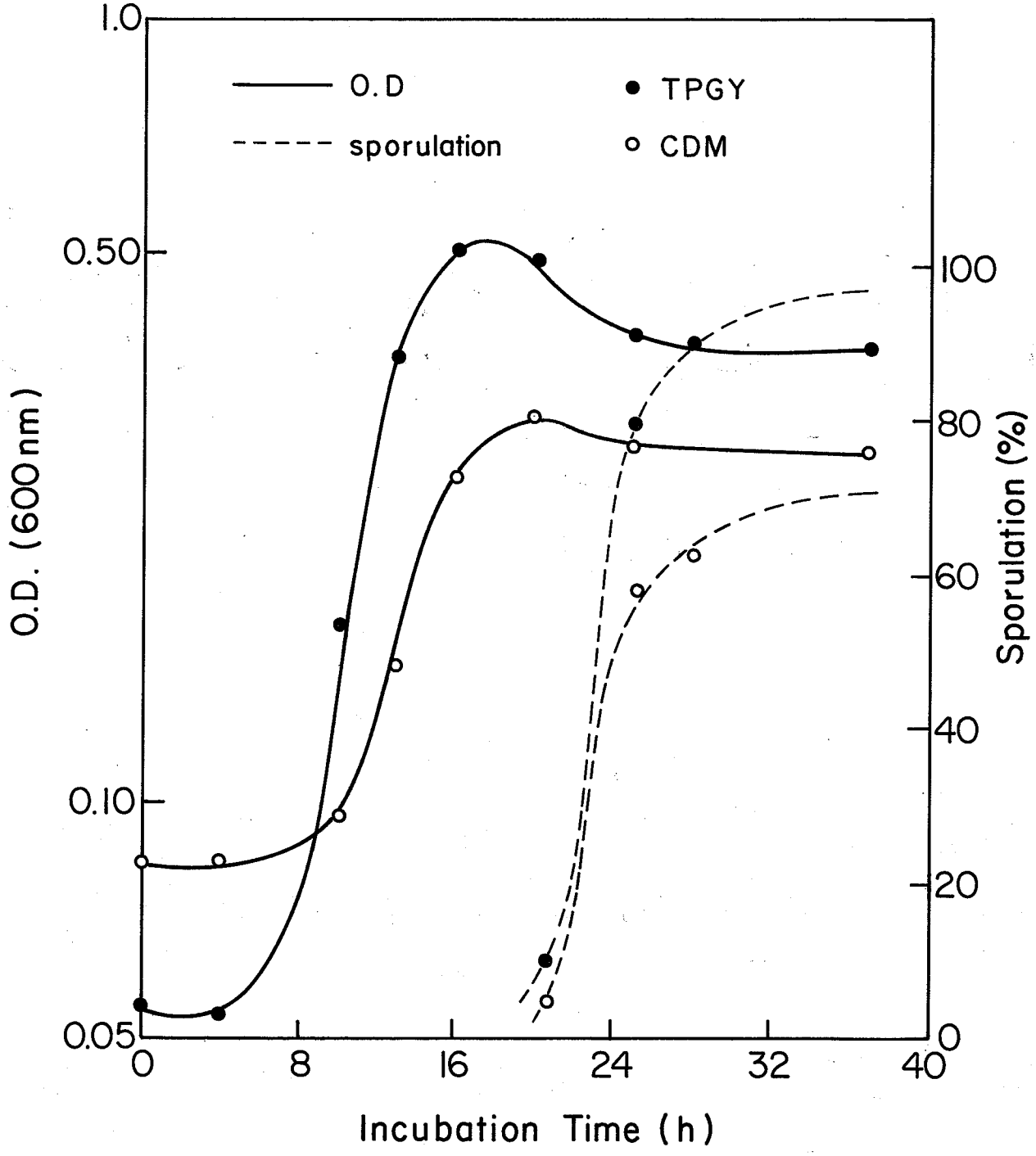
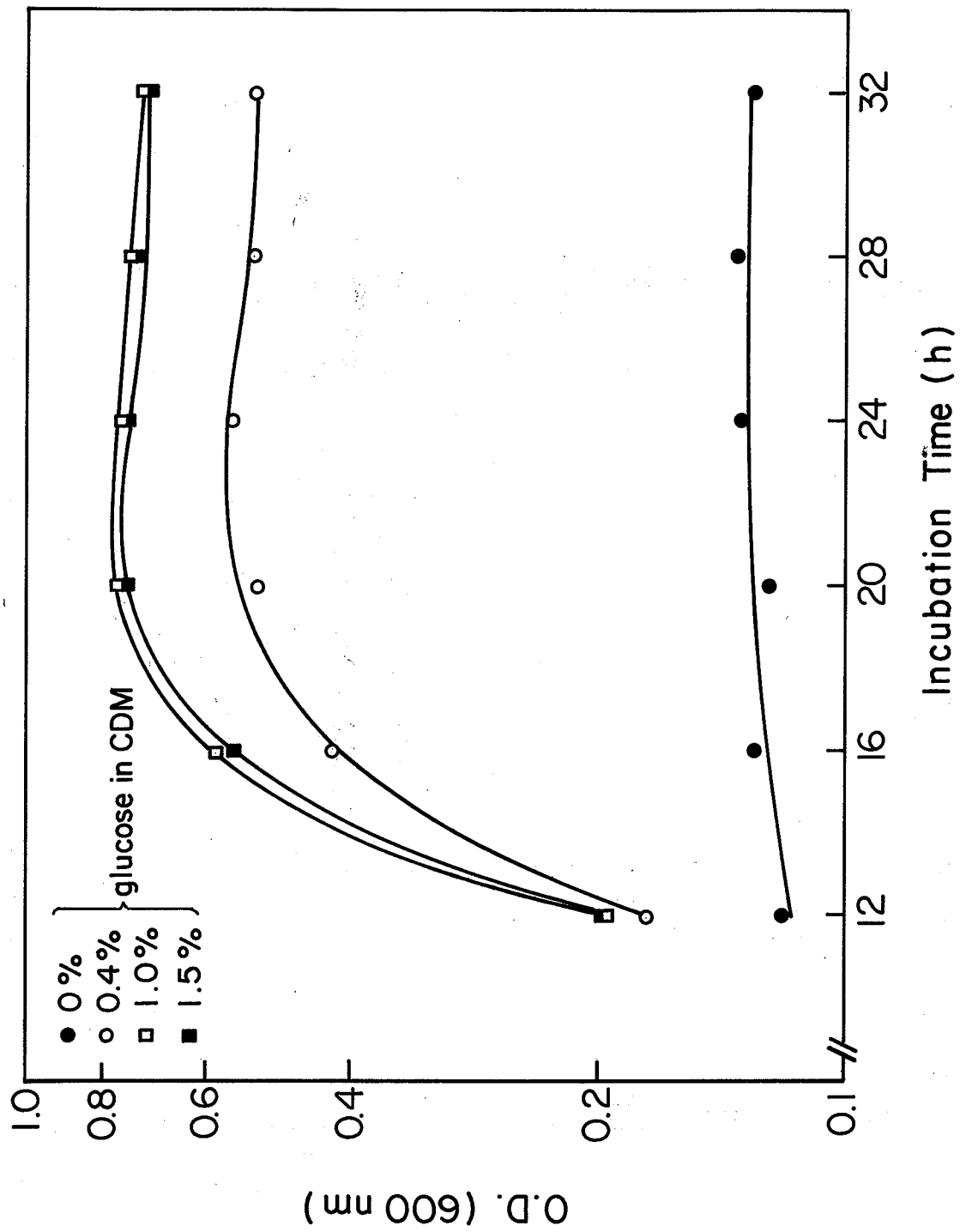

1.0
0.50
0.10
0.05
O.D. (600nm)
O.D
sporulation
TPGY
CDM
100
80
60
40
20
0
Sporulation (%)
0
8
16
24
32
40
Incubation Time (h)

Fig. 4 The effect of glucose on growth and sporulation of MSp^{+}.


O.D. (600 nm)
1.0
0.8
0.6
0.4
0.2
0.1
12
16
20
24
28
32
Incubation Time (h)
glucose in CDM
0%
0.4%
1.0%
1.5%

Amino acid requirements

Fig. 5 shows the relative amount of growth of MSp^+, RSpoIIIa and ts-25 achieved after 28 h in defined media with omission of single amino acids.

For MSp^+, growth in defined medium was reduced to less than 30% when arginine or cysteine was omitted and to about 50% without methionine or tryptophane. Deletion of glycine, histidine, isoleucine, lysine or proline did not affect growth to any significant extend.

Similar results were obtained with strain ts-25. Less than 50% of growth was obtained in defined medium when arginine, cysteine, methionine or tryptophane was deleted.

Only the absence of cysteine from CDM showed a significant reduction in growth of the RSpoIIIa strain, less than 30%. About 50% growth was obtained when arginine, proline or valine were omitted.

Data in Tables 4 and 5 indicate the effect of amino acid deletion on germination and outgrowth. After incubation for 28 h, most of the spores of MSp^+ remained refractile when isoleucine, arginine, cysteine or methionine was omitted from CDM. Some vegetative cells were observed in media lacking arginine, cysteine or methionine. Vegetative cells and endospores were observed by 36 h in media without lysine, glutamate, threonine, tyrosine or valine.

With the deletion of threonine or valine from the medium, most of the spores of ts-25 remained refractile. Some vegetative cells were observed when arginine, cysteine, isoleucine, methionine or tryptophane was omitted.

Fig. 5 The effect of single amino acid deletion in CDM on growth.

The percentage O.D. was calculated from the ratio of maximum growth in test medium compared with complete CDM.

Medium	Amino acid deleted	Medium	Amino acid deleted
1	none (CDM)	11	serine
2	isoleucine	12	leucine
3	lysine	13	threonine
4	histidine	14	tyrosine
5	glycine	15	valine
6	proline	16	phenylalanine
7	alanine	17	methionine
8	ornithine	18	tryptophane
9	aspartate	19	arginine
10	glutamate	20	cysteine

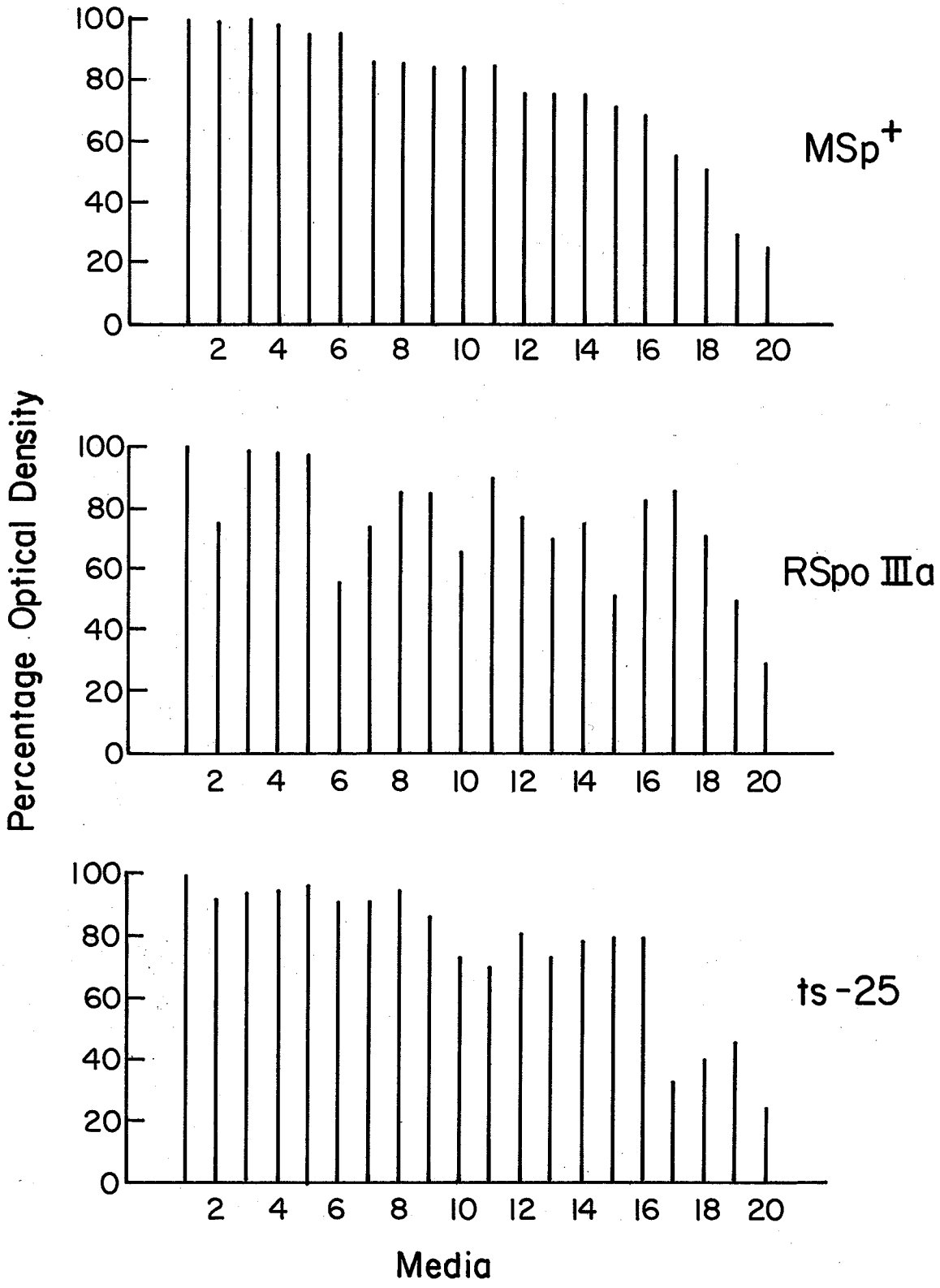

Percentage Optical Density
100
80
60
40
20
0
2 4 6 8 10 12 14 16 18 20
MSp+
RSpo IIIa
ts-25
Media

Table 4. The effect of single amino acid deletion in CDM on germination and outgrowth of spores of MSp^+.

