THE UNIVERSITY OF MANITOBA

SOME PHYSIOLOGICAL ASPECTS OF THE DEVELOPMENTAL PHASES OF GERMINATING SPORES OF CLOSTRIDIUM BOTULINUM, Msp^+

BY

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

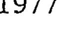
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

WINNIPEG, MANITOBA

CANADA



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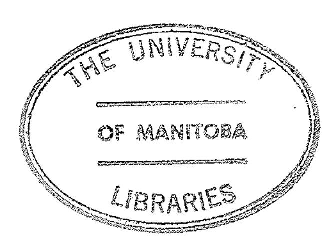

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BY

PRAVIN KUMAR BHATNAGAR

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

MASTER OF SCIENCE

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ABSTRACT

The effect of rifampicin on the developmental phases of germinating spores of Clostridium botulinum, a non-toxigenic sporogenic mutant strain has been studied. Heat-activated spores were incubated, in the absence or the presence of rifampicin, in trypticase-peptone-glucose-yeast extract broth and their developmental phases and growth were determined by phase microscopy and A_{600} measurement, respectively. In untreated cultures, germination and outgrowth (Phase I) occurred within 4 h of inoculation followed by vegetative growth (Phase II, 4-12 h), initiation of sporulation (Phase III, 12-14 h) and formation of mature endospores (Phase IV, 14-23 h). At sublethal concentration (0.05 ng/ml) rifampicin prolonged the time period for phases I and II to 8 (0-8 h) and 17 (8-25 h) h respectively. due to its inhibitory effect on RNA and protein synthesis. Rifampicin had essentially no effect on DNA synthesis. In both treated and untreated cultures growth, as measured by absorbance, continued to increase during spore formation.

Studies on the utilization of glucose showed that only 50% of the glucose was exhausted when sporulation had began during the mid-log growth phase, suggesting that the presence of glucose was required for continuous vegetative growth but its absence was not essential for sporulation. However, when log-phase cells were used as inoculum, phase-bright endospores were observed and glucose was exhausted, 6 h after the end of logarithmic growth phase. Contrary to the present day concept it would appear that, under certain conditions, both

vegetative growth and sporulation can occur at the same time.

TO MY PARENTS

ALL THAT A MAN ACHIEVES

AND ALL THAT HE FAILS TO ACHIEVE

IS THE DIRECT RESULT OF HIS OWN THOUGHTS

ACKNOWLEDGEMENTS

The author expresses sincere gratitude to Dr. R. Z. Hawirko, Professor of Microbiology, whose patience, sincerity, and understanding guidance have yielded inspiration on countless occasions. Many thanks are offered to Drs. C. T. Chow and P. Y. Maeba for their valuable advice and discussions during this project.

The fine example of my brother and sister in-law, Om and Shashi, was a constant source of inspiration.

It is a great pleasure to acknowledge with thanks the valuable help of Miss Barbra MacDonald and Mr. Charlie Naccarato. Thanks are also extended to all those whose assistance, in one way or another , was sought for the completion of this thesis.

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ABBREVIATIONS

A_{600}	-	absorbance at 600 nm
ATCC	-	American Type Culture Collection
CPM	-	counts per minute
DPA	-	dipicolinic acid
DNA	-	deoxyribonucleic acid
EMP	-	Embden-Meyerhof-pathway
HPUra	-	6-(p-hydroxyphenylazo)-uracil
Msp^+	-	sporogenic mutant of C. botulinum type E
NTG	-	N-methyl-N'-nitro-N-nitrosoguanidine
PHB	-	poly-β-hydroxybutyrate
RNA	-	ribonucleic acid
m RNA	-	messenger RNA
r RNA	-	ribosomal RNA
t RNA	-	transfer RNA
sp^-	-	asporogenous mutant, that will not sporulate under any condition
sp^+	-	sporogenic mutant, that sporulates at high frequency
t_0	-	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
TPGY	-	Tripticase peptone glucose yeast-extract broth
V/V	-	volume per volume
W/V	-	weight per volume

INTRODUCTION

INTRODUCTION

One of the main challenges posed by modern biology is the clarification of the mechanisms which lead to cell differentation. The complexity of the changes, in the embryo of multicellular organisms, has led many investigators to look for simple unicellular systems as models in the study of cell differentation. One of these is the developmental cycle of spore-forming bacteria and the process of outgrowth and sporulation (25,42).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 new vegetative cell.

The development of dormant spores into actively metabolizing cells have been extensively studied in Bacillus spp. (42, 43, 79) Three sequential processes are known to be responsible for the changing of a dormant bacterial spore into a vegetative cell : activation, germination and outgrowth. They are fundamentally different from each other, and the occurrence of each stage is dependent on the previous process having taken place. They are induced by different external factors, inhibited by different inhibitors and mediated by different kinds of biochemical reactions (31).

In many cases, especially among the anaerobic clostrida, fresh spores will not germinate even under the optimal environmental conditions, unless they are preheated or aged. The process of "conditioning the spore to germination" has been called "activation".

Activation is reversible ,in most cases, in the sense that the germination rate of spores ,if stored after activation, will decrease as a function of time. Activation is not inhibited by the presence of metabolic inhibitors and there is no evidence that it is metabolism-mediated. When activated spores are exposed to the appropriate environment ,an irreversible step called "germination" occurs. Germination is essentially the conversion of a resistant and dormant spore into a sensitive and metabolically active form. Germination may , also, occur in the presence of inhibitors of macromolecular synthesis (actinomycin D, chloramphenicol), thus not dependent on RNA and protein synthesis (77). During this process i.e. germination, 30% of the dry weight of a spore (including DPA, calcium and cortical materials) is excreted into the medium. Germination seems to be a process during which several spore specific substances are broken down ,the spore state is irreversible terminated and metabolism is activated. It does not consist of ,nor is it dependent on, the synthesis of new macromolecules. It must ,therefore, be viewed as a process of biochemical degradation responsible for the termination of the cryptobiotic state and not as a growth or differentation process.

After germination is completed "outgrowth" occurs, the process of emergence of the first vegetative cell from the germinated spore is called "outgrowth". Outgrowth is repressed by inhibitors of macromolecular synthesis ,particularly RNA and protein, which is absent in the spore state. In outgrowing spores the initiation of protein synthesis seems to depend on transcriptional events. This

is the most simple explanation for the fact that Bacillus cereus spores germinated in the presence of actinomycin D do not incorporate amino acids and their ability to form new proteins is blocked (85). This led most workers to assume that spores have no m-RNA and new m-RNA has to be synthesized before protein synthesis can start. The rapid synthesis of RNA and DNA during the exponential phase of growth ceases near the end of log phase (99). Usually no net synthesis of RNA or DNA occurs during the sporulation phase which covers a period of several hours after the end of growth. In some cases a slow synthesis of DNA occurs during sporulation, but a constant level is reached before endospore formation (98).

The biochemical changes occurring during the spore formation in Bacillus have been studied extensively whereas only a limited number of reports have been published on Clostridium spp. One of the most important determining factors of sporogenesis is the level of available carbohydrate, mainly glucose. 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 (15). Hence Day and Costilow (14) suggested that acetate , which accumulated during glucose catabolism ,was utilized during early sporulation, which is reminiscent of the fate of acetate during aerobic spore formation (5, 32).

In a recent report (35) it has been shown that when sublethal doses of rifampin are added to log-phase cells, sporulation (Fig.1) is blocked at stage III to V and mature cells were unable to form

to form division septa between the daughter cells, resulting in the formation of excessively elongated cells with immature spores at each pole.

Since the process of germination and outgrowth also requires macromolecular synthesis, it was of interest therefore, to study the effect of rifampicin on the post-germinative developmental phases, kinetics of macromolecular synthesis, chemical analyses of macromolecules during the developmental phases. The work also included the comparison of the developmental phases of heat-activated spores with log-phase cells.

HISTORICAL

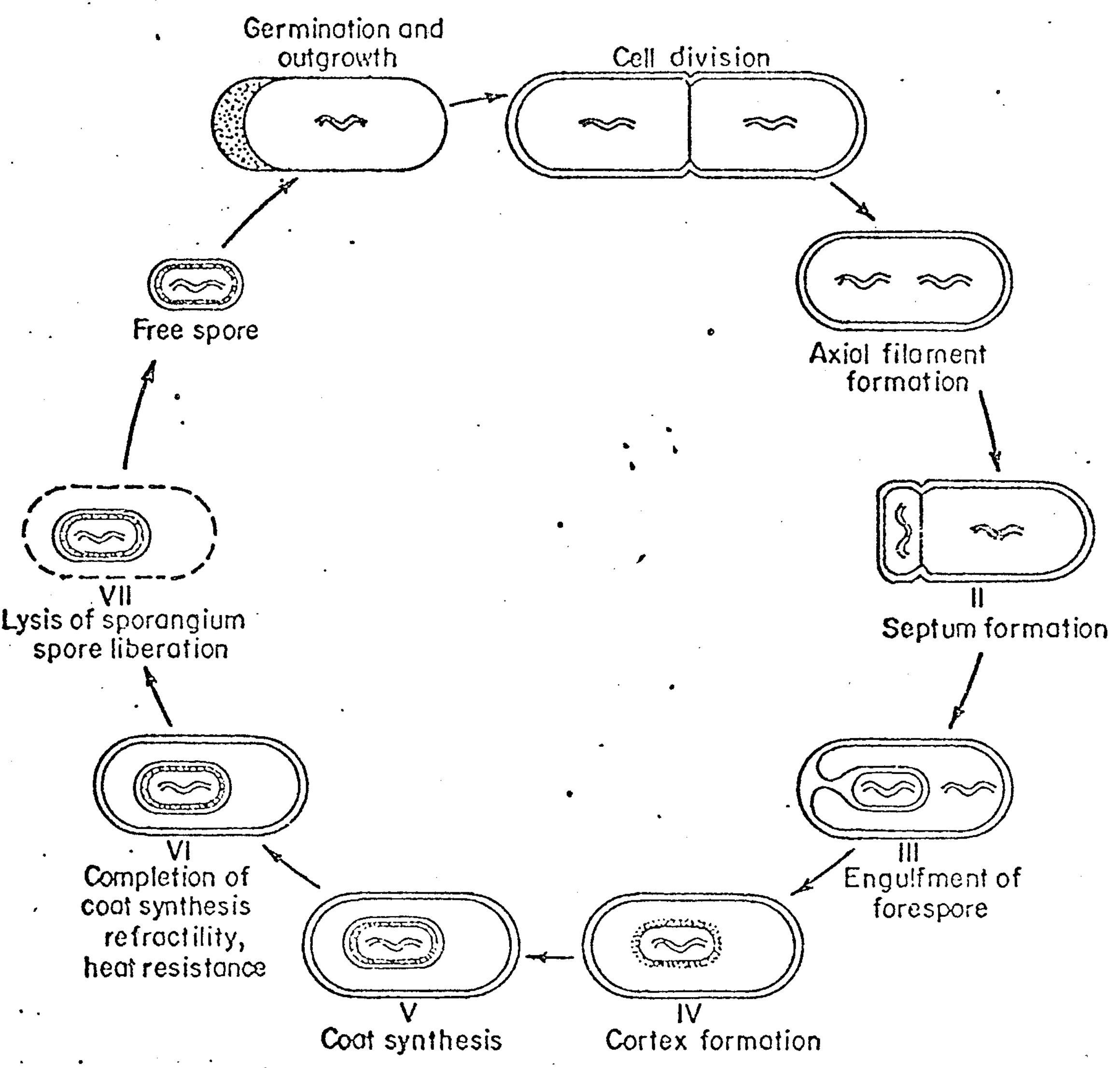
HISTORICAL

The morphological stages which occur during outgrowth have been described for several species of Bacillus (24,57). Hoeinger and Headley (38) have studied the process of spore germination in Clostridium pectinovorum by phase-contrast and electron microscopy. Unlike most other Bacillaceae ,germination of this species takes place within sporangium. Under phase-contrast ,the spore darkens and swells slightly and then vegetative rod slips out through the end opposite the collar-like extension of the sporangium. The time required to proceed through outgrowth to the first cell division is dependent upon the composition of the medium. If the germinating medium is insufficient to support vegetative growth (i) development is arrested (88) or (ii) the outgrowing cell may proceed to form a second spore without intervening cell division (a process called "microcycle") (92).

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 (98 ,99) and for Clostridium pectinovorum by Fitz-James (23).

The cytological changes which occur during sporulation have been defined on a temporal sequence. Seven morphological steps have been described (Fig. 1). The process is essentially the same in Bacillus and Clostridium species (24,41). The end of lograthemic growth phase (Stage 0) is followed by the formation of an axially disposed filament of condensed chromatin (Stage I). Stage I is

Figure 1. Life cycle of sporulating Bacillus spp. and morphological stages (Modified drawing from Kornberg et al (49).


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

completed when 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 mother cell and forespore cytoplasmic units. During stage III ,the forespore protoplast is engulfed as a result of unidirectional growth of 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 fine structure of the cytoplasm of the spore protoplast occur that causes it to appear more homogenous and electron-dense. Stage VII consists of the liberation of the mature free spore via autolysis of the mother cell. Subsequent to the maturation of the endospore a lytic enzyme is synthesized or activated which brings about its release from the sporangium.

Biochemically, one can follow the various stages of outgrowth by examining the synthesis of RNA, protein,and DNA. It has been established that outgrowth is dependent upon a repair in the protein synthesizing system and an ordered synthesis of proteins (46). It is emphasized that the macromolecular composition of vegetative cell ,i.e. the product of outgrowth ,is dependent upon the growth rate imposed by

the environment. If germination is carried out in the presence of actinomycin D ,the germinated spore will neither produce RNA nor protein ,indicating that no preformed functional m-RNA exists in the spore (33), since the antibiotic specifically attaches to DNA and prevents DNA-dependent RNA synthesis without interfering with the functioning of m-RNA once it is formed i.e. protein synthesis. Thus, no protein synthesis will occur if actinomycin D is present during germination of bacterial spores because m-RNA cannot be synthesized. The relationship between growth rate and cellular RNA content arises because ,the faster the bacteria grow, faster they must synthesize protein, and RNA is involved in protein synthesis.

Torriani and Levinthal (85) have reported that the first macromolecular synthesis being synthesized is RNA ,which seems to begin immediately on germination. Protein synthesis lags behind RNA synthesis ,the lag time being around two minutes. Net synthesis of new DNA starts very late during outgrowth in B. cereus and B. subtilis In synchronously sporulating cells net synthesis of DNA starts suddenly 120-160 minutes after germination (42). Some incorporation of radioactive precursors into DNA occurs immediately after germination but this has been attributed to repair synthesis (97). Repair synthesis of DNA is usually not inhibited by the addition of chloramphenicol but replication of DNA is inhibited (59). The incorporation of radioactive precursors during outgrowth does not occur in the presence of chloramphenicol ,therefore Steinberg and Halvorson (77) suggested that this synthesis is not 'repair' but must be DNA replication. Lately Lammi and Vary (53), repeating these experiments ,suggested

that although this synthesis is inhibited by chloramphenicol, it still might be repair reaction and chloramphenicol prevents this reaction indirectly by preventing the synthesis of enzymes necessary for DNA replication. In order to test this hypothesis, Lammi and Vary have used a pyrimidine analogue (HPVra) which has been shown by Brown (6) to block replication but not repair. This analogue stopped incorporation of precursors immediately, showing that the incorporation of radioactive precursors into DNA before net synthesis occurs is replication and not repair.

The various sequential macromolecular synthesis occuring during outgrowth do not depend on DNA synthesis or replication, since Steinberg and Halvorson (77) have shown that time-ordered protein synthesis occurs also in the presence of an inhibitor of DNA synthesis or in a thymidine auxotroph outgrowing in the absence of thymidine.

The macromolecular composition of vegetative cell i.e. the product of outgrowth, is dependent upon the growth rate imposed by the environment. A large proportion of the biomass of all bacteria is protein which may account for more than 70% of the bacterial dry weight, although 50 - 60% is more usual in Bacillus spp. The protein content of vegetative bacteria varies slightly with growth rate in a given medium but is significantly decreased when endogenous reserve materials are accumulated. In contrast RNA content of vegetative bacteria varies 3 - 4 fold with growth rate and RNA may account for 25% of the dry weight of rapidly growing bacteria but only 8% of slow growing bacteria.

The DNA content of vegetative organism varies less and in Bacillus subtilis vegetative cell, for example, DNA accounted for about 2% of the dry weight at all growth rates from 0.1 to 0.6 hr^{-1} in magnesium-limited chemostat cultures (83). The amount of protein per unit of DNA varies little in vegetative bacteria, whereas the amount of RNA per unit of DNA varies by a factor of 3-4 fold at different growth rates (83). To a large extent then the pattern of macromolecular synthesis during outgrowth will depend on the physiochemical conditions of the environment.

Growth and spore formation of Clostridium botulinum usually require complex media, such as meat and serial infusions (7, 89), and strict anaerobic conditions. Cooked-meat medium supports spore formation of many clostridial spp (70). Sugiyama (80) obtained spores of C. botulinum in a medium containing 5% casitone and 0.5% peptone. TPG medium (5% trypticase, 0.5% peptone and o.4% glucose).had been used successfully for the production of spore crops of type E strains of C. botulinum (18, 69), over 80% spores were obtained for Msp^+ (18) and about 60% for other five strains. Roberts (69) showed that over 80% spores of C. botulinum type 7272 A were formed in TPG medium supplimented with 0.1% yeast extract and also 1% ammonium sulfate (TPAY-GT).

Nucliec acid changes of sporulating cells have a general pattern which allows the cell to carry out the necessary functions for spore formation and ensures both a complete genome for the dormant spore and synthetic machinery necessary for its germination.

At the same time, sporulation involves the synthesis of spore

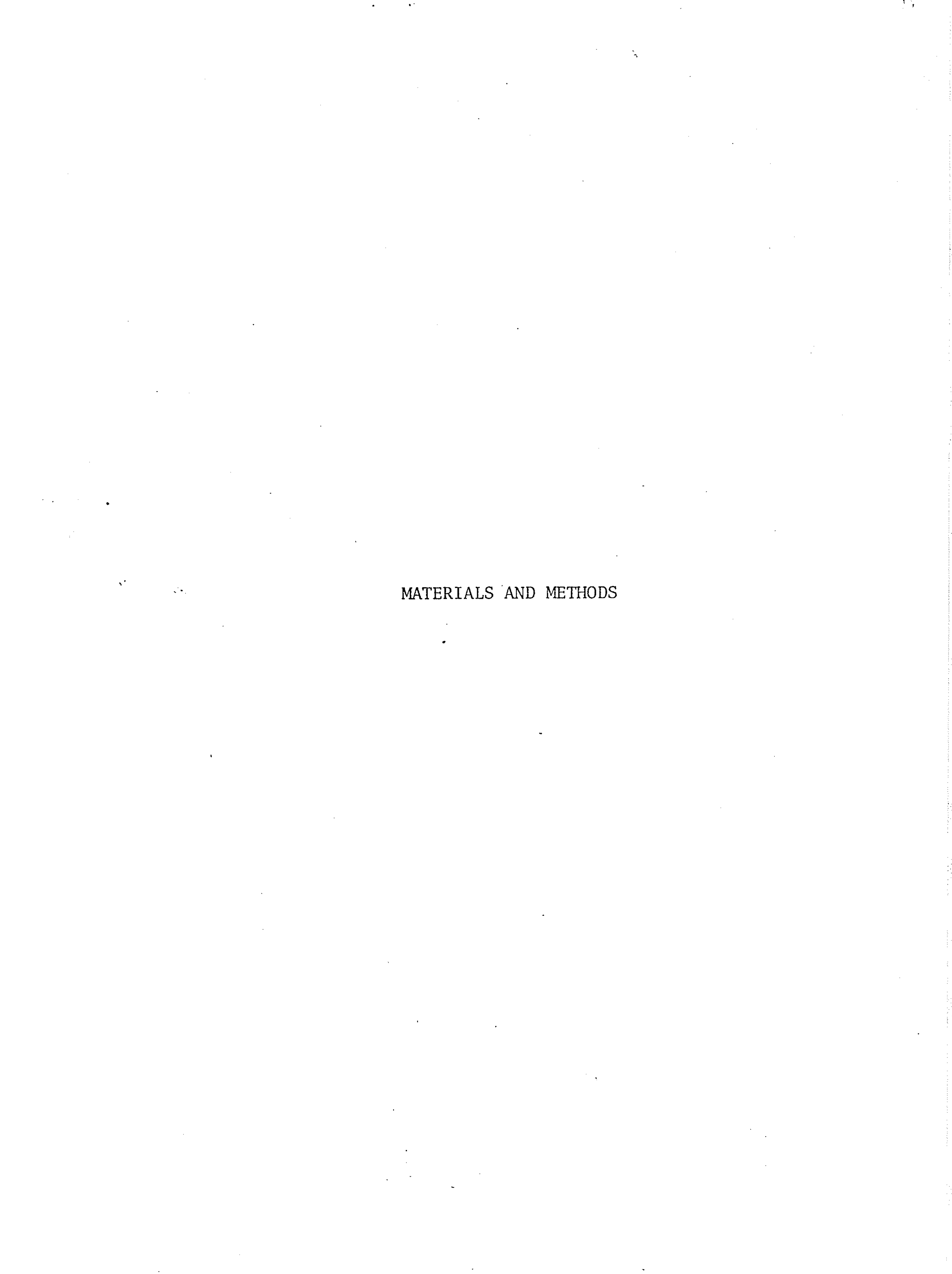
components and the controlled degradation of some RNA and protein fractions, since sporulation generally takes place when medium is deficient for growth. The rapid synthesis of RNA and DNA during the exponential phase of growth ceases near the end of the log-phase (98). Usually no net synthesis of RNA or DNA occurs during the sporulation phase which covers a period of several hours after the end of growth. In some cases a slow synthesis of DNA occurs during sporulation, but a constant level is reached before endospore formation (99). In most cases RNA synthesis occurs actively during sporulation, but the total RNA of sporulating cells remains constant and then decreases as the endospore matures and lysis of the sporangium commences. Both DNA and RNA are present in the metabolically inert spore (20). They are present in lesser amounts than that found in vegetative cells. The ratio of RNA to DNA is reduced in spores when compared to the ratio found in vegetative cells and this is consistent with the relationship found between RNA content and growth rates of bacterial cells. The general pattern of nucleic acid synthesis and content is correlated therefore with the metabolic status of sporulating cells and spores.