Amino acid deleted	O.D. 28h	O.D. 36h	final pH	Microscopic Observation 28h	Microscopic Observation 36h
None (CDM)	0.15	0.20	5.8	veg. cells endospores	free spores
Isoleucine	0.03	0.02	7.4	bright and dark spores	bright and dark spores
Arginine	0.09	0.09	6.1	bright and dark spores veg. cells	bright and dark spores veg. cells
Methionine	0.10	0.10	6.5	bright and dark spores veg. cells	bright and dark spores veg. cells
Cysteine	0.10	0.12	6.0	bright and dark spores veg. cells	veg. cells and dark spores
Valine	0.02	0.11	6.8	dark spores	veg. cells endospores
Tyrosine	0.03	0.13	6.1	dark spores	veg. cells endospores
Threonine	0.04	0.17	6.1	dark spores	veg. cells
Glutamate	0.02	0.20	6.1	bright and dark spores	veg. cells endospores
Lysine	0.06	0.33	5.7	dark spores	veg. cells endospores
Tryptophane	0.16	0.16	5.8	veg. cells	veg. cells endospores
Serine	0.08	0.16	6.0	veg. cells	veg. cells
Aspartate	0.17	0.18	6.2	veg. cells	endospores
Proline	0.14	0.18	5.9	veg. cells	endospores
Leucine	0.11	0.19	5.9	veg. cells	veg. cells
Phenylalanine	0.20	0.19	6.0	veg. cells endospores	endospores

Table 4 continued.

Histidine	0.21	0.22	5.8	veg. cells endospores	endospores
Ornithine	0.16	0.23	5.9	veg. cells	lysed cells
Alanine	0.18	0.26	5.8	veg. cells endospores	veg. cells endospores
Glycine	0.18	0.29	5.6	veg. cells	veg. cells endospores

Table 5. The effect of single amino acid deletion in CDM on germination and outgrowth of spores of ts-25

Amino acid deleted	O.D. 28h	O.D. 36h	final pH	Microscopic Observation 28h	Microscopic Observation 36h
None (CDM)	0.04	0.32	5.5	veg. cells dark spores	veg. cells endospores
Isoleucine	0.01	0.03	6.8	dark spores	dark spores veg. cells
Arginine	0.01	0.02	6.4	dark spores veg. cells	dark spores veg. cells
Methionine	0.06	0.06	6.3	bright and dark spores veg. cells	bright and dark spores veg. cells
Cysteine	0.01	0.08	6.2	dark spores	dark spores veg. cells
Valine	0.01	0	7.2	bright spores	bright spores
Tyrosine	0	0.16	6.0	bright spores	veg. cells
Threonine	0	0	7.1	bright and dark spores	bright and dark spores
Glutamate	0	0.3	5.8	dark spores	veg. cells
Lysine	0.03	0.25	5.9	dark spores veg. cells	veg. cells cell lysis
Tryptophane	0.01	0.07	6.0	dark spores veg. cells	veg. cells cell lysis
Serine	0.01	0.17	6.2	dark spores	veg. cells
Aspartate	0.07	0.33	5.2	dark spores veg. cells	veg. cells endospores
Proline	0.03	0.34	5.3	dark spores veg. cells	veg. cells endospores
Leucine	0.01	0.15	5.7	dark spores veg. cells	veg. cells cell lysis
Phenylalanine	0.18	0.36	5.6	veg. cells	veg. cells

Table 5 continued.

Histidine	0.03	0.29	5.4	dark spores veg. cells	veg. cells endospores
Ornithine	0.06	0.30	5.6	dark spores veg. cells	veg. cells
Alanine	0.05	0.33	5.2	dark spores veg. cells	veg. cells cell lysis
Glycine	0.07	0.26	5.6	veg. cells	veg. cells cell lysis

Effect of Tween 80 and ethanol in CDM

The effect of Tween 80 and ethanol on sporulation of MSp^+ is shown in Table 6. With 0.1% Tween 80 in CDM, cell began to sporulate at about 4 h earlier but approximately the same percentage of sporulation (64%) was achieved as compared to control (CDM).

Lysis of the cells occurred in CDM containing ethanol by 28-32 h and the percentage sporulation was lower.

Growth of C. botulinum strains in CDM

Table 7 shows the sporulation (%) of C. botulinum spp. in CDM and TPGY medium. Ten strains of type E including MSp^+ were able to grow and sporulate in CDM. In general, the percentage of cells forming spores was lower in CDM than in TPGY medium, ranging from 60% for MSp^+ to under 10% for FDA 5191. Two strains, FDA PM-15 and FDA 070 formed tiny round refractile bodies, "defective" spores, in CDM.

Other serotypes of C. botulinum and C. sporogenes did not sporulate in CDM, however, normal vegetative growth was observed.

Table 6. Effect of Tween 80 and Ethanol on sporulation of strain MSp^+

Media	Sporulation (%)				
	16h	20h	24h	28h	32h
CDM + 0.01 % Tween 80	0	13	32	36	45
CDM + 0.05 % Tween 80	<5	18	47	52	(lysis)
CDM + 0.1 % Tween 80	20	34	63	(lysis)	-
CDM + 0.1 % ethanol	0	18	30	38	(lysis)
CDM + 0.5 % ethanol	0	19	30	(lysis)	-
CDM + 1.0 % ethanol	0	17	49	(lysis)	-
CDM	0	31	47	64	(lysis)
TPGY	0	35	68	88	93

Table 7. Sporulation of *Clostridium* spp. in CDM and TPGY media.

Clostridium spp.	Sporulation (%)		Time[a] (h)	
	CDM	TPGY	CDM	TPGY
C. botulinum type E strains :				
MSp[+]	60-65	90-95	28	32
ATCC 9564	35-40	60-65	28	32
FDC Minnesota	45-50	80-85	40	28
FDA D8	40-45	> 95	48	48
ATCC 17786	20-25	35-40	64	72
FDA Beluga	10-15	60-65	32	32
Ts-25	< 10	30-35	32	32
FDA 5191	< 10	70-75	36	42
FDA PM-15	30-35[b]	75-80	80	28
FDA 070	30-35[b]	65-70	80	32
C. botulinum strains:				
type A (AG 270)	-	45-50	-	120
type B (BG 272)	-	45-50	-	24
type C	-	10-15	-	120
type D (ATCC 17851)	-	55-60	-	24
type F (FG 273)	-	90-95	-	72
C. sporogenes (ATCC 19404)	-	15-20	-	120

[a] Time required to achieve maximum sporulation.

[b] 'defective' spores

ATCC = American Type Culture Collection
FDC = Food and Drug Directorate, Ottawa, Ontario, Canada
FDA = Food & Drug Administration, Washington, D.C., U.S.A.

PART II Biochemical studies in TPGY medium

Effect of rifampin on growth and sporulation of sporogenic strains

The amount of growth (expressed in O.D. value) and sporulation obtained after addition of rifampin are shown in Table 8. Rifampin was first added to the culture at 11 h when most of the spores of MSp^+ had germinated into young vegetative cells. The growth obtained after 24 h was markedly reduced in cultures which were treated with rifampin at 11-14 h and refractile spores were not observed. In cultures treated with rifampin at 15 h, many forespores were observed by 24 h. An increasing number of refractile endospores were observed at 24 h in cultures treated with rifampin after 15 h.

Similar results were obtained with ATCC 9564 except that growth and sporulation occurred 2h earlier and maximum sporulation was 59% compared with 86% by MSp^+.

Table 8. Effect of Rifampin on growth and sporulation of MSp$^+$ and ATCC 9564

Time[a](h)	O.D.[b]		Sporulation[b](%)	
	MSp$^+$	ATCC 9564	MSp$^+$	ATCC 9564
11	0.17	0.20	0	0
12	0.28	0.25	0	0
13	0.40	0.30	0	(forespores)
14	0.40	0.31	0	< 5
15	0.44	0.36	(forespores)	10
16	0.45	0.38	< 5	36
17	0.46	0.42	18	52
18	0.47	0.45	37	55
19	0.49	0.46	58	57
20	0.50	0.49	67	59
21	0.50	0.51	70	59
22	0.50	-	76	-
23	0.50	-	86	-

[a]Time of addition of rifampin (1.5 μg/ml) during incubation of cultures.

[b]After a total of 24 hours of incubation.

Incorporation of labeled compounds during the growth cycle of sporogenic strains of C. botulinum

The incorporation of labeled compounds during germination, growth and sporulation of heat-activated spores of MSp^+ is shown in Fig.6. During the lag period (0-4 h) heat-activated spores became non-refractile and germinated into vegetative cells. The incorporation of uracil and thymidine was rapid whereas that of methionine was very gradual. During logarithmic growth phase (4-14h), the labeled compounds were incorporated at a linear rate. The level of methionine incorporated into cells remained constant during the stationary phase while that of uracil and thymidine dropped and then remained relatively constant. At 16h (T_2), most of the cells were granular and swollen and by 18h about 10% had formed refractile endospores.

The incorporation of labeled thymidine and uracil by the parent strain, ATCC 9564, peaked some 5-6 hours earlier than MSp^+ as shown in Fig.7. The pattern in the incorporation of labeled compounds was similar to that of the MSp^+.

Incorporation of labeled compounds during sporulation

Fig.8 shows the incorporation of labeled uracil by MSp^+ during sporulation. When rifampin (1.5 μg/ml) was added at 14h (T_0), the level of radioactivity in the TCA-precipitate remained low during the entire stationary phase and most of the cells in the culture remained granular at 24h (T_{10}). In the absence of rifampin, cells continued

to incorporate ^{14}C-uracil up to T_2. During maturation of spores (T_4-T_{10}) as the percentage of refractile endospores increased from less than 5% to over 70%, the level of labeled uracil in culture remained constant. When rifampin was added at T_3 (17 h), uracil incorporation was comparable to the control (i.e. without adition of rifampin). However, only 20% of the cells formed endospores when observed at 24 h (T_{10}).

When chloramphenicol (50 μg/ml) was added at T_0 (14 h), the level of ^{14}C-uracil incorporated into the culture was unexpectedly higher. O.D. of the culture started to drop at T_5 and cell lysis was observed. At T_{10}, less than 10% of the cells were in the forespore or non-refractile endospore stages.

Similar results in incorporation of ^{14}C-uracil were obtained with the parent strain ATCC 9564 as shown in Fig. 9.

^{3}H-thymidine in MSp^{+} culture was not incorporated during stationary phase (Fig. 10). On the other hand, ^{14}C-methionine was gradually incorporated into the cells (Fig. 11). Less than 10% and over 70% refractile endospores were observed at T_4 (18 h) and T_{10} (24 h) respectively.

Fig. 6 Incorporation of radioactive precursors during germination and outgrowth of MSp^+ spores.

^3H-thymidine (25 μCi/15ml), ^{14}C-uracil (15 μCi/15ml) or ^{14}C-methionine (15 μCi/15ml) was added to TPGY medium inoculated with heat-activated spores. Samples were withdrawn at intervals and assayed for radioactivity.

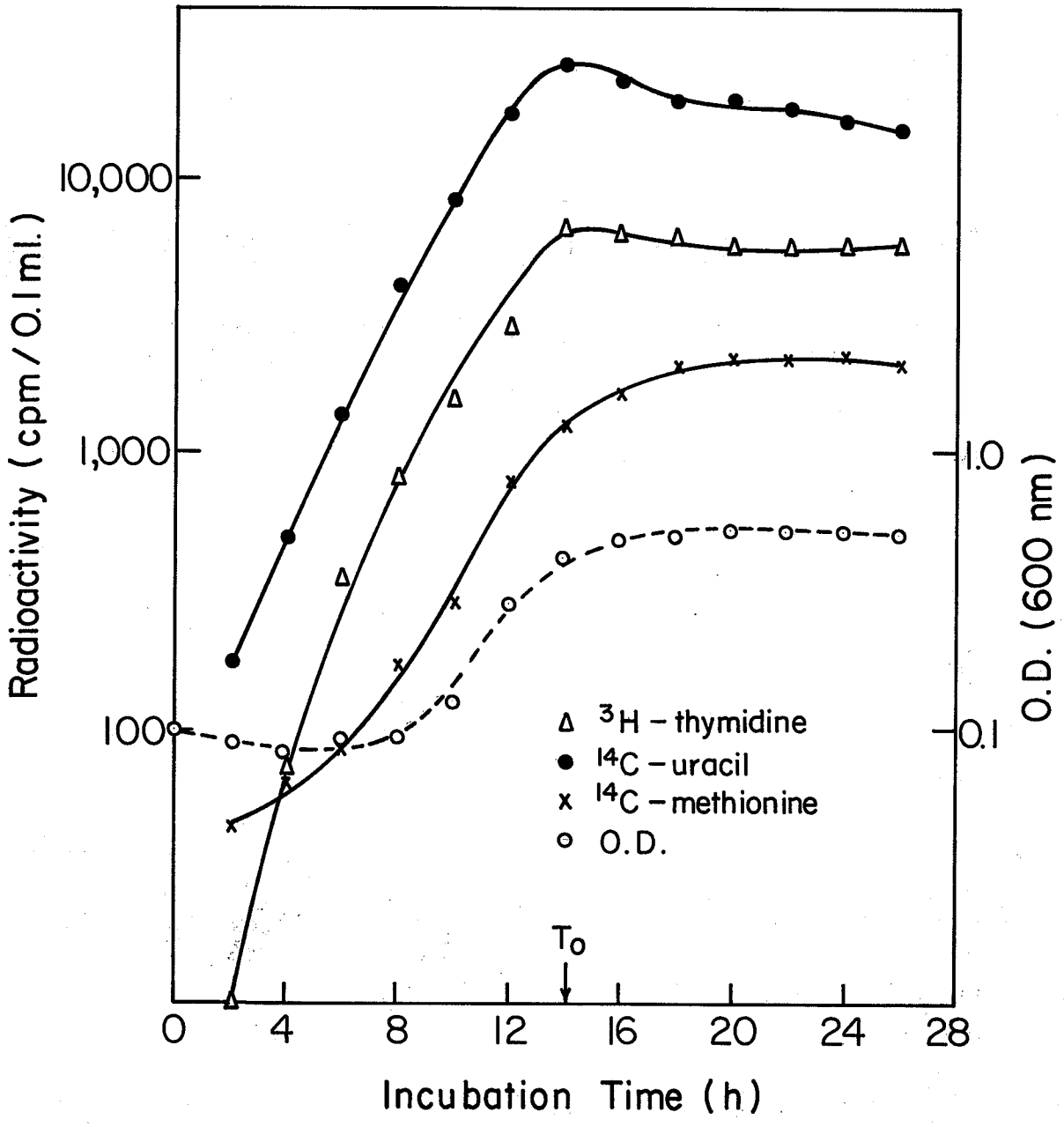

10,000
1,000
100
Radioactivity (cpm / 0.1 ml.)
1.0
0.1
O.D. (600 nm)
Δ ³H – thymidine
● ¹⁴C – uracil
x ¹⁴C – methionine
o O.D.
T₀
0
4
8
12
16
20
24
28
Incubation Time (h)

Fig. 7 Incorporation of radioactive precursors during germination and outgrowth of ATCC 9564 spores.