It is widely accepted that sporulation is characterstic of stationary-phase cells. The level of available carbohydrate, mainly glucose, is an important factor in determining the sporogenesis. Investigation of the metabolic and regulatory role of glucose before and during sporogenesis (5,63) stimulated the interest in quantitative modification in the pathway of glucose catabolism in relationship to

the separate stages of development of aerobic spore formers. In Bacillus spp (62) 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 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 (4) . This pattern of events is fairly general among Bacillus spp. (30).

Emeruwa (18) has reported that glucose adapted cells of the sporogenic Msp^{+} strains of Clostridium botulinum catabolize glucose via Embden-Meyerhof pathway to yield acetate which serves as a precursor for poly-β-hydroxybutyrate granules. The enzymes of acetate catabolism as well as those of butyric acid fermentation may be partially susceptible to induction of 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.

MATERIALS AND METHODS



MATERIALS AND METHODS

Organism

A non-toxigenic sporogenic mutant, Msp^+,which was derived from Clostridium botulinum type E, ATCC 9564, by treatment with N-methyl -N'-nitrosoguanidine (NTG) (19) was used in this study.

Media

The media used was a trypticase-peptone-glucose-yeast broth (TPGY) containing 5% trypticase (BBL), 0.5% protease peptone (Difco), 0.4% glucose, 0.4% yeast extract (Difco) and 0.2% sodium thioglycollate (Difco) as reducing agent.

Preparation of stock spore suspension

A stock spore suspension of Msp^+ (1 ml) which was heat-activated at 65 °C for 10 min was added to 9 ml TPGY medium in screw-cap tubes and incubated at 28 °C for about 10-12 h until most of the spores had germinated into young vegetative cells. A 10% inoculum of the young culture was added to fresh TPGY medium (100 ml vol.) and incubated for 24 to 36 h. Cultures showing > 90% spores were centrifuged for 15 min at 4080xg, at 5 °C. The pellet was washed three times with deionized water and then treated with a solution of 0.02 M phosphate buffer, pH 7.0, containing 100 µg/ml trypsin and 200 µg/ml lysozyme 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 min, 4,000xg for 10 min and 10,000xg for 5 min. The cleaned spores were resuspended in 0.02 M

phosphate buffer, pH 7.0, to an absorbance of about 0.5 at 600 nm, containing approximately 10^6 spores/ml. This stock spore suspension was dispensed into screw-cap tubes, 5 ml/tube, and stored at 0° to 2° C.

Growth conditions

Spores from a stock spore suspension were heat-activated in H_2O at 65^oC for 10 min and a 10% (v/v) inoculum was added to the media (TPGY) in stoppered test tubes. Rifampicin (Sigma Chemical Company) was added at 0.05 or 0.1 ng/ml to other test cultures and the cultures were incubated at 28^oC. Samples for phase-contrast microscopy and electron microscopy were withdrawn with a hypodermic syringe to maintain anaerobic conditions. Growth was measured spectrophotometrically (600 nm, Coleman Junior II A, model 6/20 A). For quantitative studies a 10% inoculum of heat-activated spores was added to 90 ml TPGY in Nephelo-culture flask (Fig. 2).

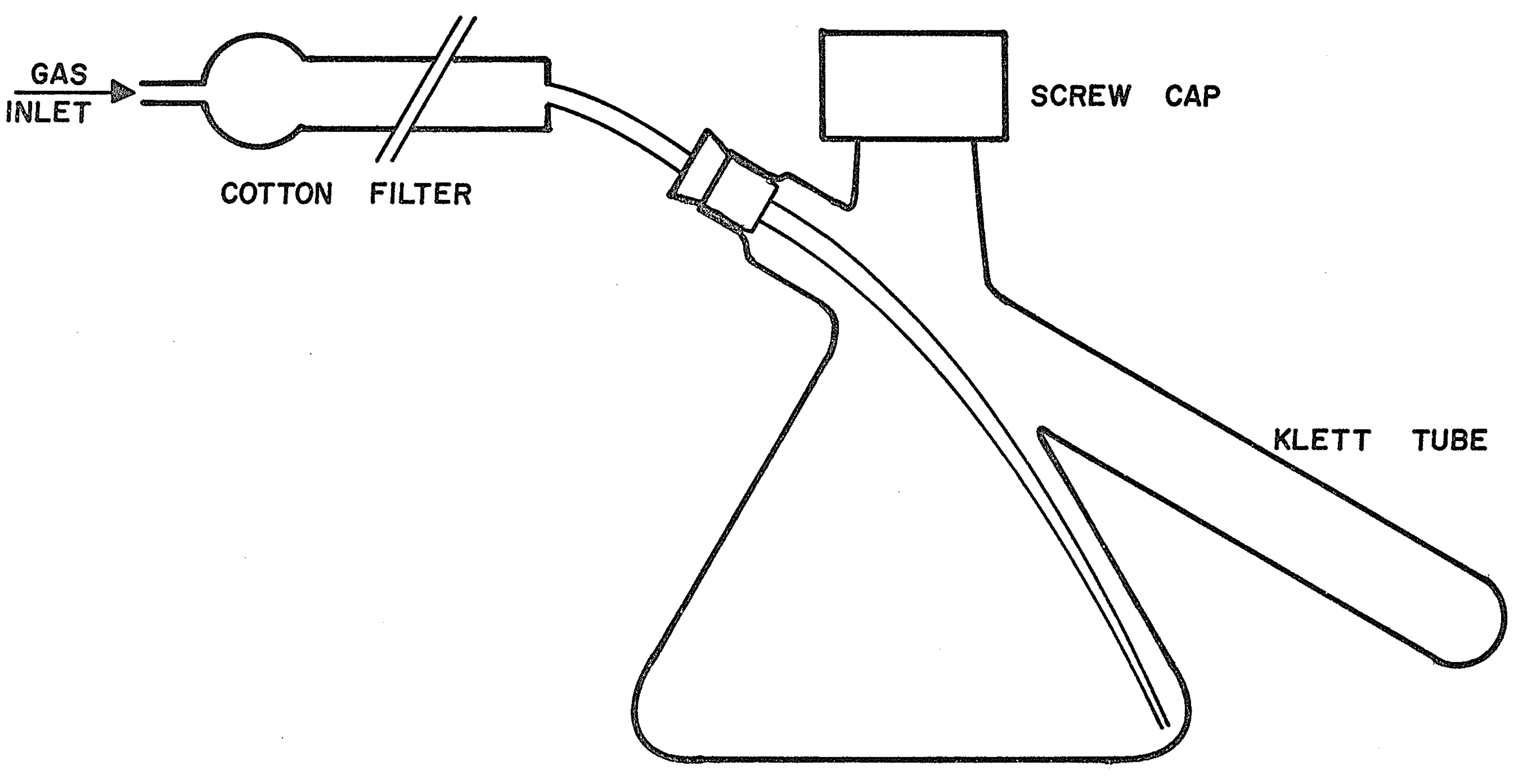
Log-phase cells of the Msp^+ mutant were prepared from an inoculum of heat-activated spores, grown in TPGY medium, for 10 h. After three successive transfers, log-phase cells (10% v/v) of 10 h culture were inoculated into fresh medium.

Synthesis of DNA, RNA and Protein

1. Incorporation of labelled precursors. -

L-methionine-^{14}C (specific activity 56 mCi/m mol.), uracil-2-^{14}C (specific activity 59 mCi/m mol.) and thymidine-6-^3H (specific activity 5 μCi/m mol.) obtained from Amersham/Searle Corp.Arlington Heights, Illionois, were used as a measure of protein, RNA and DNA synthesis

Fig. 2 Nephelo culture flask



NEPHELO CULTURE FLASK

respectively. The labelled compounds were added separately, in the amounts of 1.5 μCi/ml, to TPGY broth with and without rifampicin (0.05 ng/ml) and then inoculated with heat-activated spores. The samples (0.2 ml) were withdrawn in duplicate, at appropriate intervals, from untreated and rifampicin treated cultures and added to an equal volume of cold 10% trichloroacetic acid (TCA) and held at $0^{o}C$ for several h. The resultant precipitate was collected on membrane filters (0.45 μm pore size, Gelman) which were presoaked with 5% TCA containing unlabelled substrate. The precipitate was, then, washed 3 X with 5% TCA and then dried with 95% cold ethanol. The membrane filter was placed in a counting vial with 10 ml Scinti-Verse solution (Fisher Scientific Company). Radioactivity (cpm) was counted (5 min) with a Beckman liquid scintillation counter, model Sc-230.

2. Chemical Analysis

Protein determination -

Protein content was determined by the Folin method of Lowry *et al* (58) as outlined in Thach and Newburger (75) using crystalline bovine serum albumin as standard.

DNA determination -

The diphenylamine method of Dishe as modified by Burton (8) was used as outlined in Kwapinski (50) using calf-thymus DNA as standard.

RNA determination -

Ribonucleic acid content was assayed by the Orcinol method (76) as outlined in Thach and Newburger (75) using yeast RNA as standard.

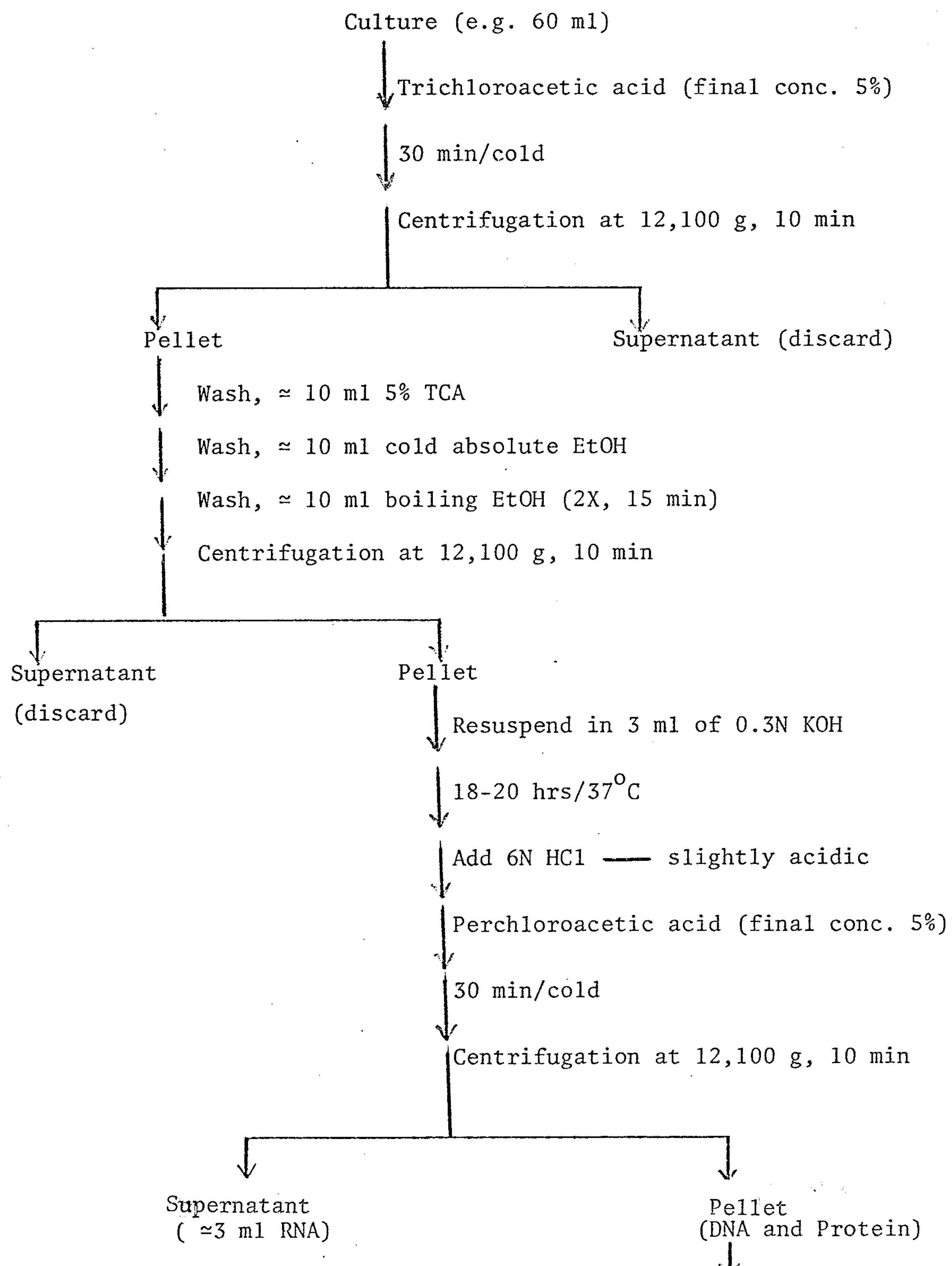
Estimation of Biomass

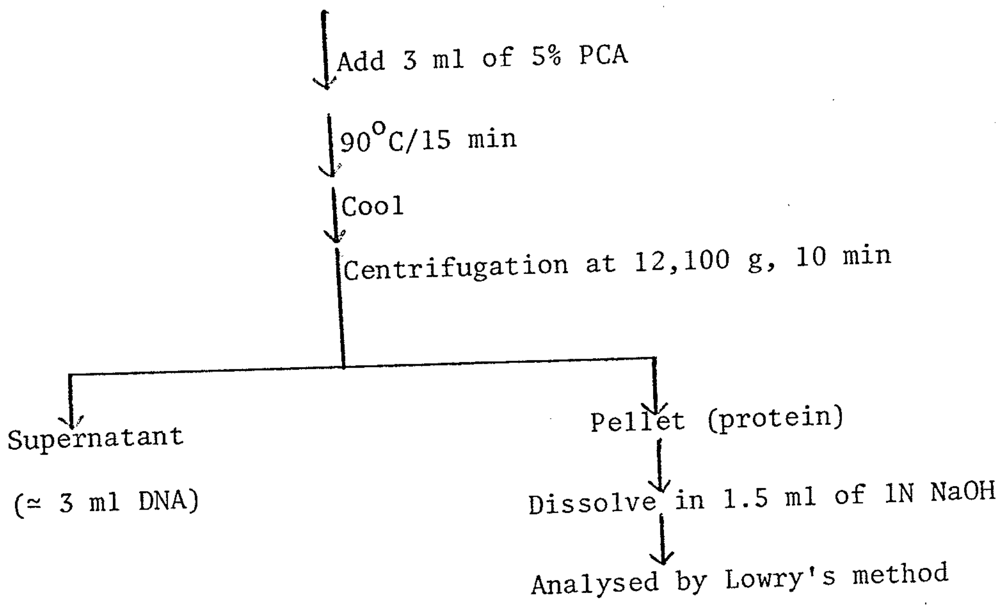
The dry weight was determined on centrifuging cells from a 10 ml of culture collected at 4,000 g for 10 min and washed 2 X DW and then cells were placed in a weighed aluminum foil dish, dried overnight at 80°C, then it was placed in desiccator before reweighing. The drying process was repeated until a constant weight was obtained.

Electron microscopy

Cultures treated with 0.1 ng/ml rifampicin were collected at 22 h, and fixed by the method of Kellenberger *et al* (47).

Schematic summary of procedures used for the extraction of DNA, RNA and Protein





Add 3 ml of 5% PCA
90°C/15 min
Cool
Centrifugation at 12,100 g, 10 min
Supernatant
(≃ 3 ml DNA)
Pellet (protein)
Dissolve in 1.5 ml of 1N NaOH
Analysed by Lowry's method

Effect of Glucose on Growth and Sporulation

The effect of glucose on growth and sporulation was examined. Heat-activated spores were inoculated into TPGY, 22 mM glucose, or in the absence of glucose (TPY). Samples were tested for growth and sporulation and pH changes.

Effect of inoculum on glucose (22 mM) utilization and sporulation

The effect of inoculum, heat-activated spores compared with that of log-phase cells, on glucose utilization, growth and development was tested. Heat-activated spores (10% v/v) were inoculated into 90 ml TPGY and in other experiment log-phase cells were used as inoculum. Samples were removed at various time intervals for phase microscopy, pH changes, residual glucose and growth.

Glucose was estimated enzymatically with glucostat reagent (Worthington-Biochemical Corp., Freehold, N.J.) as outlined in Nowtony (66) using D-glucose as standard.

Cultures grown in the absence of yeast-extract

The effect of yeast-extract was determined by growing heat-activated spores in 10 ml TPGY, 0.4% yeast-extract, or in the absence of yeast-extract. Samples were tested for growth, pH changes and phase microscopy.

RESULTS

RESULTS

Rifampicin effect on Clostridium botulinum life cycle-

Heat-activated spores of Clostridium botulinum were incubated in the absence or the presence of rifampicin and their developmental phases and growth were determined by phase microscopy and A_{600} measurement, respectively. In the untreated cultures (Fig 3 a, Table 2) germination and outgrowth (Phase I) begins 4h after inoculation, followed by vegetative growth, 4-12h, (Phase II), initiation of sporulation, 12-14h, (Phase III) and the formation of mature endospores, 14-23h, (Phase IV). The extent of development and growth of the treated cultures was dependent on the concentration of rifampicin used. In this study, outgrowth of heat-activated spores was inhibited by > 0.2 ng/ml rifampicin (Table 1), and some vegetative cells were observed after 36h of incubation with 0.1 ng/ml (Fig. 3 c). However, at 0.05 ng/ml the time required for the developmental phases I, II, III and IV had been prolonged to 8 (0-8h), 17 (8-25h), 3 (25-28h), and 10 (28-38h) h, respectively (Fig. 3 b, Table 2). Rifampicin concentrations of 0.05 and 0.1 ng/ml were defined as the sublethal and lethal doses.

Figure 3. Effect of rifampicin- *Clostridium botulinum* spores were incubated in the presence of 0 (a), 0.05 (b) and 0.1 (c) ng/ml rifampicin. Phase-microscopy was used to determine their developmental phases ; I= germination and outgrowth (emergence of vegetative cells), II= vegetative growth (98% vegetative cells), III= initiation of sporulation (1% endospores) and IV= formation of mature endospores (98% endospores).

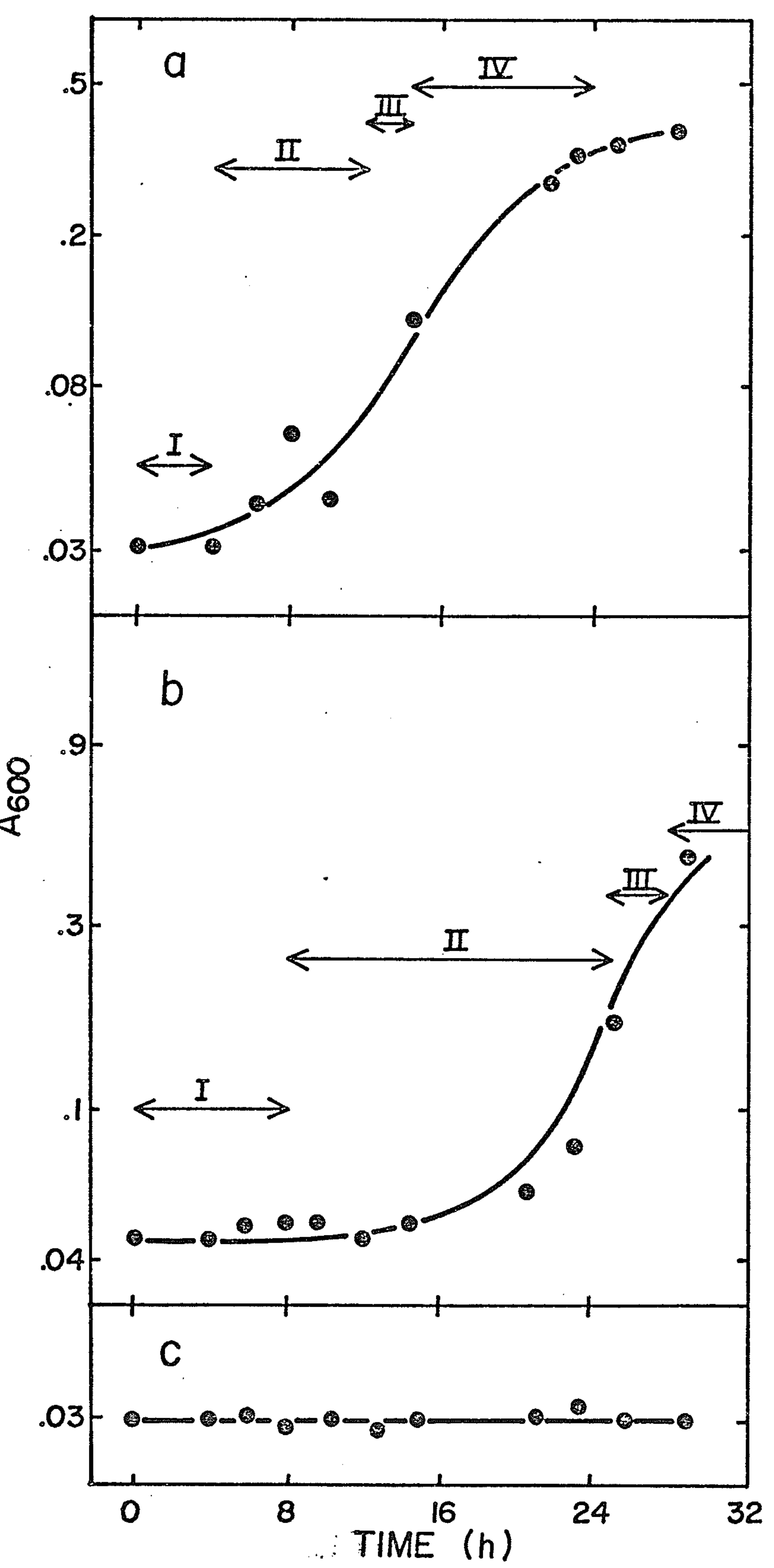

a
.5
.2
.08
.03
I
II
III
IV
b
.9
.3
.1
.04
A600
c
.03
0
8
16
24
32
TIME (h)

Table 1.

Effect of rifampicin concentration on post-germinative development of *Clostridium botulinum*

Doses of rifampicin (ng/ml)	Time (h)								
	4	8	12	16	20	24	28	32	36
0	1 Δ	70	100 ←← S P O R U L A T I O N						
0.1	-	-	-	-	-	-	-	-	1
0.05*	-	1	20	60	80	100 ←← S P O R U L A T I O N →→→			

Δ % vegetative cells

* Sublethal dose

\- No vegetative cell

Table 2.