^{3}H-thymidine (25 μCi/15ml), ^{14}C-uracil (15 μCi /15ml) or ^{14}C-methionine (15 μCi/15ml) was added to TPGY medium inoculated with heat-activated spores. Samples were withdrawn at intervals and assayed for radioactivity.

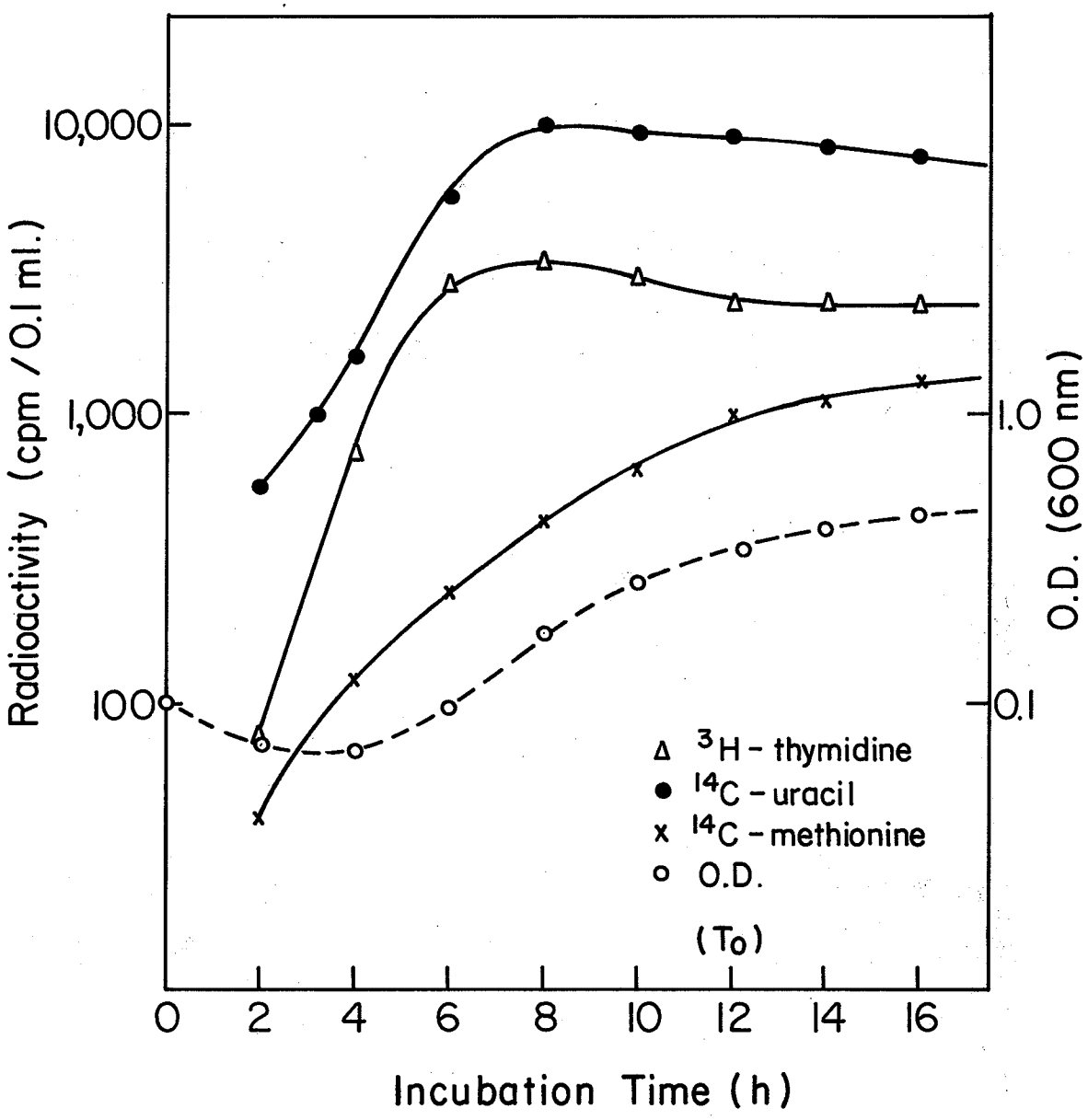

10,000
1,000
100
Radioactivity (cpm / 0.1 ml.)
1.0
0.1
O.D. (600 nm)
Δ ³H - thymidine
● ¹⁴C - uracil
x ¹⁴C - methionine
o O.D.
(T₀)
0 2 4 6 8 10 12 14 16
Incubation Time (h)

Fig. 8 ^{14}C-uracil incorporation in MSp^{+} during sporulation.

^{14}C-uracil (15 μCi/15ml) was added to culture in TPGY medium at the end of log phase. Cell suspensions were sampled at intervals and radioactivity of the TCA-precipitate was counted.

conc. of chloramphenicol (CM) = 50 μg/ml

conc. of rifampin = 1.5 μg/ml

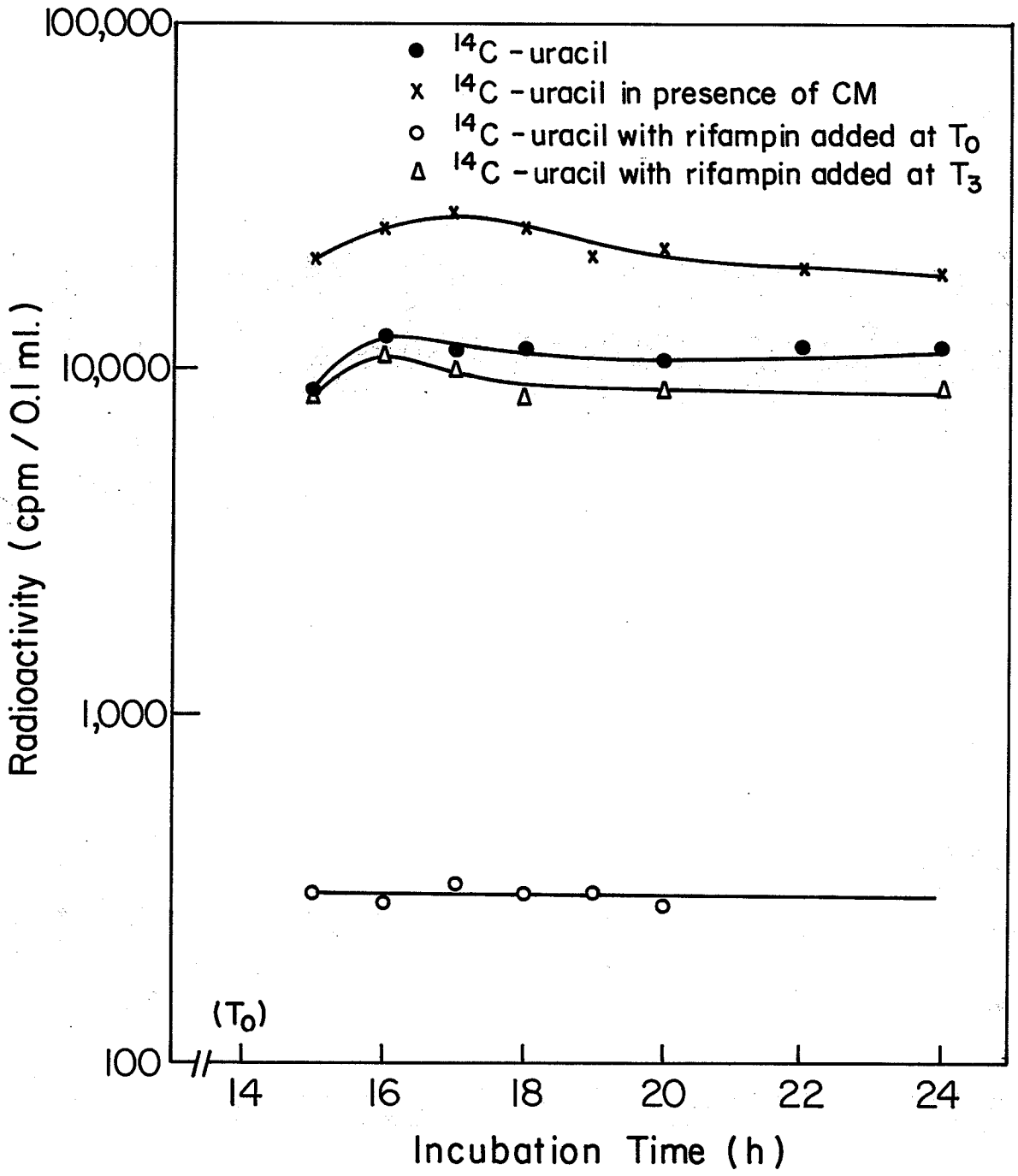

100,000
10,000
1,000
100
Radioactivity (cpm / 0.1 ml.)
● ^{14}C - uracil
x ^{14}C - uracil in presence of CM
o ^{14}C - uracil with rifampin added at T_0
Δ ^{14}C - uracil with rifampin added at T_3
(T_0)
14
16
18
20
22
24
Incubation Time (h)

Fig. 9 ^{14}C-uracil incorporation in ATCC 9564 during sporulation.

^{14}C-uracil (15 μCi/15ml) was added to culture in TPGY medium at the end of log phase. Cell suspensions were sampled at intervals and radioactivity of the TCA-precipitate was counted.

conc. of chloramphenicol (CM) = 50 μg/ml

conc. of rifampin = 1.5 μg/ml

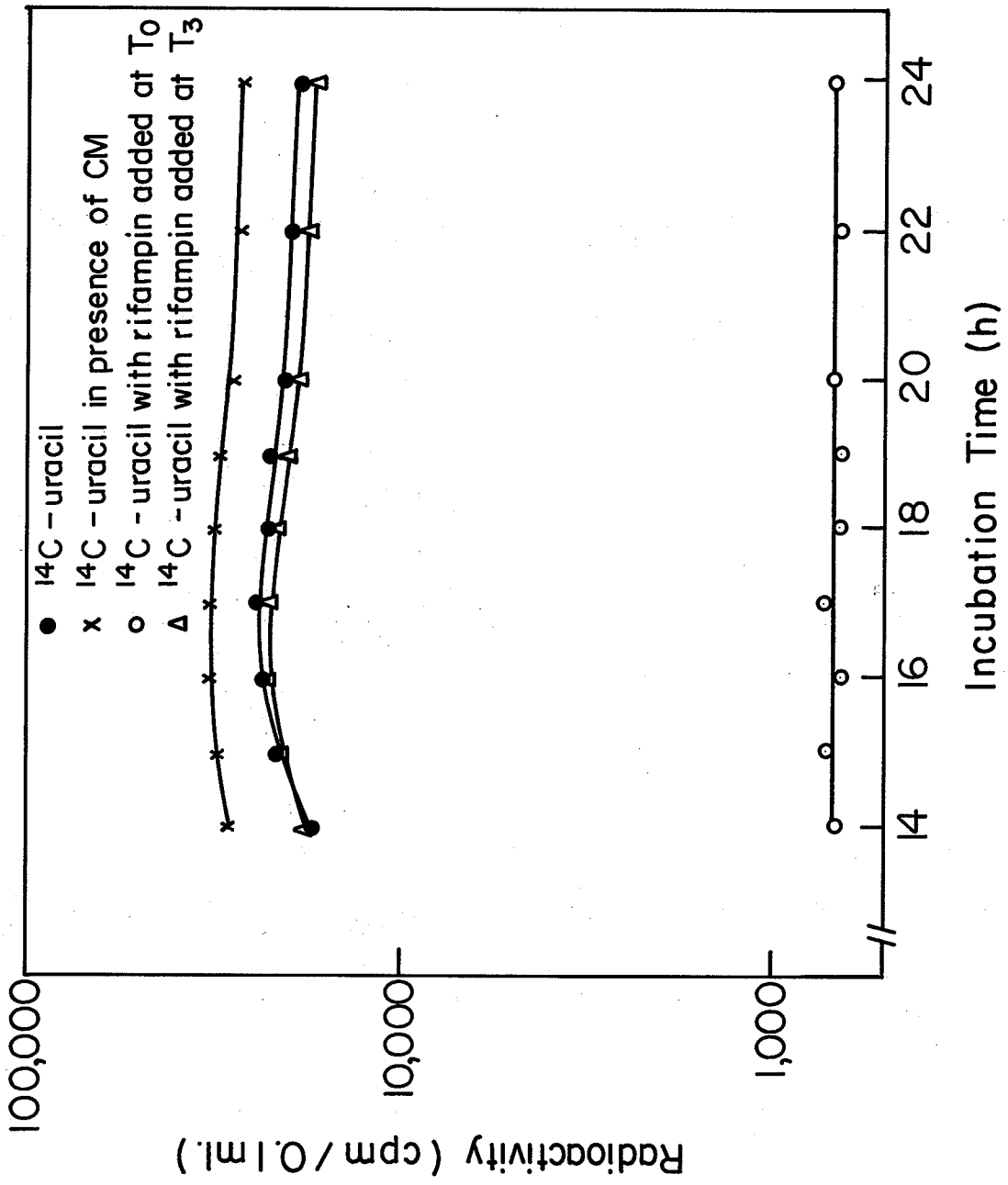

● ^{14}C – uracil
x ^{14}C – uracil in presence of CM
o ^{14}C – uracil with rifampin added at T_0
Δ ^{14}C – uracil with rifampin added at T_3
100,000
10,000
1,000
Radioactivity (cpm / 0.1 ml.)
14
16
18
20
22
24
Incubation Time (h)

Fig. 10 ^{3}H-thymidine incorporation by MSp^{+} during sporulation.

^{3}H-thymidine (25 μCi/15ml) was added to the culture in TPGY medium at the end of log phase. Cell suspensions were sampled at intervals and radioactivity of the TCA-precipitate was counted.

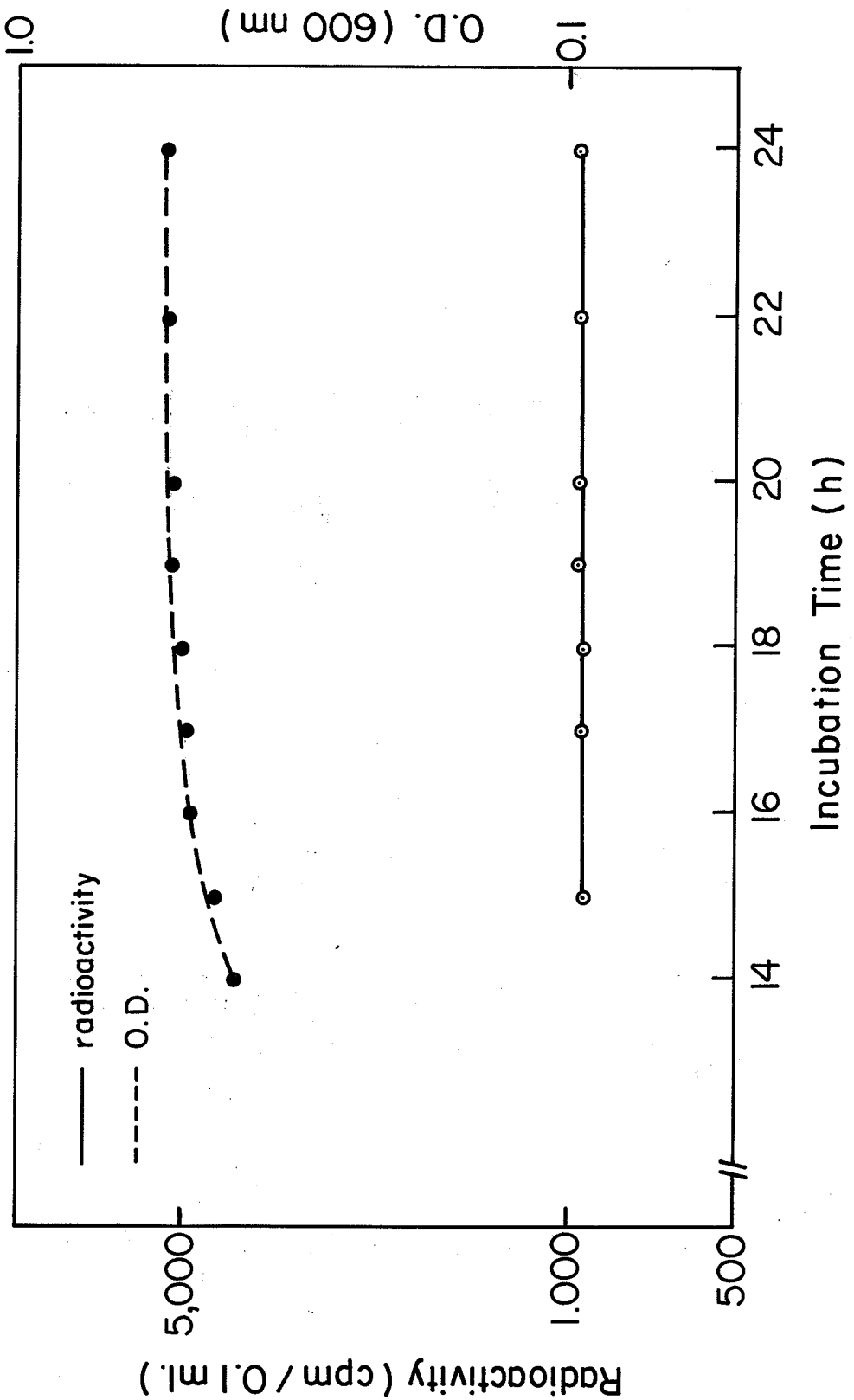
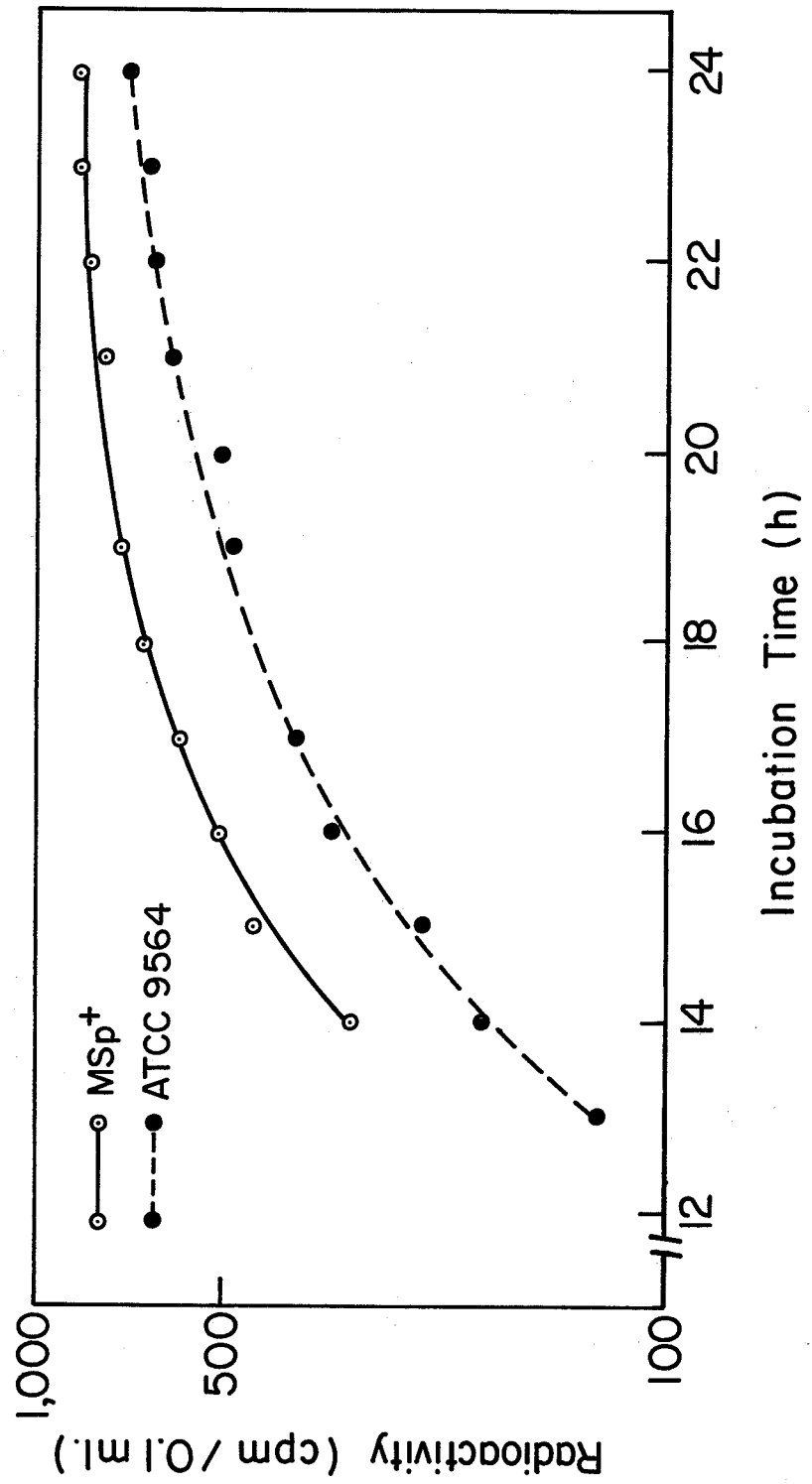

Radioactivity (cpm / 0.1 ml.)
5,000
1,000
500
14
16
18
20
22
24
Incubation Time (h)
1.0
0.1
O.D. (600 nm)
radioactivity
O.D.

Fig. 11 ^{14}C-methionine incorporation by MSp^{+} and ATCC 9564 during sporulation.

^{14}C-methionine (15 μCi/15ml) was added to culture in TPGY medium at the end of log phase. Cell suspensions were sampled at intervals and radioactivity of the TCA-precipitates was counted.


Radioactivity (cpm / 0.1 ml.)
1,000
500
100
12
14
16
18
20
22
24
Incubation Time (h)
MSp+
ATCC 9564

Incorporation of labeled compounds during the growth cycle of an asporogenic mutant

There was a lag of six hours after inoculation of stationary phase cells of RSpoIIIa into fresh TPGY medium (Fig. 12). Growth began after that and during the logarithmic growth phase (6-18 h) incorporation of the labeled thymidine, uracil and methionine by cells was rapid. During the stationary phase (18 - 28h), the level of labeled thymidine in culture showed a slight decline. Vegetative cells appeared granular at the end of log phase and lysis was observed at 24 h.

Comparison of ^{14}C-methionine incorporation between sporogenic and asporogenic mutants with the parent strain

Result in Fig. 13 shows that the asporogenic mutant, (RSpoIIIa), in contrast to the sporogenic mutant (MSp^{+}) and the parent strain (ATCC 9564), did not show incorporation of ^{14}C-methionine after T_3.

Fig. 12 Incorporation of radioactive precursors by RSpoIIIa during growth and stationary phase.

^{3}H-thymidine (25 μCi/15ml), ^{14}C-uracil (15 μCi/15ml) and ^{14}C-methionine (15 μCi /15ml) were added separately to TPGY broth inoculated with stationary phase cells of RSpoIIIa.