The effect of sublethal dose of rifampicin on the developmental phases and growth of <u>C. botulinum</u>

	Rifampicin (ng/ml)	I {Spores-1% veg.cells}	II {1% - 98% veg.cells}	III {98% veg.cells-1% Esp.}	IV {1% - 98% Esp.}
Time(h)	0	0 - 4	4 - 12	12 - 14	14 - 23
	0.05	0 - 8	8 - 25	25 - 28	28 - 38
A_{600}	0	.047	.295	.480	.503
	0.05	.05	.281	.470	.520

Incorporation of ^{14}C-uracil

In untreated cultures, a marked fluctuation of uracil incorporation occurred during the first half of phase I (0-2h) and after that incorporation proceeded at a linear rate (Fig. 4). During elongation and cell division the incorporation of uracil into newly synthesized RNA was very rapid reaching a maximum at about 12h and after a slight drop, it remained relatively constant during the sporulation phase (Fig.5).

On the other hand, in cultures treated with a sublethal dose of rifampicin, the pattern of ^{14}C-uracil incorporation, for about 3h, was the same as in untreated cultures (Fig.4) but during the course of germination and outgrowth the synthesis of RNA appeared to be repressed. After a prolonged " fluctuation period " (0-8h) and a period of no net increase (8-14h), uracil was incorporated at a rate similar to that of untreated culture until the end of phase II (14-25h) (Fig.5).

The ability of ^{14}C-uracil to be specifically incorporated into RNA was confirmed by alkaline hydrolysis (0.5N NaOH for 6 to 8h followed by trichloroacetic acid precipitation). Greater than 95% of the label incorporated at 25 min was alkali labile.

Incorporation of 6-^{3}H-thymidine

DNA synthesis was followed by the incorporation of 6-^{3}H-thymidine. In untreated cultures, net synthesis of DNA started at about 20 min after inoculation and continued at a rapid rate until the mid point of log-phase (Fig. 6). During outgrowth and

first few cell divisions, the incorporation of thymidine increased by 14 fold. DNA synthesis continued and reached a maximum at about 10 h and began to decrease once sporulation was initiated.

In cultures treated with sublethal dose of rifampicin, the pattern of thymidine incorporation was similar to that of untreated cultures for the first 6-8 h. An inactive period of net synthesis occurred during phase II, followed by a period of very rapid synthesis during the sporulation phase (Fig. 7).

Incorporation of ^{14}C-L-methionine

The kinetics of protein synthesis during the life cycle of *Clostridium botulinum* was investigated by following the incorporation of ^{14}C-L-methionine and a mixture of ^{14}C-amino acids. Results obtained with the two precursors were very similar. In untreated cultures incorporation of ^{14}C-L-methionine was minimal during germination and outgrowth (phase I) and showed a logarithemic increase during phase II and a gradual increase during the sporulation phase i.e. during the phases III and IV (Fig. 8).

In the rifampicin treated cultures, the pattern of protein synthesis was retarded but similar to that of untreated cultures during the phases I, II and III.

In general protein synthesis started shortly after the initiation of RNA synthesis in both treated and untreated cultures.

A summary of the results of chemical analysis of cells collected during the developmental phases are shown in Table 3 and Fig. 9.

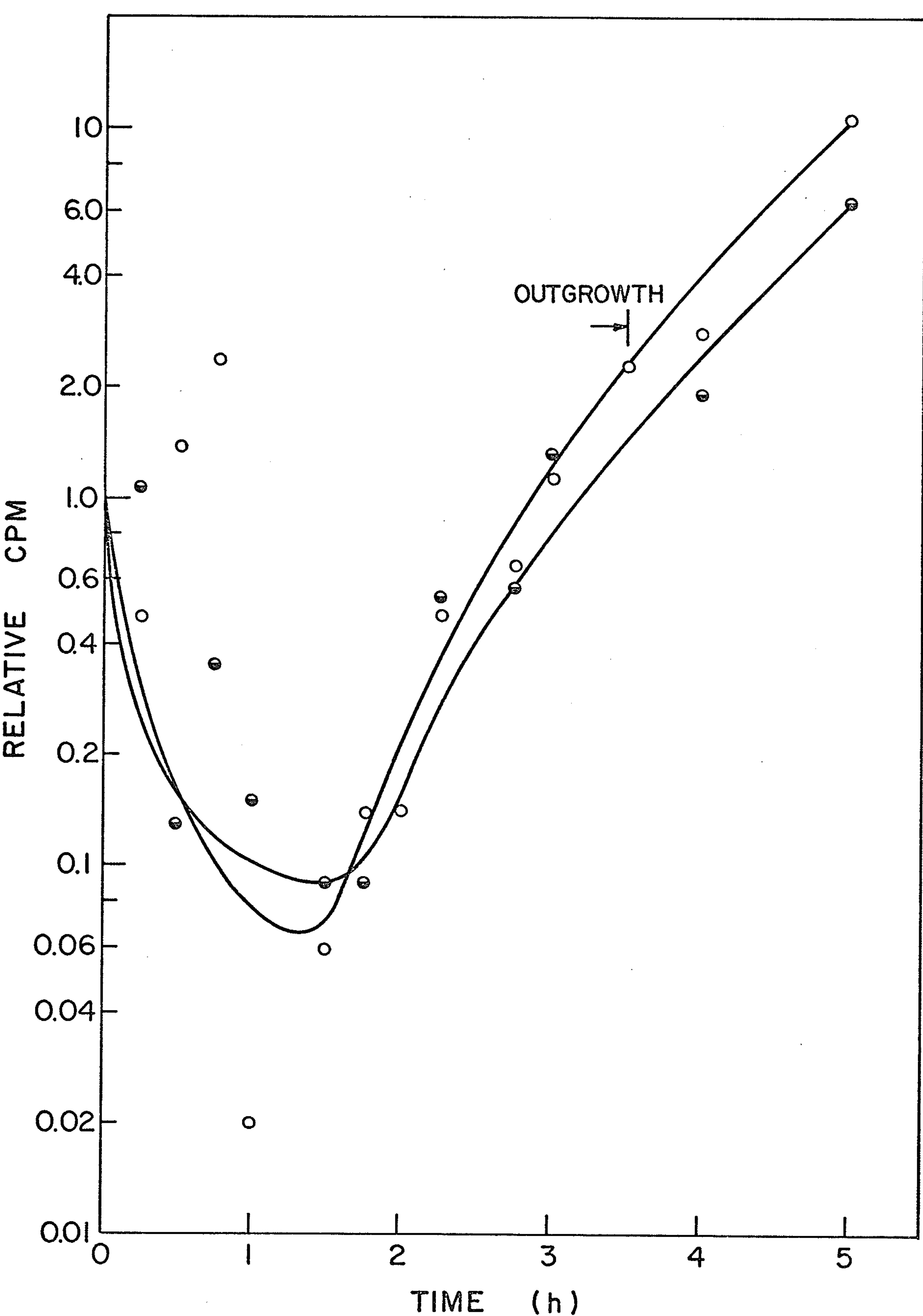
Our results (Fig. 3-8) suggested that in untreated cultures, macromolecules are synthesized during or shortly after phase I, a result agrees well with our A_{600} and phase microscopic findings and chemical data (Table 3) whereas in rifampicin treated cultures, although outgrowth was seen by phase microscopy, growth and synthesis of macromolecules (RNA and protein) did not start until 14-16 h (Figs. 5 and 8).

Figure 4. Incorporation of ^{14}C-uracil during germination and outgrowth of Msp^{+} spores.

^{14}C-uracil (1.5 µCi/ml) was added to TPGY medium incubated with heat-activated spores. Samples were withdrawn at intervals and assayed for radioactivity.

O TPGY

● TPGY + rifampicin



10
6.0
4.0
2.0
1.0
0.6
0.4
0.2
0.1
0.06
0.04
0.02
0.01
RELATIVE CPM
OUTGROWTH
0
1
2
3
4
5
TIME (h)