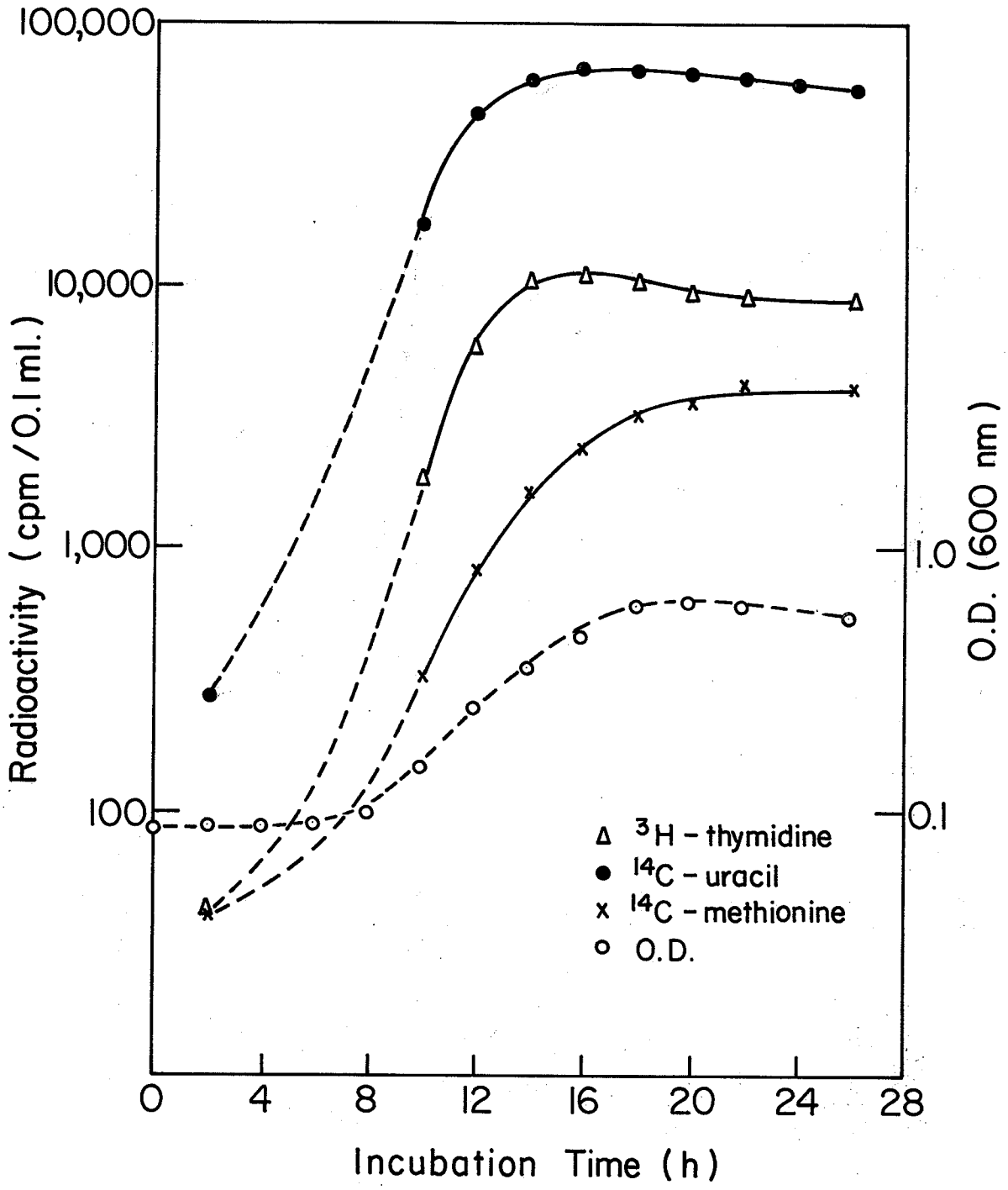
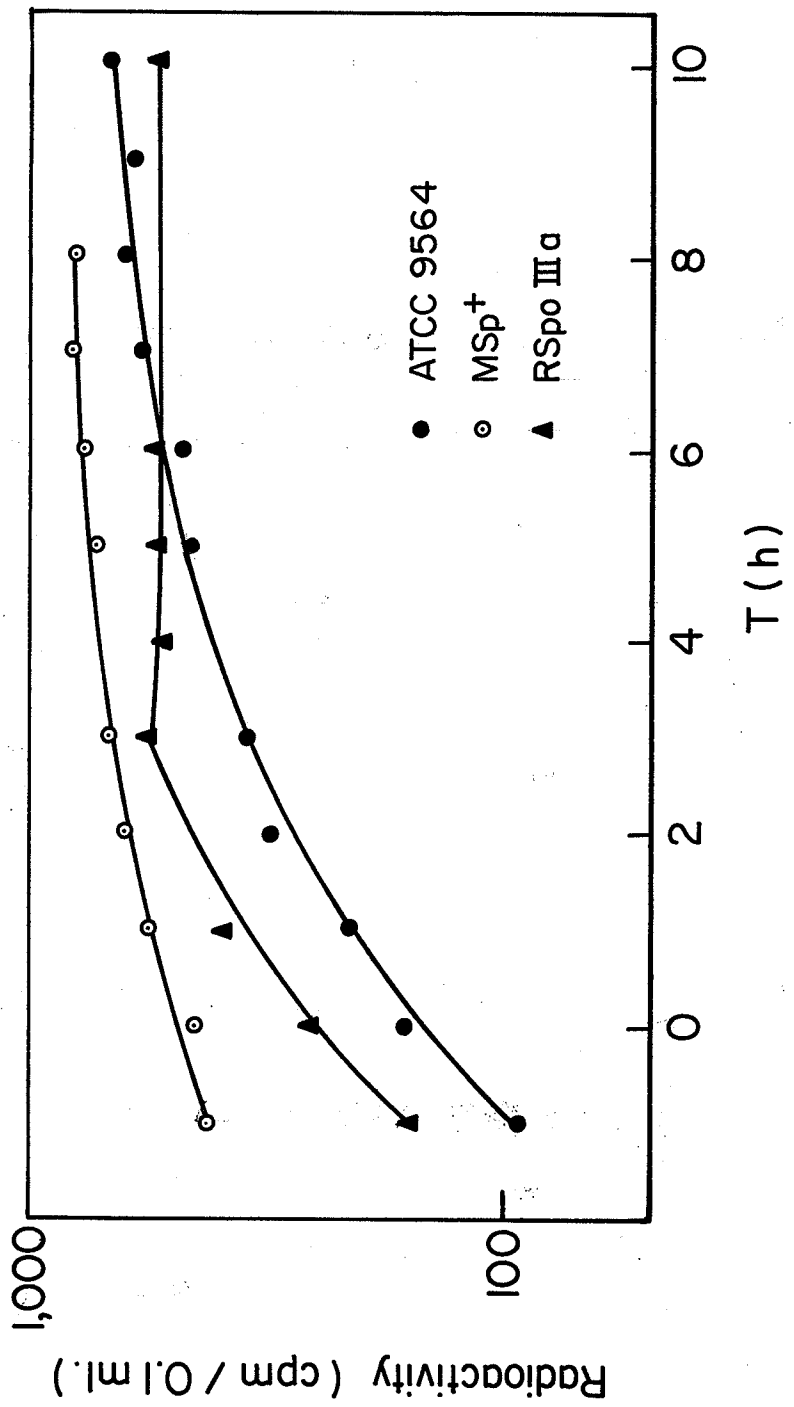

100,000
10,000
1,000
100
Radioactivity (cpm / 0.1 ml.)
1.0
0.1
O.D. (600 nm)
0
4
8
12
16
20
24
28
Incubation Time (h)
Δ ³H – thymidine
● ¹⁴C – uracil
x ¹⁴C – methionine
o O.D.

Fig. 13. Comparison of ^{14}C-methionine incorporation by the test strains during stationary phase.

^{14}C-methionine (15 μCi/15ml) was added at about an hour before the end of logarithmic growth phase (T_0). T represents the time after the end of logarithmic growth phase.


Radioactivity (cpm / 0.1 ml.)
1,000
100
0
2
4
6
8
10
T (h)
ATCC 9564
MSp+
RSpo III a

Growth and sporulation of temperature-sensitive mutant, ts-25 and the parent strain (Beluga)

Growth and sporulation of ts-25 and Beluga at 28°C and at 37°C are shown in Fig. 14 and 15. In culture of ts-25 grown at 28°C for 27 h (T_5), approximately 5% refractile endospores were observed and greater than 30% was observed after incubation for 35 h. Spores were not formed by ts-25 culture grown at 37°C and cells appeared granulated at 22 h (T_0) and lysis began at 27 h (T_5). The Beluga strain formed greater than 70% refractile endospores at 28°C and at 37°C after incubation for 40 h (T_{18}).

Temperature-sensitive period of ts-25

In the shift "up" experiment, spores were not observed if cultures were transferred to 37°C on or before 22 h (T_0) whereas in cultures transferred after 22 h sporulation was the same as at 28°C (Fig. 16a). In the shift "down" experiment, spores were produced in cultures transferred on or before 12 h (Fig. 16b).

Temperature dependence of ^{3}H-thymidine incorporation during growth of ts-25

There was no significant difference in the incorporation of ^{3}H-thymidine at 28°C and at 37°C during logarithmic growth phase (Fig. 17a). By changing incubation temperature to 37°C betwen 17 h and 19 h did not facilitate further incorporation of ^{3}H-thymidine (Fig. 17b).

Fig. 14 Growth and Morphological changes of ts-25 in TPGY medium at 28 and 37°C.

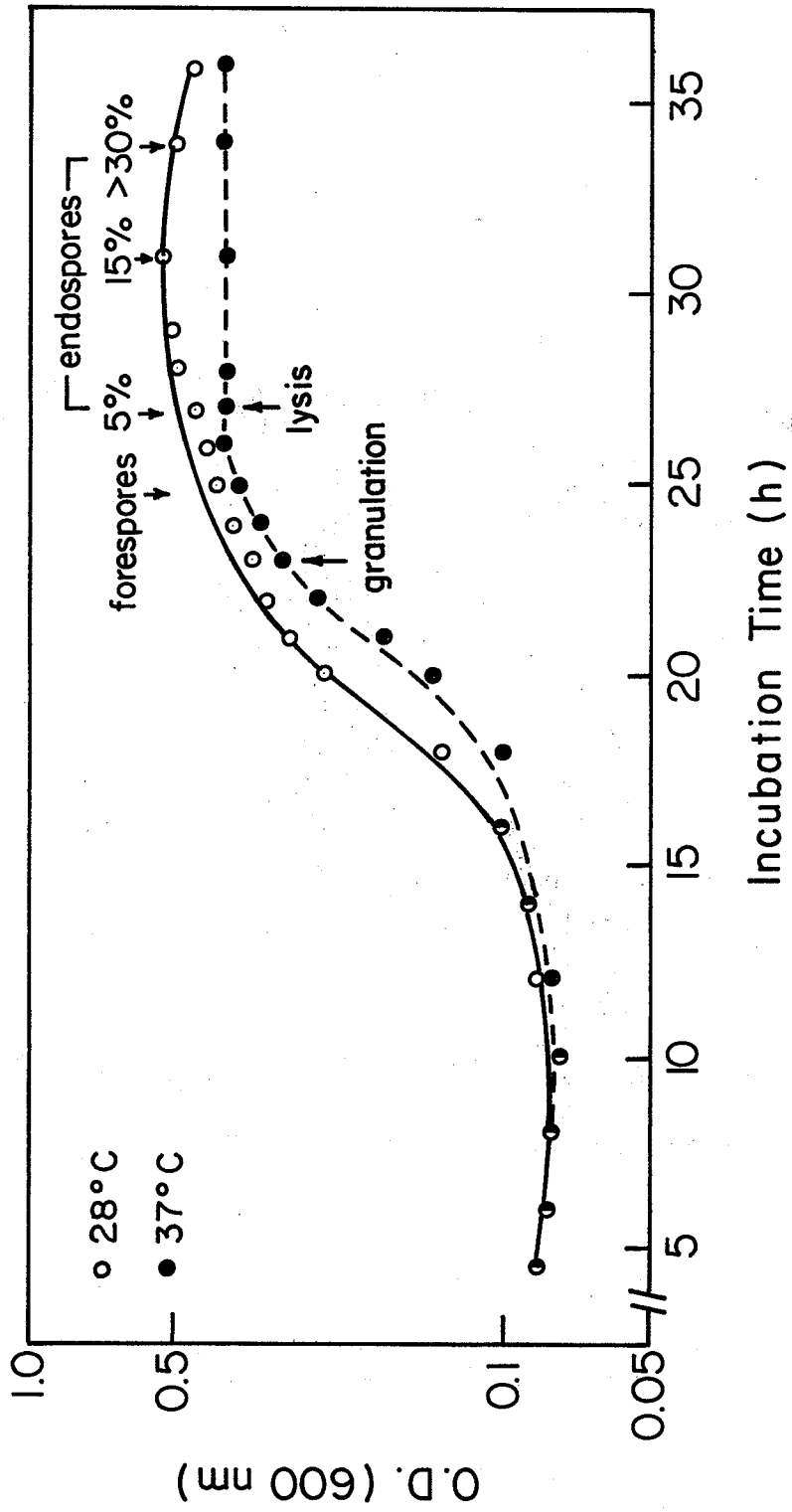

endospores
forespores 5%
15% >30%
lysis
granulation
o 28°C
• 37°C
1.0
0.5
0.1
0.05
5
10
15
20
25
30
35
O.D. (600 nm)
Incubation Time (h)

Fig. 15 Growth and Morphological changes of strain Beluga in TPGY medium at 28 and 37°C.

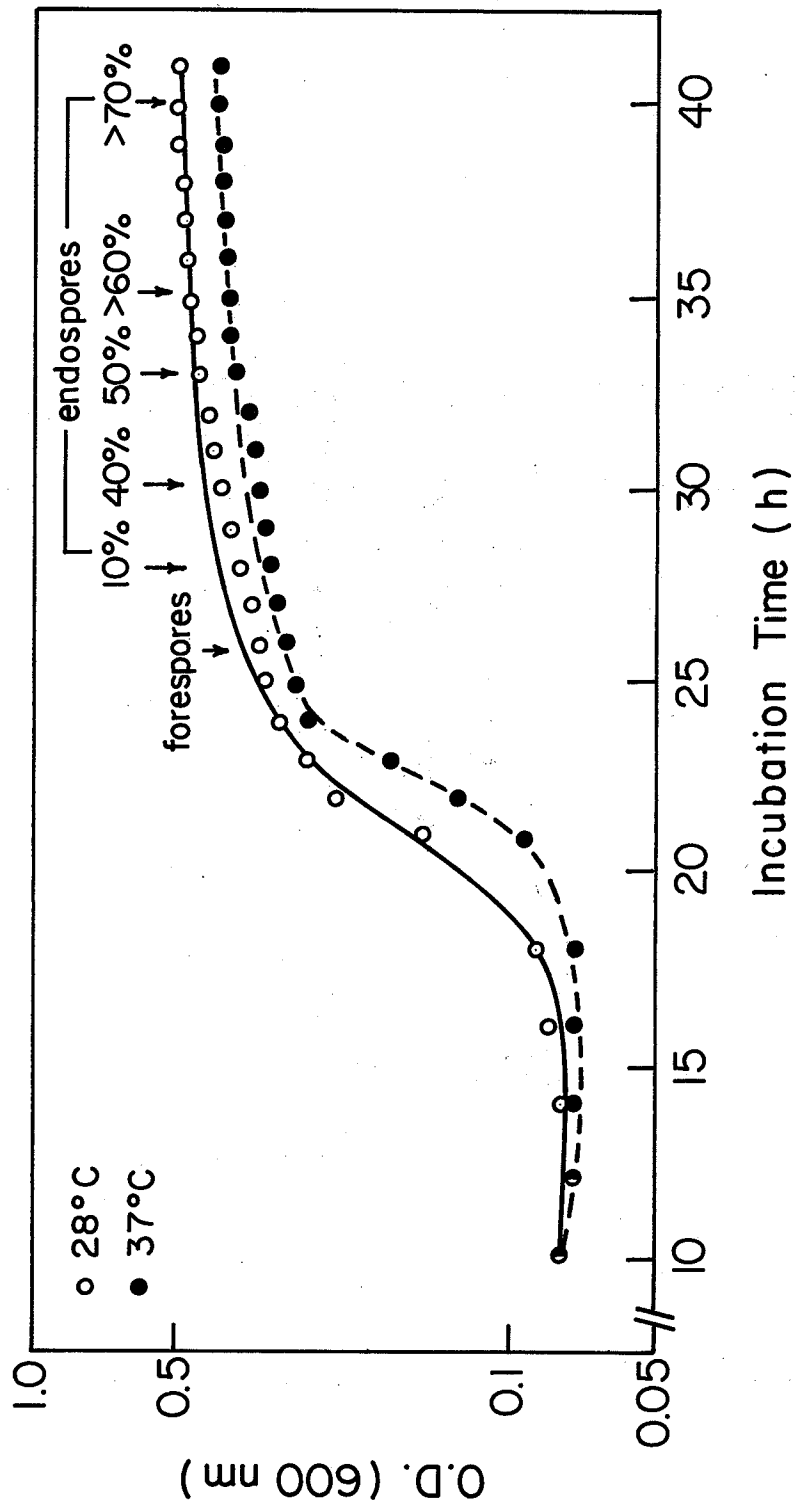
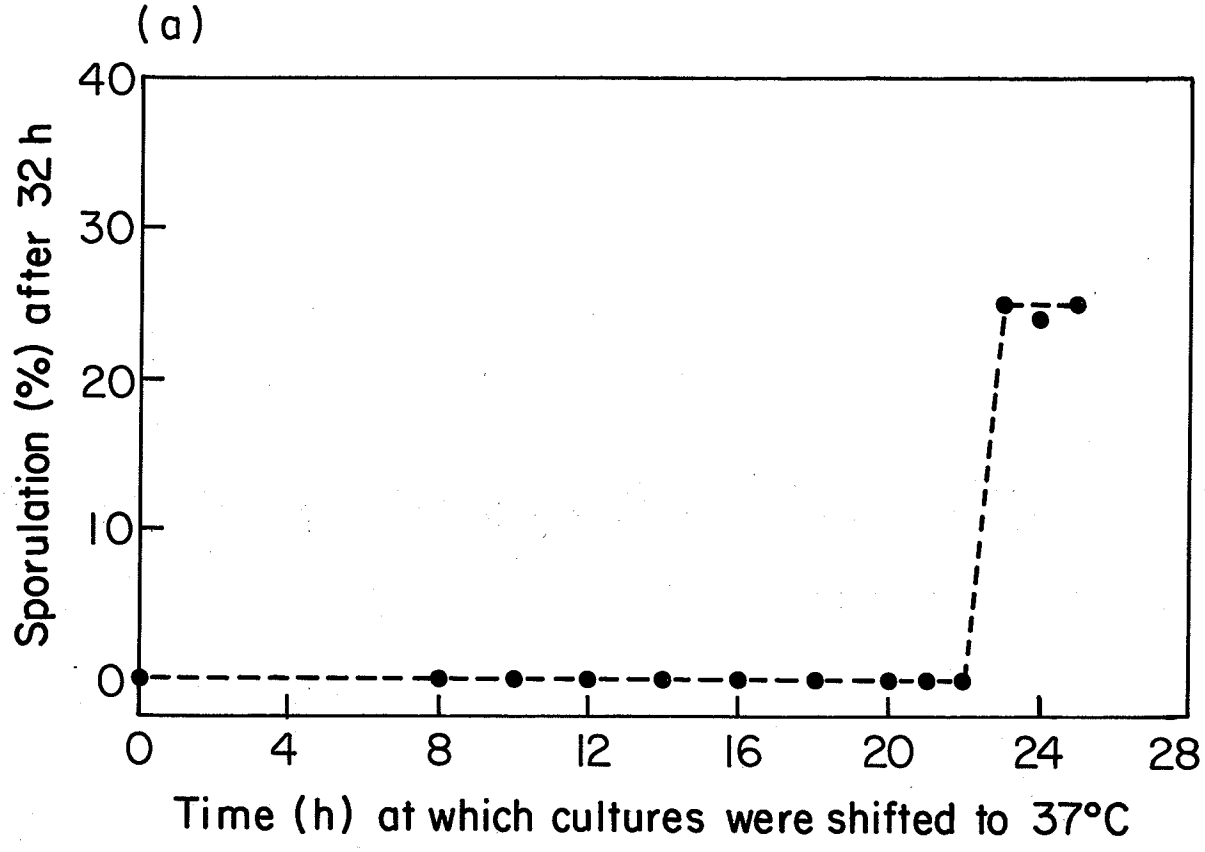