Figure 5. Incorporation of ^{14}C-uracil during phases I, II, III and IV

O TPGY

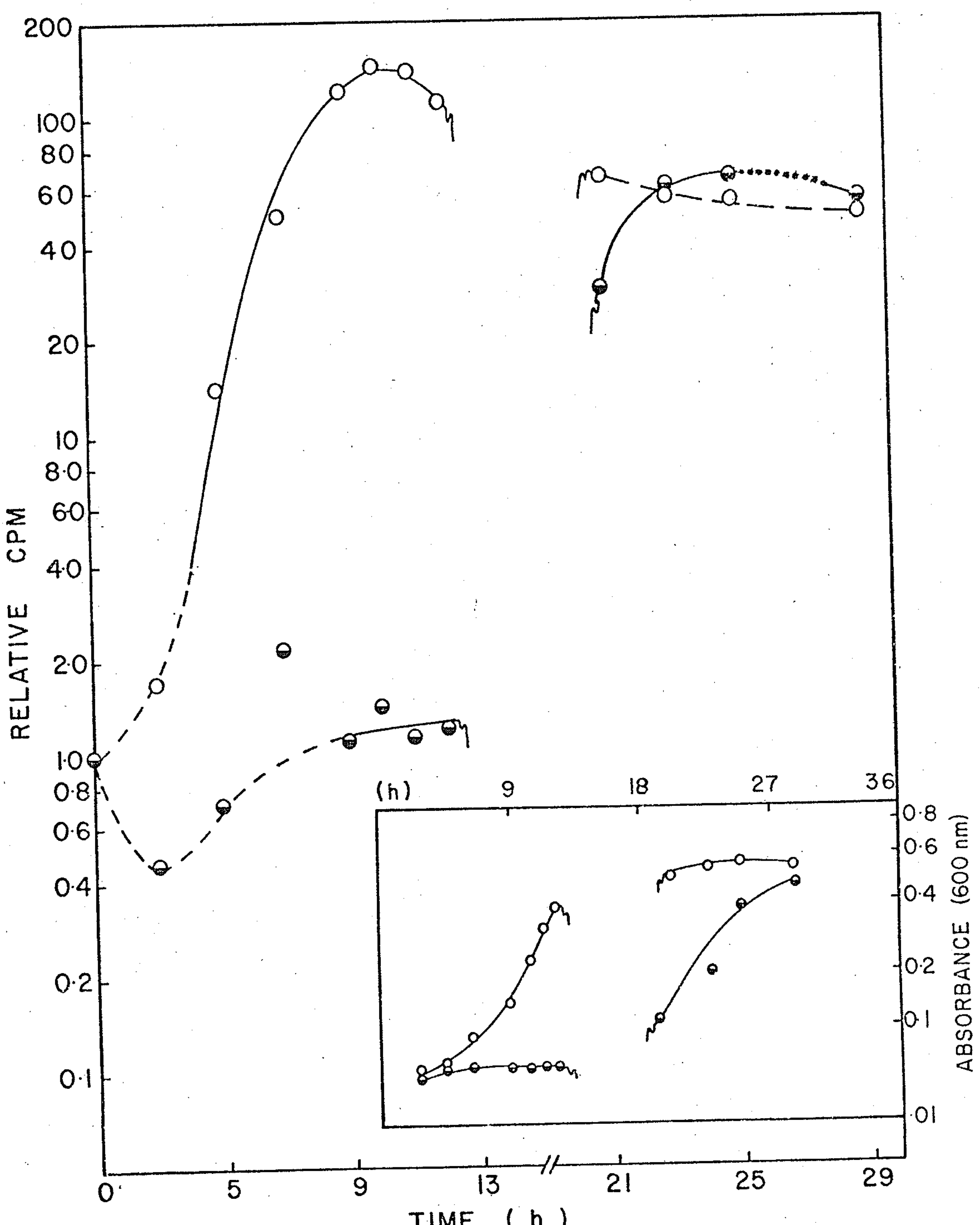
⊖ TPGY + rifampicin

----- Phase I

——— Phase II

..... Phase III

—–— Phase IV



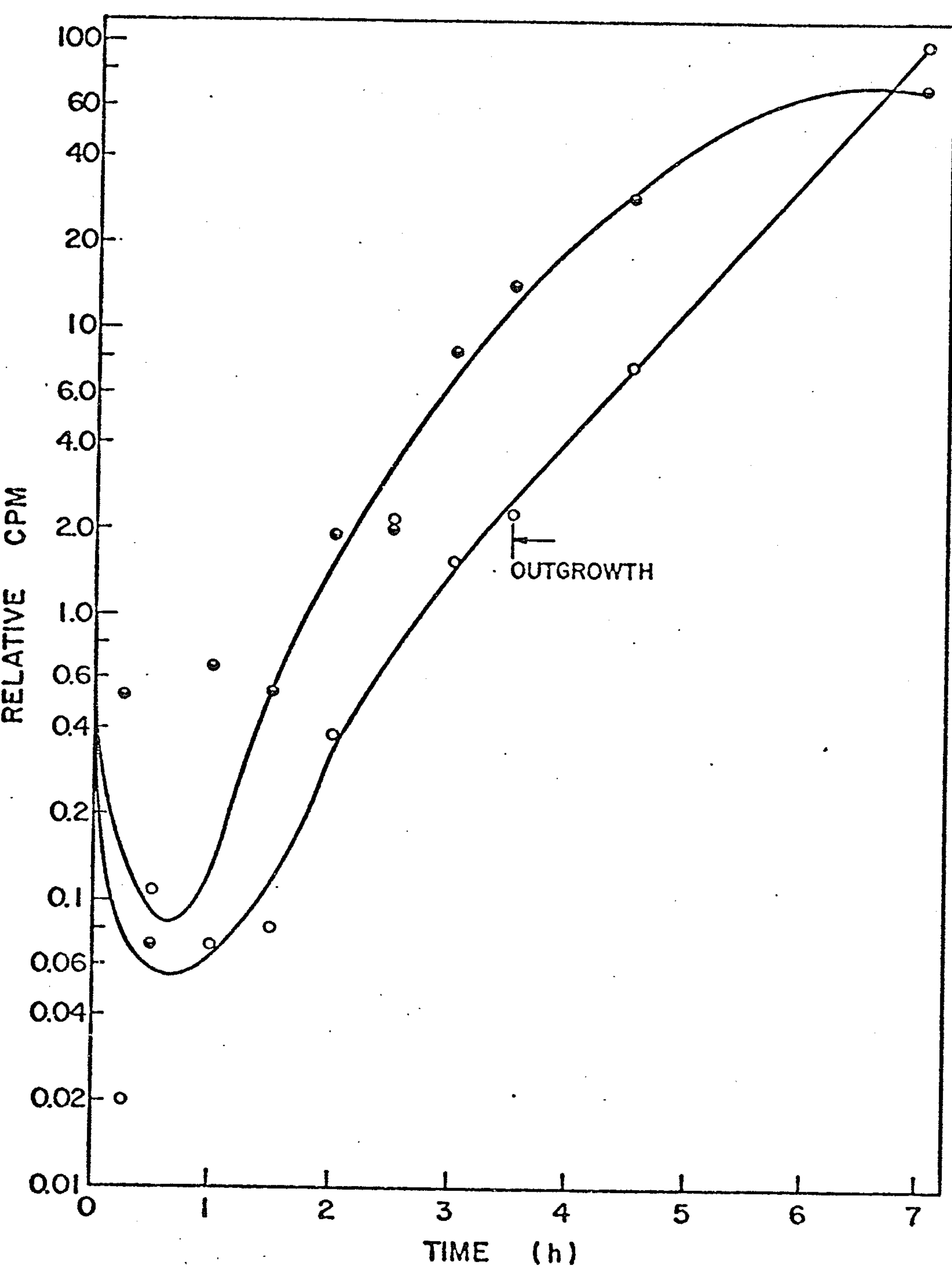
200
100
80
60
40
20
10
8·0
6·0
4·0
2·0
1·0
0·8
0·6
0·4
0·2
0·1
RELATIVE CPM
0
5
9
13
21
25
29
TIME (h)
(h)
9
18
27
36
0·8
0·6
0·4
0·2
0·1
·01
ABSORBANCE (600 nm)

Figure 6. Incorporation of 6-^{3}H-thymidine during germination and outgrowth of Msp^{+} spores

6-^{3}H-thymidine (1.5 μCi/ml) was added to TPGY medium incubated with heat-activated spores. Samples were withdrawn at intervals and assayed for radioactivity.

O TPGY

● TPGY + rifampicin



100
60
40
20
10
6.0
4.0
2.0
1.0
0.6
0.4
0.2
0.1
0.06
0.04
0.02
0.01
RELATIVE CPM
OUTGROWTH
0
1
2
3
4
5
6
7
TIME (h)

Figure 7. Incorporation of 6-^{3}H-thymidine during phases I, II, III and IV

O TPGY

ө TPGY + rifampicin

---- Phase I

—— Phase II

.... Phase III

— — Phase IV

300
200
100
80
60
40
20
10
8
6
4
2
1

RELATIVE CPM

(h) 6 12 20 26

0.8
0.6
0.4
0.2
0.1
.01

ABSORBANCE (600 nm)

0 4 8 12 23 27

TIME (h)

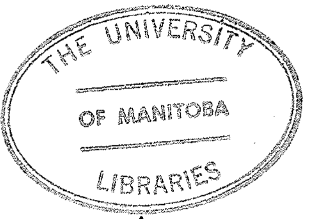


Figure 8. Incorporation of ^{14}C-L-Methionine during phases I, II, III and IV in rifampicin treated and untreated cultures.

O TPGY

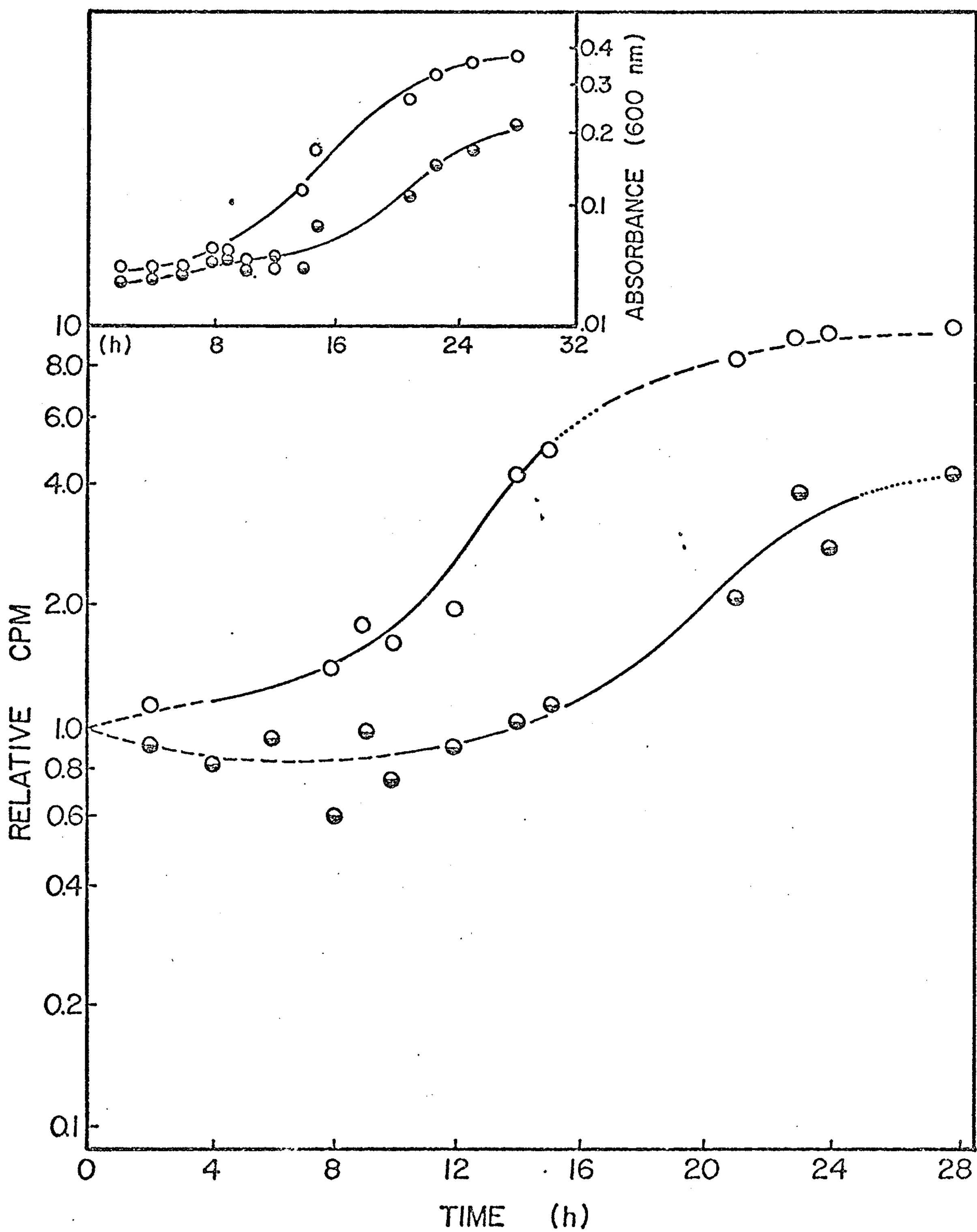
⊖ TPGY + rifampicin

----- Phase I

——— Phase II

..... Phase III

_ __ _ Phase IV



0.4
0.3
0.2
0.1
.01
ABSORBANCE (600 nm)
(h)
8
16
24
32
10
8.0
6.0
4.0
2.0
1.0
0.8
0.6
0.4
0.2
0.1
RELATIVE CPM
0
4
8
12
16
20
24
28
TIME (h)

Table 3.