o 28°C
● 37°C
endospores
10% 40% 50% >60% >70%
forespores
1.0
0.5
0.1
0.05
O.D. (600 nm)
10
15
20
25
30
35
40
Incubation Time (h)

Fig.16 Temperature sensitivity of sporulation of ts-25.

(a) In the "shift up" experiment, cultures were grown at 28°C for varying periods of time and then shifted to 37°C for a total incubation period of 32 h.

(b) In the "shift down" experiment, cultures were grown at 37°C for varying periods of time and then shifted to 28°C for a total incubation period of 32 h.


(a)
Sporulation (%) after 32 h
40
30
20
10
0
0
4
8
12
16
20
24
28
Time (h) at which cultures were shifted to 37°C

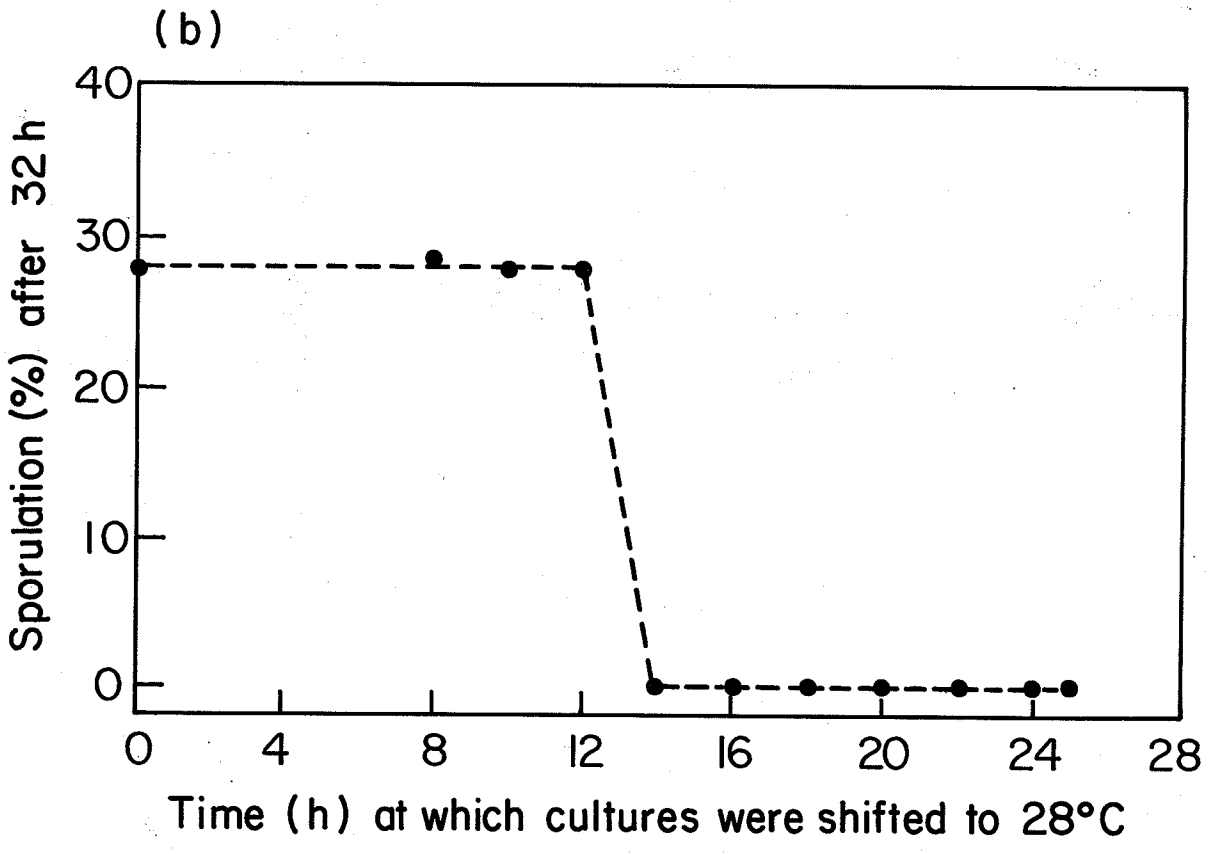
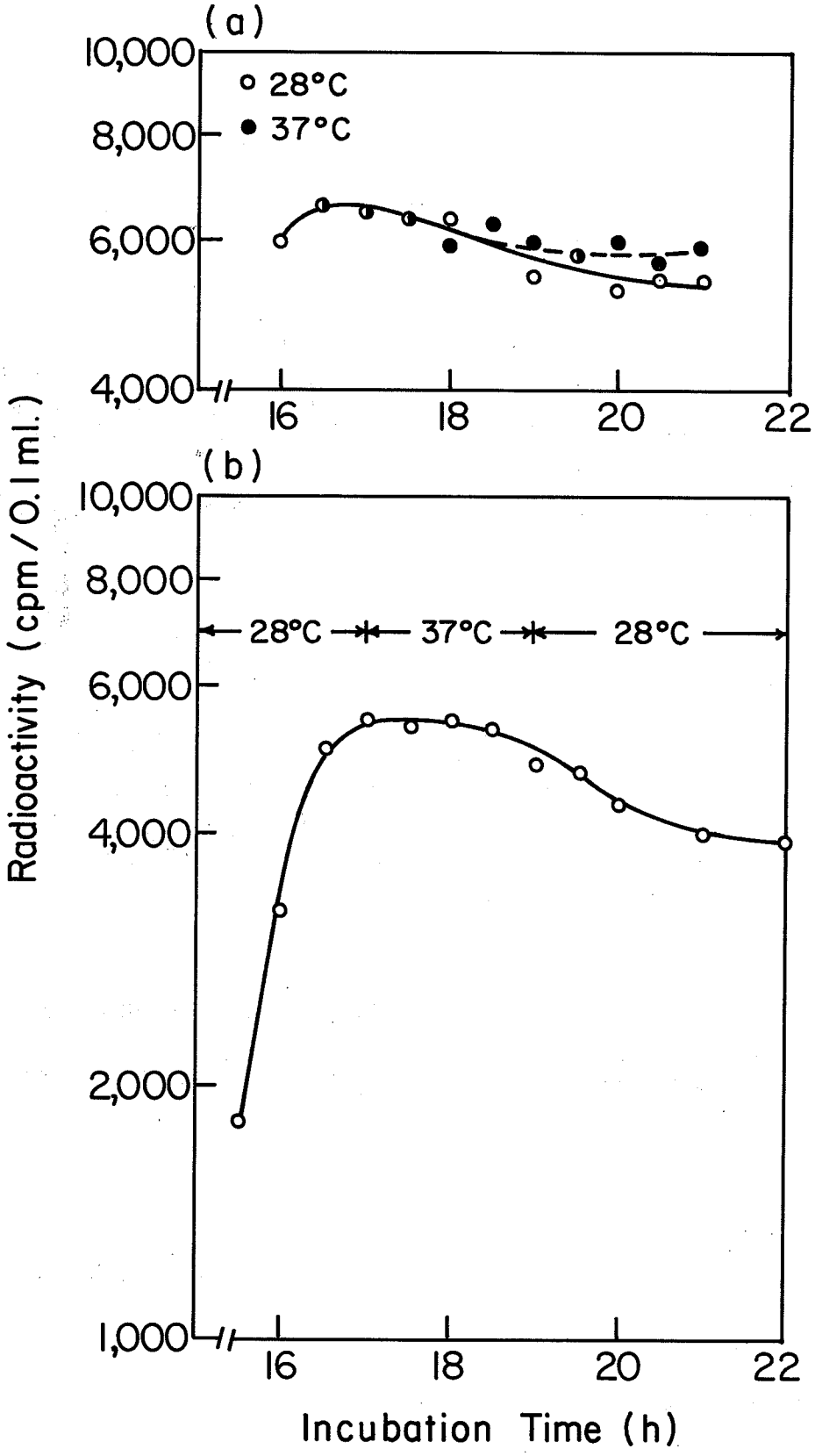

(b)
Sporulation (%) after 32 h
40
30
20
10
0
0
4
8
12
16
20
24
28
Time (h) at which cultures were shifted to 28°C

Fig.17 Temperature dependence of ^{3}H-thymidine incorporation during growth of ts-25.

(a) ts-25 was grown in 30 ml ^{3}H-thymidine-TPGY medium (50 μCi/30ml) at 28°C. At 16 h, the culture was divided into two equal portions, one was incubated at 28°C and the other at 37°C. Samples were withdrawn for radioactivity measurement.

(b) ts-25 was grown in 15 ml TPGY at 28°C. At 15 h, ^{3}H-thymidine (25 μCi/15ml) was added to the culture and incubation was continued for 2 h at 28°C, 2 h at 37°C and then 5 h at 28°C again. Samples were assayed for radioactivity.


(a)
10,000
8,000
6,000
4,000
○ 28°C
● 37°C
16
18
20
22
(b)
10,000
8,000
6,000
4,000
2,000
1,000
28°C
37°C
28°C
16
18
20
22
Radioactivity (cpm / 0.1 ml.)
Incubation Time (h)

Incorporation of labeled compounds by a temperature-sensitive mutant and its parent strain during their growth cycles

The incorporation of ^{14}C-uracil and ^{14}C-methionine by ts-25 at 28°C and at 37°C is shown in Fig.18. An inverse relationship was observed for the incorporation of methionine and uracil at both temperatures. At 37°C, the incorporation of methionine was higher whereas that of uracil was lower. During stationary phase (22 h onward) incorporation of methionine continued at 28°C but stopped at 37°C. The level of ^{14}C-uracil in culture at 28°C remained constant while that of the culture at 37°C dropped.

Incorporation of radioactive compounds by the Beluga strain during the stationary phase was similar at 28°C and at 37°C except for uracil which showed a higher rate of incorporation at 28°C than 37°C (Fig.19).

Incorporation of ^{14}C-methionine in CDM by sporogenic and asporogenic strains of C. botulinum

An increase in incorporation of ^{14}C-methionine resulted when MSp^{+}, ATCC 9564 and RSpoIIIa were incubated in CDM instead of TPGY medium (Fig. 20).

Fig. 18 Incorporation of radioactive precursors by ts-25 at 28°C and at 37°C.

Ts-25 cultures in TPGY (30ml) were incubated at 28°C or at 37°C. At early log phase (17h) each culture was divided into 2 parts, ^{14}C-methionine (15 μCi/15ml) was added to one and ^{14}C-uracil (15 μCi/15ml) was added to the other. Radioactivity was assayed at intervals.