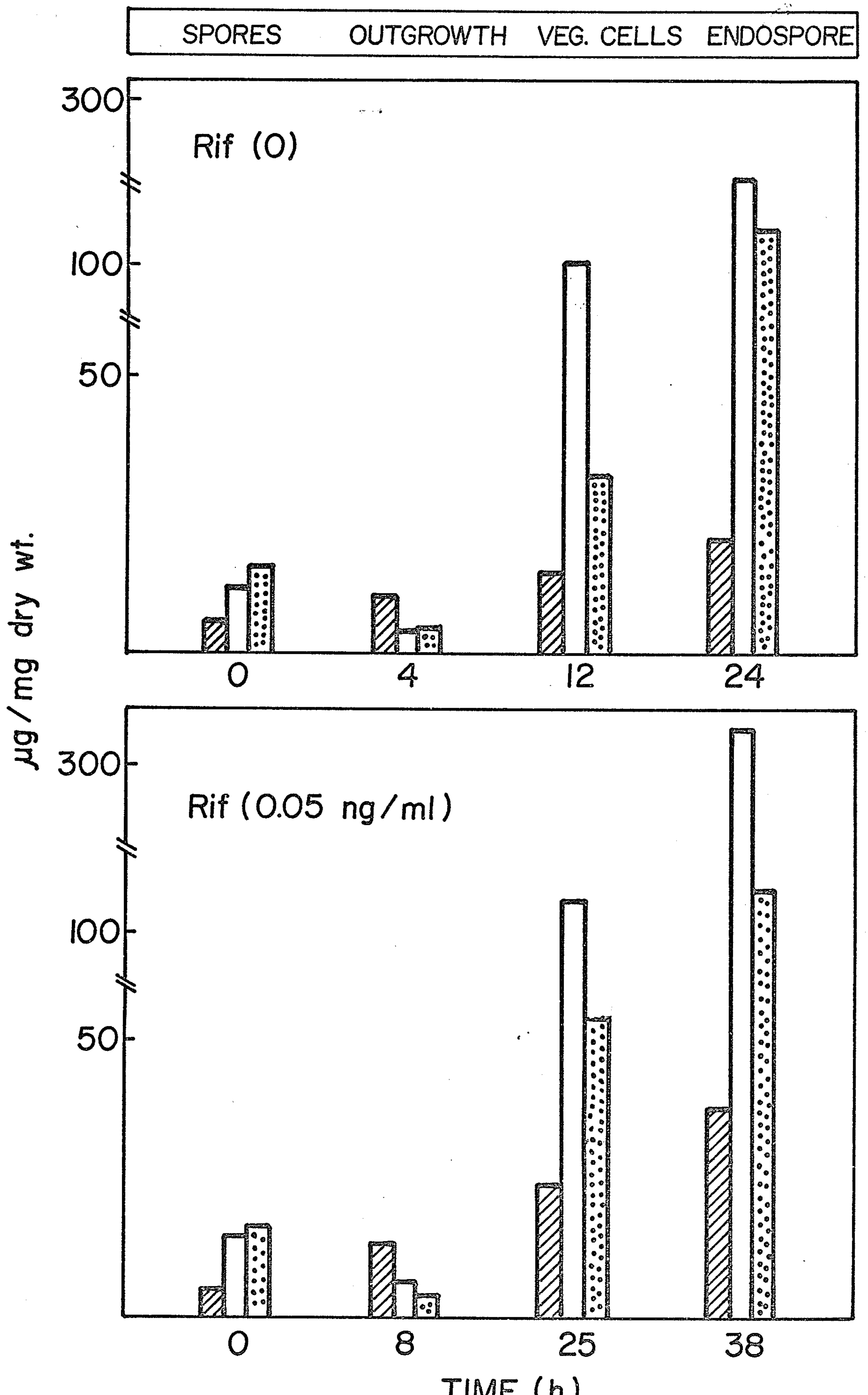
Chemical analyses of macromolecules during the developmental phases of rifampicin treated and untreated cultures of *C. botulinum*.

Stages from spore to spore cycle	DNA		RNA		PROTEIN	
	Rif (-)	Rif (+)*	Rif (-)	Rif (+)	Rif (-)	Rif (+)
Zero time	6.23#	5.46	11.30	15.95	16.21	17.17
outgrowth	9.61	14.04	3.68	6.36	4.74	4.56
100% veg.cells	15.23	25.20	105.23	140.55	32.06	59.04
100% Endo-spores	20.48	38.25	201.0	337.71	157.68	155.31

µg/mg dry weight , * 0.05 ng/ml

Figure 9. Histogram showing the changes in Macromolecules at various stages of growth cycle (data obtained from Table 3).

DNA	(hatched)
RNA	(blank)
Protein	(dotted)


SPORES
OUTGROWTH
VEG. CELLS
ENDOSPORE
300
Rif (0)
100
50
μg / mg dry wt.
0
4
12
24
300
Rif (0.05 ng/ml)
100
50
0
8
25
38
TIME (h)

PART II

The kinetics of growth and pH changes of Clostridium botulinum spores grown in the absence or the presence of glucose are shown in Fig. 10. Cultures grown in trypticase-peptone-yeast extract broth without glucose showed no signigicant changes in pH and very little increase in absorbance. However, only a few spores germinated into vegetative cells and endospores were not observed within the time period tested (25h).

Cultures grown in the presence of 0.4% glucose showed normal growth and a rapid drop in pH. Sporulation occurred at about 14h.

Effect of glucose on sporulation

The relationship between the onset of sporulation and the level of glucose in TPGY medium was investigated. When log-phase cells were used as an inoculum (Fig. 12), maximum absorbance was reached at about 10h, indicating the end of exponential phase followed by stationary phase. At the start of stationary phase, by 12h, the glucose was no longer detected and a minimum pH of 5.8 was reached. Phase bright spores (1%) were observed 6h after the begining of stationary phase.

On the other hand, when an inoculum of heat-activated spores was used (Fig. 13),although the growth rate appeared to be the same as with log-phase cell inoculum, the absorbance of the culture continued to rise throughout the life cycle. The rate of glucose utilization and resulting pH changes were much more gradual. Phase-bright spores (1%) were first observed at about 14h, long before the

end of log-phase, when only about 50% of the glucose was exhausted and 98% endospores were observed at about 24 h, by this time glucose was all used up.

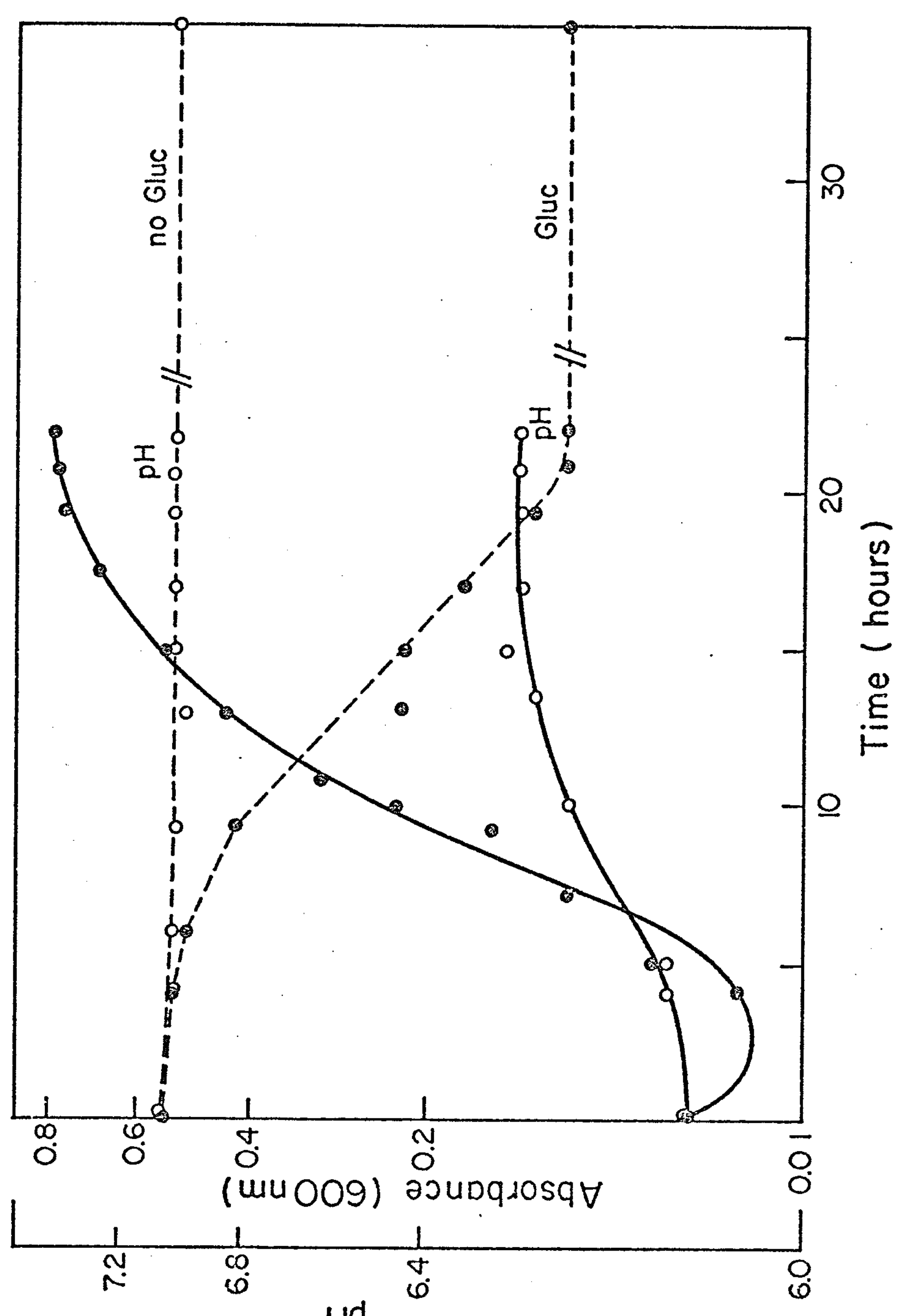
Cultures grown in the absence of yeast-extract.

When yeast-extract was omitted from the TPGY, the rate and amount of growth was reduced and sporulation was delayed for about 4 h (Fig. 11) compared with cultures containing 0.4% yeast extract.

Figure 10. Growth and pH changes in the presence or the absence of glucose.

●——● TPGY

○——○ TPY



pH
6.0
6.4
6.8
7.2
Absorbance (600 nm)
0.0
0.2
0.4
0.6
0.8
Time (hours)
10
20
30
no Gluc
pH
Gluc
pH

Figure 11. Growth and pH changes in the presence or the absence of yeast-extract.