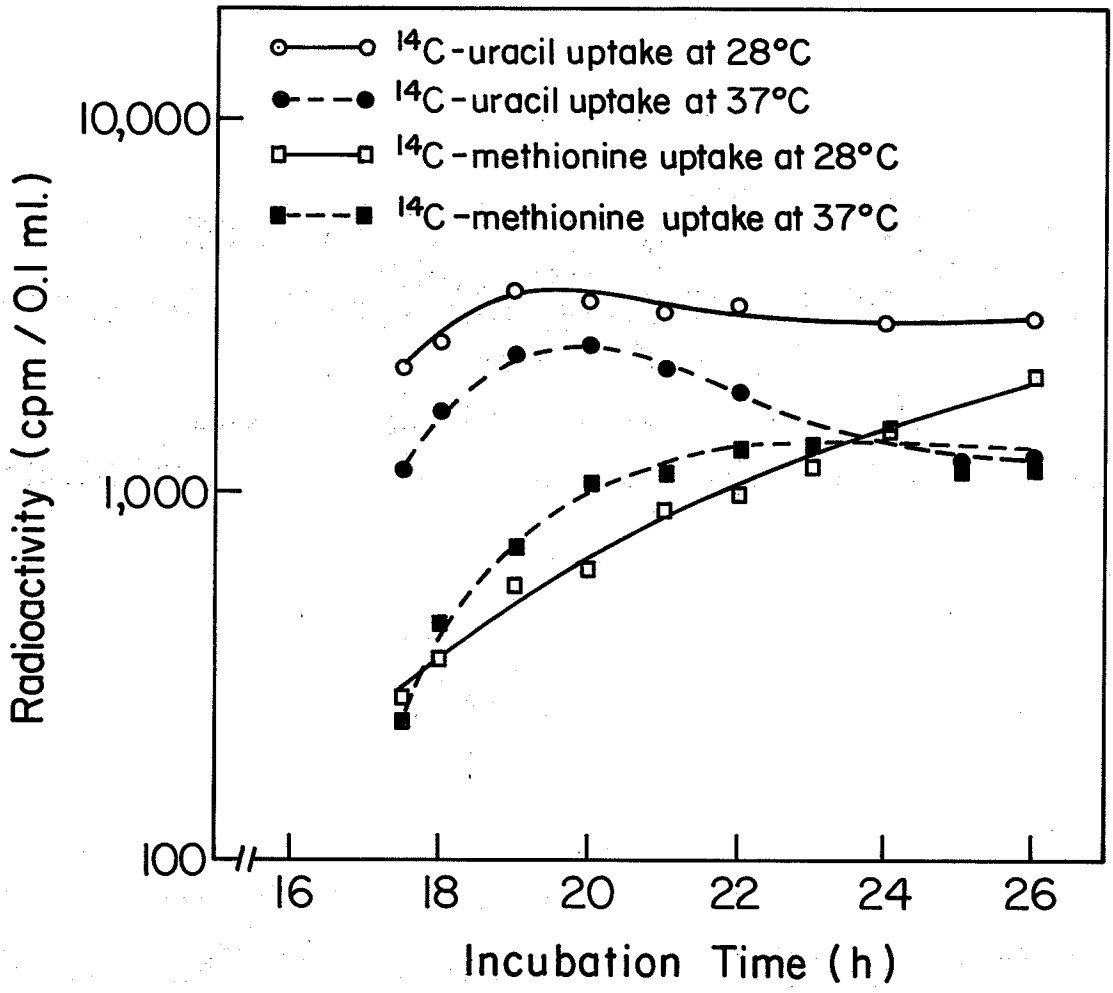

^{14}C-uracil uptake at 28°C
^{14}C-uracil uptake at 37°C
^{14}C-methionine uptake at 28°C
^{14}C-methionine uptake at 37°C
10,000
1,000
100
Radioactivity (cpm / 0.1 ml.)
16
18
20
22
24
26
Incubation Time (h)

Fig. 19 Incorporation of radioactive precursors by strain Beluga during stationary phase at 28 and 37°C.

^{3}H-thymidine (25 μCi/15ml), ^{14}C-uracil (15 μCi/15ml) and ^{14}C-methionine (15 μCi /15ml) were added to cultures in TPGY medium near the end of log phase.

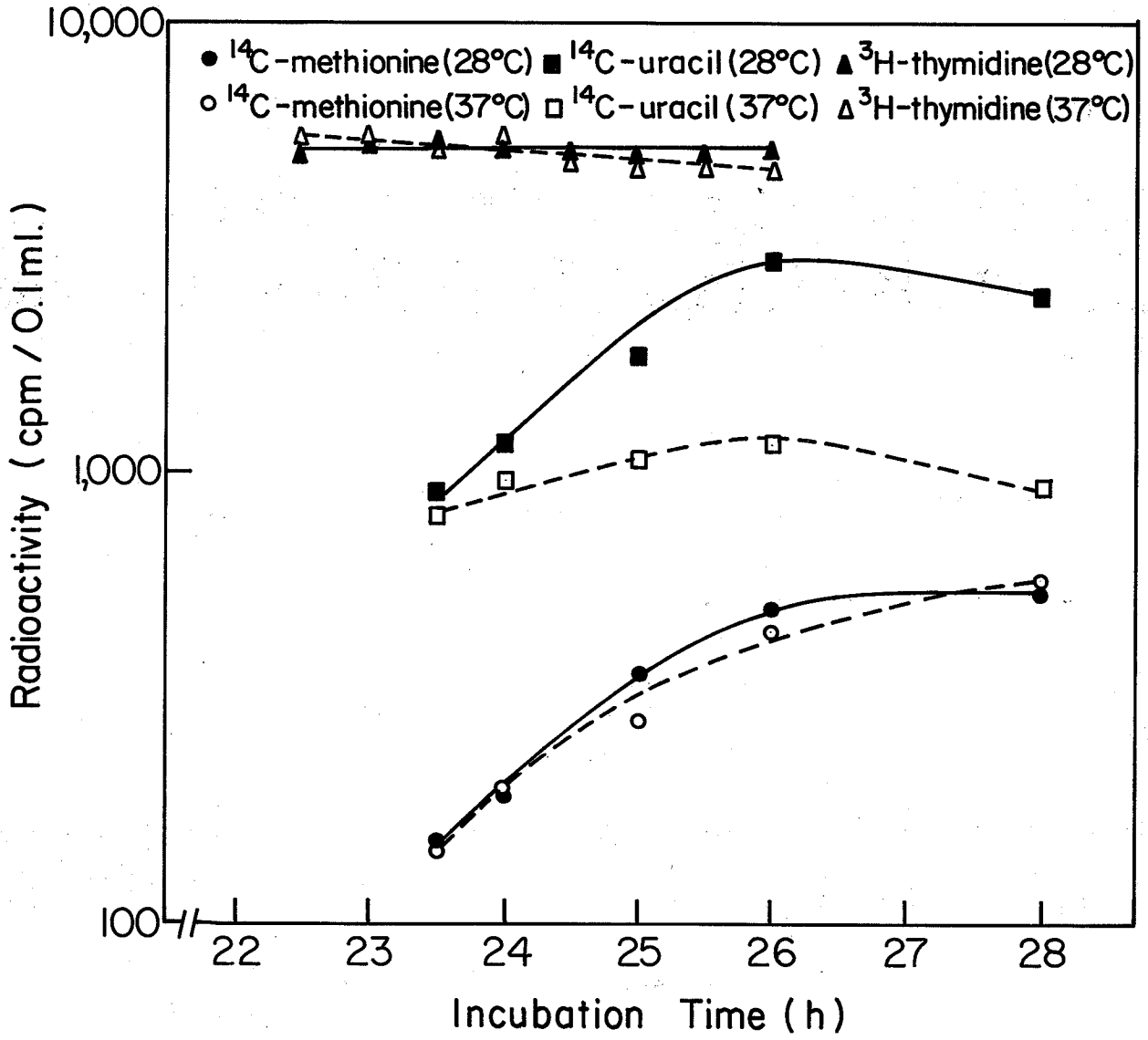
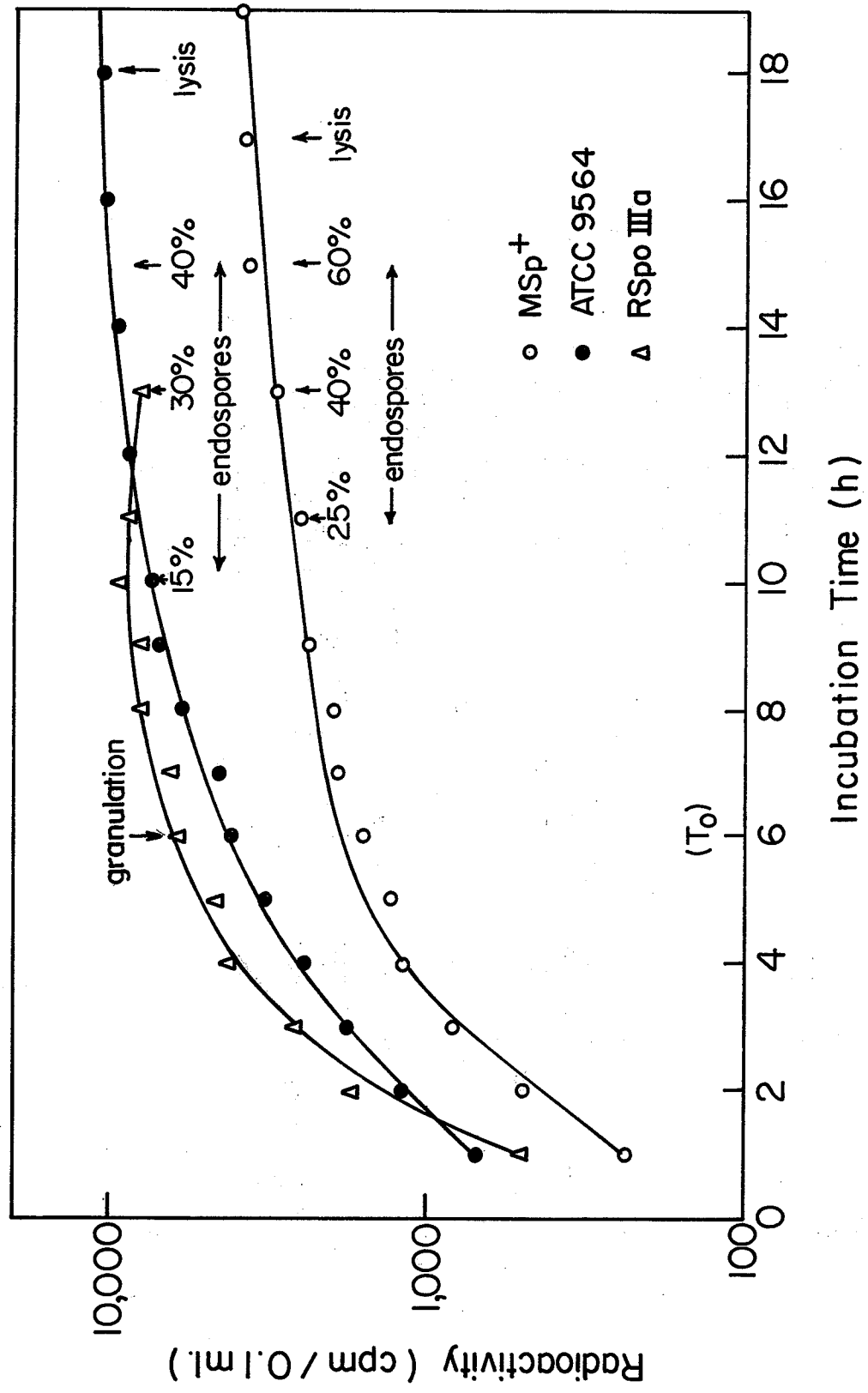

10,000
1,000
100
Radioactivity (cpm / 0.1 ml.)
● ^{14}C-methionine(28°C) ■ ^{14}C-uracil(28°C) ▲ ^{3}H-thymidine(28°C)
○ ^{14}C-methionine(37°C) □ ^{14}C-uracil(37°C) △ ^{3}H-thymidine(37°C)
22
23
24
25
26
27
28
Incubation Time (h)

Fig. 20 Incorporation of ^{14}C-methionine during growth and sporulation in CDM.

Spores or cells were grown up in sealed tubes containing TPGY medium to achieve an O.D. of 0.2. The cultures were centrifuged and TPGY was replaced by CDM containing ^{14}C-methionine (15 μCi/15ml). O.D. and radioactivity in cultures were measured at hourly intervals.


Radioactivity (cpm / 0.1 ml.)
10,000
1,000
100
0
2
4
6
8
10
12
14
16
18
(T0)
Incubation Time (h)
granulation
15%
30%
40%
lysis
endospores
25%
40%
60%
lysis
endospores
MSp+
ATCC 9564
RSpo IIIa

D I S C U S S I O N

DISCUSSION

The basal medium originally designed for growth and sporulation of the Minnesota strain did not allow germination of MSp^+ spores. The addition of casaminoacids and/or yeast extract indicated that an increase in the amount of the amino acids and vitamin factors was required to allow spore germination and subsequent growth and sporulation to take place. This modified basal medium was augmented with sodium bicarbonate (0.1%), purines, pyrimidines, sodium acetate (1.0%) and sodium thioglycollate (0.2%). The pre-reduced chemically defined medium, CDM, supported germination, outgrowth, growth and sporulation of type E strains of Clostridium botulinum.

Studies have shown that sodium bicarbonate enhanced the rate and extent of germination and outgrowth of botulinal spores (1, 97, 121, 133). In the complex medium supplemented with $NaHCO_3$, germination and outgrowth of the MSp^+ spores occurred earlier and when the $NaHCO_3$ was omitted from the CDM, the lag period for germination was significantly prolonged. It is likely that the $NaHCO_3$ dissociates in the medium to release CO_2 which is required for germination and outgrowth (121, 132). It has been suggested that $NaHCO_3$ may function as a

chelating agent to enhance spore germination (91), however, the growth-initiating effect of CO_2 on clostridia is not yet understood.

Purines and pyrimidines have been shown to be stimulatory for the growth of C. perfringens (14, 45) and C. parabotulinum type A (76) but not for C. botulinum type E strain D8 (128). Our results indicated that growth of MSp^+ was somewhat increased when purines and pyrimidines were included in the initial CDM.

In this study the addition of Na-acetate to the initial CDM increased the percentage sporulation of MSp^+. Previously it was found that acetate was involved in sporogenesis of Bacillus cereus (83) and C. botulinum (22, 30). Frank et al (41) found that Na-acetate along with proline and histidine was a stimulant for sporulation of the anaerobic strain PA3679. Emeruwa and Hawirko (30) reported that ^{14}C-acetate was readily incorporated into poly-β-hydroxybutyrate (PHB) granules of both sporogenic and asporogenic mutants (MSp^+ and RSpoIIIa) of C. botulinum type E and they also observed that most of the PHB granules which had accumulated in the sporogenic mutant was catabolized during spore development whereas in the asporogenic mutant it remained essentially unchanged.

The pre-reduced state of the CDM was maintained by sodium thioglycollate, a redox potential reducing

agent. The chief value of this reductant is its use in fluid cultures of anaerobes which are cultivated without the need for other precautions. Na-thioglycollate was routinely added to TPGY medium for the cultivation of C. botulinum strains in our laboratory. Besides functioning as a reducing agent on the medium, Na-thioglycollate has been reported to function as chelating agent to stimulate germination of clostridial spores (55, 97, 123). However, germination of C. botulinum 62A (121) and C. bifermentans (48) spores in hydrolysates of casein was found to be inhibited by addition of Na-thioglycollate.

Glucose was essential as its omission, for the most part, prevented outgrowth and spore formation of MSp^+ in CDM. The stimulatory effect of glucose on growth was shown to be concentration-dependent with an upper limit of 1%. On the other hand spore formation was repressed as the glucose concentration of the CDM was increased to 1.0 - 1.5%. This finding is in accordance with that of Emeruwa and Hawirko (33, 53) using tryptic-case-peptone glucose broth. Catabolite repression of sporogenesis by glucose has been reported to occur in B. subtilis (100) and other Clostridium spp. The amount of glucose in the medium which was required to repress sporulation varied with different types of clostridial species. In the proteolytic strain sporulation was repressed by glucose concentration as

low as 0.2 - 0.5% (47, 69, 79, 139). In type E strains which were weakly saccharolytic, the concentration was higher, for example 2.4% for MSp^+ and RSpoIIIa (53), with optimum sporulation occurring in 0.4% glucose (32, 53, 111). In strongly saccharolytic clostridia such as C. pasteurianum, 3% glucose was required for optimal sporulation (13). Although 1% glucose in CDM supported maximum growth, the percentage sporulation was reduced. In order to obtain good growth with optimal sporulation, 0.4% glucose was recommended for use in CDM. At such concentration the amount of glucose in the medium was limited and sporulation would occur once the monosaccharide had been depleted (2).

The concentrations of most of the vitamin factors in the CDM were twice that of the basal medium. The choline-chloride concentration had been increased to 5 mg/100 ml medium as recommended by Gullmar and Molin (50) since at such concentration in synthetic medium the type E strain 1537/62 attained normal shape and no longer formed long chains. The role of choline in cell wall synthesis and cell division has been discussed (50, 111). All the vitamin factors included in the CDM have been shown to be essential or stimulatory for Clostridium spp. (87).

Hydrated $CuSO_4$ and $ZnSO_4$ was added to the trace minerals in the basal medium and the quantity of potassium

phosphate was reduced by one half to avoid precipitation of the large quantities of insoluble phosphate. Ethylenediamine tetra-acetate (EDTA) (approx. 0.0002 M final concentration) was added to the trace mineral-phosphate solution to dissolve the precipitates. EDTA has been shown to be a stimulant for growth (0.0006M-0.0012M) of B. sphaericus (19) and a "germinant" (0.001M-0.1M) for spores of the anaerobe, PA 3679 (15),. Since the effect of EDTA on the growth of microorganisms often varies between genera and species and also the concentration of EDTA in the CDM was very low(approx. 0.0002M) it is reasonable to assume that effect of EDTA on spore germination and growth of MSp^+ in the CDM is negligible.

Besides increasing the concentrations for each of the amino acids, three additional L-amino acids —— aspartate, ornithine HCl and tyrosine —— were included in the basal medium. There were qualitative differences in minimal amino acid requirements among species as well as among strains of the same species. Single amino acid deletion experiments showed that arginine, cysteine, isoleucine and methionine are required for germination and subsequent outgrowth of spores of MSp^+ and ts-25 with ts-25 having additional requirements for threonine, tryptophane and valine. In accordance with Ward and Carroll (128), there was no absolute requirement for any single amino acid for growth of the

MSp^+, RSpoIIIa and ts-25 strains. Omission of arginine, cysteine, methionine or tryptophane from CDM retarded the growth of MSp^+ and ts-25 as O.D. was reduced to 50% or less. Deletion of arginine, cysteine, proline or valine restricted the growth of RSpoIIIa. In general, alanine, arginine, aspartate, cysteine, glutamate, leucine, methionine, phenylalanine, serine, threonine, tryptophane, tyrosine and valine were necessary for maximum growth of the three mutants, with MSp^+ showing additional requirement for ornithine, RSpoIIIa for ornithine, proline and isoleucine and ts-25 for proline and isoleucine. The amino acid requirements for sporulation of MSp^+ and ts-25 has not been evaluated in the report because it is difficult to define nutritional requirements for sporulation since once growth has occurred the medium is no longer definded. A replacement sporulation technique was used to study the role of amino acids in sporulation and the minimal requirements for commitment to sporulation in Bacillus subtilis (89).

The CDM was non-restrictive in the sense that it supported spore outgrowth and spore formation of all ten type E strains tested. The degree of sporulation and the time required to achieve maximum sporulation varied among the strains. Generally, a higher percentage sporulation was observed in TPGY medium than in CDM. For example, MSp^+ formed over 90% spores in TPGY medium

with about 65% in the CDM. Such an occurence may be due to the limitation of certain nutrients required for sporulation in the CDM. Since derepression of catabolite in the medium initiates sporulation (100) and endospore formation in bacteria has been recognized as a response to depletion of nutrients it is logical to assume that spore-formers may take a longer time to achieve maximum sporulation in complex medium than in chemically defined medium which is usually not as rich as the complex medium. Vegetative cells will propagate for a longer period in the complex medium before certain of the nutrients become depleted to allow initiation of sporulation (Table 7). The longer periods required by the strains Minnesota, FDA PM-15 and FDA 070 to achieve maximum sporulation in CDM could be explained by a delay in germination.

The "defective" spores observed for FDA PM-15 and FDA 070 in CDM were quite similar to those found in cystine-inhibited sporulation of Bacillus megaterium (125) and in glucose-deficient medium of PA 3679 (41). The two strains may have special requirements for sporulation not provided by the CDM.

Other serotypes of C. botulinum and C. sporogenes grew but did not sporulate in the CDM. The deficiency in sporulation could be attributed to the lack or insufficient amount of essential nutrients other than glucose. In a

preliminary experiment, sporulation occurred in types A and F when the amount of arginine in the CDM was increased to 110 mg/100 ml medium (88).

Tween 80 (0.1%) added to CDM seemed to accelerate both growth and spore cycle of MSp^+. It is possible that it acts as a germination inducer like other chelating agents by increasing the permeability of spore coat and thus the uptake of nutrients.

Growth of log phase cells of MSp^+ and ATCC 9564 was abruptly stopped upon the addition of rifampin, an inhibitor of DNA-dependent RNA polymerase (92), at hourly intervals. In addition only vegetative cells were observed in these cultures after 24 h of incubation indicating that rifampin was also an effective inhibitor for the synthesis of RNA required for spore formation. Addition of rifampin to cultures at the end of log phase and during the first two hours after the beginning of sporulation prevented development beyond the forespore stage with most of the cells in the cultures remaining in either axial filament or forespore stage. It appears that synthesis of RNA continued during the early stages of sporogenesis. The gradual increase in the percentages of refractile endospores observed at 24 h in the series of samples with rifampin added at hourly intervals during the stationary phase is evidence of continued RNA synthesis for spore maturation and also asynchrony in the sporulat-

ion cycle. Our results generally agreed with the findings of Leighton and Doi (70) who suggested that continuous RNA synthesis was necessary during sporulation and that the m-RNA produced was not any more stable than that found during vegetative growth.

The temporal sequence and pattern of macromolecular syntheses was followed using radioactive precursors. Similar to observations reported for Bacillus spp. (3, 46, 84, 95 ,105), during germination and outgrowth of MSp^+ spores, RNA was the first macromolecule to be synthesized, followed by protein synthesis and then the synthesis of DNA which began at 2 h after inoculation. Our results showed that the rates of uptake of the labeled thymidine, uracil and methionine were similar during log phase. In MSp^+, DNA synthesis ceased at the end of log phase (T_0) whereas in B. subtilis Szulmajster and Canfield (115) found that it ended at $T_{1.5}$ and Aubert et al (6) noted that it stopped at T_3. However, Ryter and Aubert (99) using autoradiography to study sporulation in individual cells reported that DNA synthesis of the Marburg 168 strain had almost completely stopped by stage I, i.e. between T_0 and T_1. This provided evidence of a close relationship between the arrest of DNA synthesis and commitment to sporulation. However, Young and Fitz-James observed that with B. cereus a last burst of DNA synthesis occurred after

stage II (135). Day and Costilow (23) using replacement sporulation technique with C. botulinum 62A found that there was no apparent DNA synthesis after the cells were obviously swollen, i.e. at stage II.

In this study, net RNA synthesis in MSp^+ culture was stopped at T_2 compared to T_0 reported for Bacillus spp. (11) but with the addition of rifampin at T_0, net RNA Synthesis did not continue. When rifampin was added at T_0, endospores were not observed but when treated at T_3, 20% of the cells formed endospores. Although net synthesis of RNA had stopped when rifampin was added at T_3 the level of labeled uracil in the cells did not drop, presumably because they were retained in the cells in a precursor state. It is difficult to explain why the total uptake of uracil in the chloramphenicol-treated cells was higher than in the control (Fig. 8).

Net synthesis of protein in MSp^+ continued during early stationary phase but the rate of synthesis gradually decreased during the period T_0 to T_7 and then ceased. In B. subtilis, the net synthesis of protein was not observed after T_0 (11). The discrepancy could be due to generic traits and/or the inherent difficulty in obtaining synchronous sporulating-cultures of Clostridium spp. During stationary phase, the asporogenic mutant RSpIIIa, deviated from the sporogenic and parent strains in that the uptake of ^{14}C-methionine was stopped at T_3 while that of MSp^+ and ATCC 9564 continued (Fig. 13). Since the

asporogenic mutant was unable to develop beyond stage III (53), synthesis of proteins (enzymes) would not be required for the subsequent development. When the strains were allowed to grow in CDM instead of TPGY medium, a similar pattern of ^{14}C-methionine uptake was observed (Fig. 20). The radioactivity of theTCA-ppt. of cells grown in CDM was higher possibly because the amount of labeled methionine molecules in the defined medium was lower compared to the TPGY. Thus the chemically defined medium proved to be more sensitive than complex medium for biochemical studies. One disadvantage of the CDM reported here is that the spore yield was consistently lower than that attained in complex medium.

From the shift "down" experiment, the temperature sensitive period of the temperature-sensitive mutant ts-25, was found to begin at early log phase (or outgrowth phase) and from the shift "up" experiment, it was maintained until just after the end of log phase (T_1). Since ultrastructure studies showed that blockage of sporulation at the non-permissive temperature was at stage I, i.e. between T_0 and T_1 (67) and also synthesis of DNA generally stopped before T_1 (23, 99) it was reasonable to expect the effect of higher temperature was to cause structural changes of certain proteins (enzymes) in the cells which prevented spore development. Mutants of Bacillus spp. with an altered serine

protease(27) or an altered RNA polymerase (71, 73, 107) were found to block sporulation at stage 0 by interfering with the clippage of β subunit. Leighton (72) also isolated a single-site mutant of B. subtilis with a rifampin-resistant RNA polymerase and found that this mutation caused temperature-sensitive sporulation.

During growth of ts-25 the uptake of ^{3}H-thymidine, used to measure synthesis (replication) of DNA, did not show any significant difference at the permissive and non-permissive temperatures. Therefore ts-25 is unlikely to be a mutant temperature-sensitive for the initiation of DNA replication as found in B. subtilis (49, 60, 68, 80, 124, 129). At the permissive temperature, uptake of ^{14}C-uracil by ts-25 stopped before T_0 while that of ^{14}C-methionine continued. But at the non-permissive temperature, the radioactivity of the uracil incorporated into cells began to fall from just before the end of log phase whereas that of ^{14}C-methionine also dropped after achieving a maximum around T_1 (Fig. 18). These indicated that neither RNA nor protein was synthesized after sporulation was blocked at stage I by the higher growth temperature.

The Beluga parent strain sporulated at 28°C and at 37°C. The uptake of radioactive precursors at both temperature showed that DNA synthesis stopped before T_0

while net syntheses of RNA and protein continued till T_1 and T_3 respectively (Fig. 19). No explanation can be offered as to the higher level of radioactive uracil incorporated into the ts-25 and Beluga cultures when grown at the lower temperature.

In view of the strict anaerobic condition required for growth and sporulation and the difficulty in obtaining synchronous sporulating cultures of C. botulinum strains, we do not expect our results to be conclusive. Our data confirm the previous findings on the blockage of the temperature-sensitive, ts-25, and asporogenic, RSpoIIIa, mutants. The difference in timing of the cessation of net RNA and protein syntheses in our strains compared with Bacillus spp. may be attributed to generic traits. In order to varify this, more refined techniques such as replacement sporulation and autoradiography in combination with electron microscopy should be used to study the sporulating process as it occurs in individual cells. The data on sporulation in this report are pertinent to the whole cell population.

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