●——● TPGY

O——O TPG

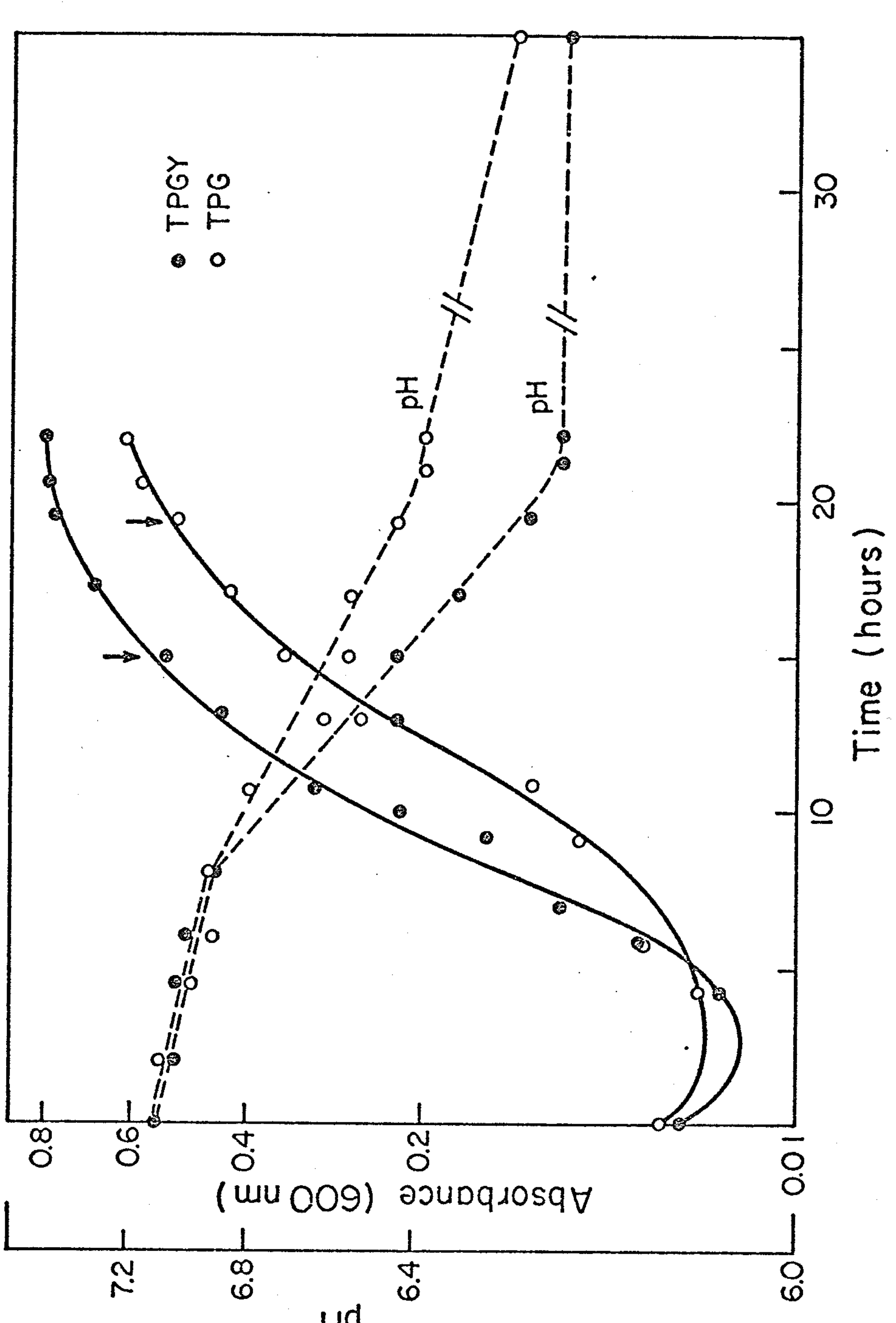
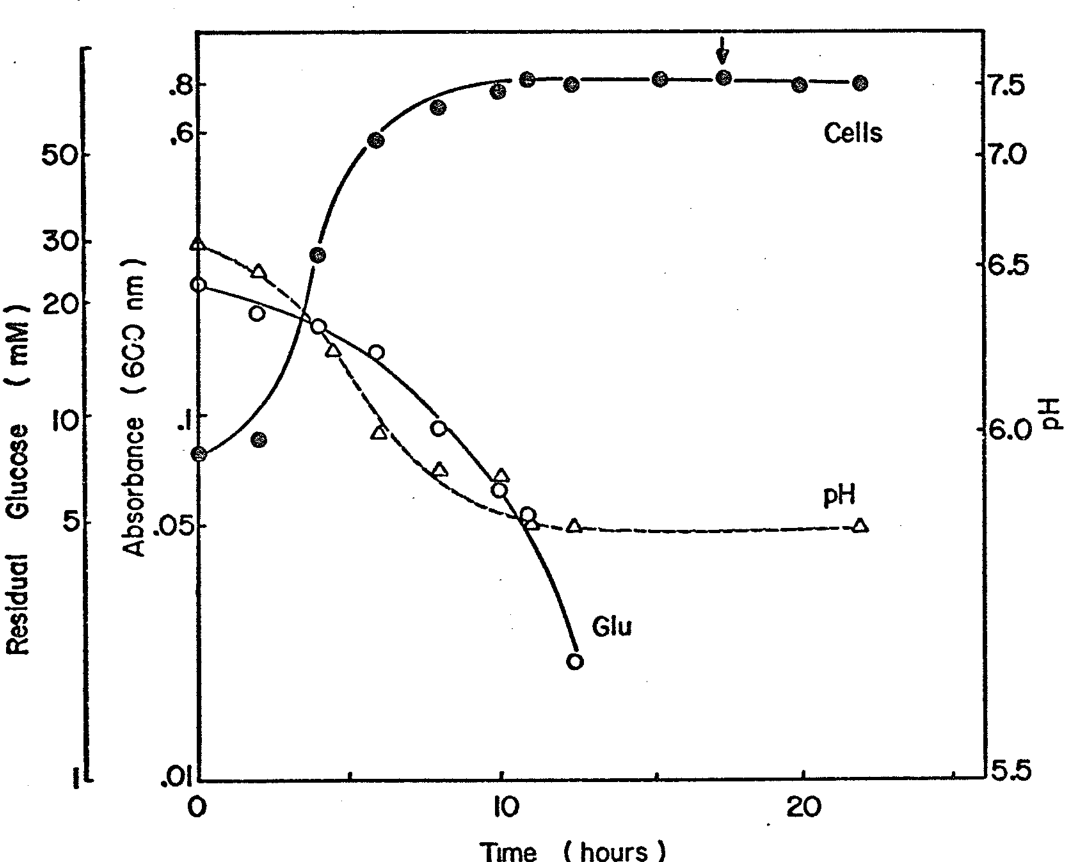

TPGY
TPG
pH
pH
Time (hours)
Absorbance (600 nm)
pH
0.01
0.2
0.4
0.6
0.8
6.0
6.4
6.8
7.2
10
20
30

Figure 12. Growth and glucose utilization of cultures of log-phase cells.

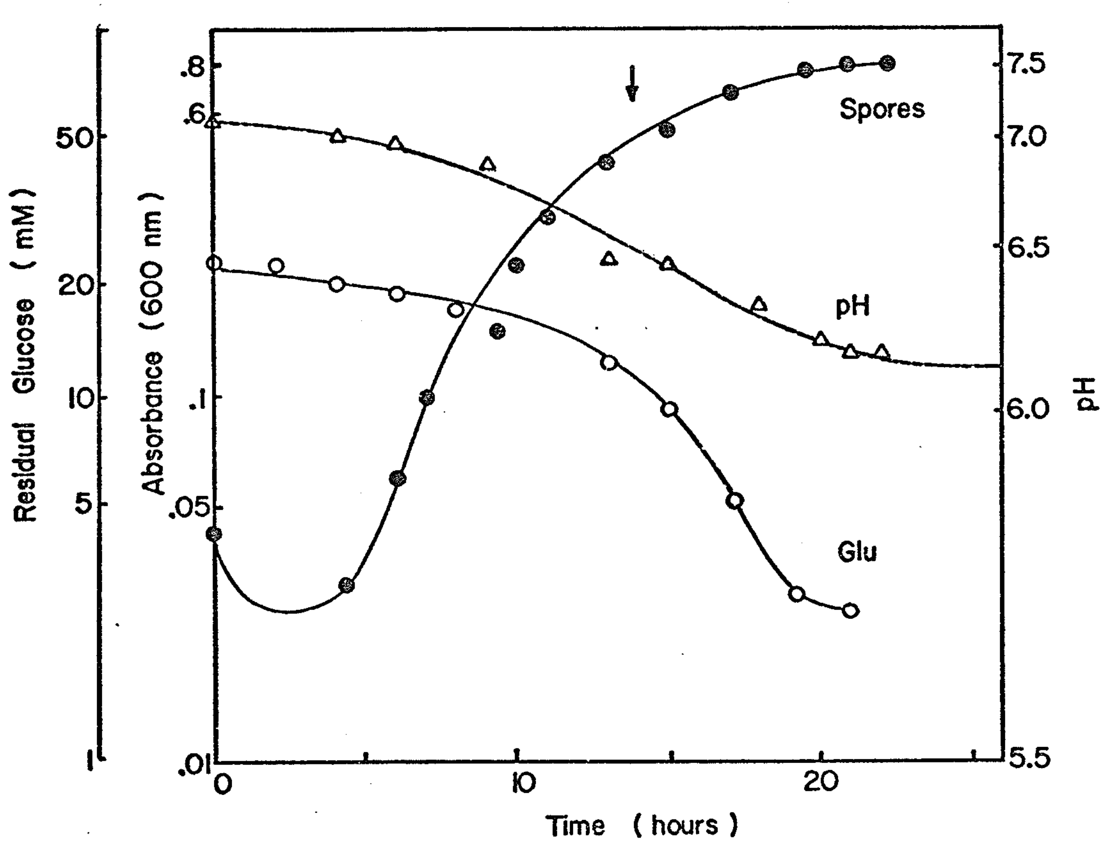
●——●	Absorbance
O——O	Residual glucose
△——△	pH changes
↓	Time of appearence of phase-bright endospores



Residual Glucose (mM)
50
30
20
10
5
1
Absorbance (600 nm)
.8
.6
.1
.05
.01
Cells
pH
Glu
7.5
7.0
6.5
6.0
5.5
pH
0
10
20
Time (hours)

Figure 13. Growth and glucose utilization of cultures of heat-activated spores

●——●	Absorbance
O——O	pH changes
Δ---Δ	Residual glucose
↓	Time of appearence of Phase-bright endospores



Residual Glucose (mM)
50
20
10
5
1
Absorbance (600 nm)
.8
.6
.1
.05
.01
Spores
pH
Glu
7.5
7.0
6.5
6.0
5.5
pH
0
10
20
Time (hours)

DISCUSSION

DISCUSSION

The activity of rifampicin, a hydrozone derivative of rifampin SV, shown to be a specific inhibitor of RNA polymerase in E.coli (34, 95) and Bacillus subtilis (26), is concentration dependent. Lancini et al (54) reported that 500 ng/ml of rifampicin completely inhibited growth of cells of E.coli K-12 whereas only 2.5 ng/ml was required to inhibit the growth of gram positive bacteria (74). Similarly, it was shown in a previous study from this laboratory that 5 ng/ml was required to inhibit sporulation of actively growing cells of Clostridium botulinum (35). However, in our experiments, outgrowth of heat-activated spores of Clostridium botulinum was completely inhibited by > 0.2 ng/ml, delayed for 36 h by 0.1 ng/ml and for ≃ 4 h by 0.05 ng/ml. It may be that the cells of the germinating spores exhibit increased sensitivity to rifampicin compared with the mature vegetative cells.

A rifampicin concentration of 0.05 ng/ml, which delayed the outgrowth for 4 h and allowed cell replication, was selected in order to monitor the selective inhibitory effect on the subsequent developmental changes.

As reported earlier (35) septum formation of some cells was blocked when the rifampin was added to exponentially growing cells. We had observed that cell replication of outgrowing cells did not occur with 0.1 ng/ml confirming that rifampicin exhibits an impairment of cell division as has been reported by Newton (65).

RIFAMPICIN

During the early part of phase I of untreated and treated cultures (Fig. 4), the "fluctuation" of ^{14}C-uracil incorporation is likely due to a rapid turnover of RNA species in germinating spores of *Clostridium botulinum* as has been reported for *Bacillus* sp. (42). The incorporation of uracil into newly synthesized RNA increased during outgrowth and cell division and then started to decrease at the end of exponential growth (Fig. 5) which is in accord with the work of Balassa (3).

The inhibition of uracil incorporation by rifamycin was reported in *Bacillus subtilis* by Calvari *et al* (9) and in *S. aureus* by Hartmann *et al* (34). As expected in rifampicin treated cells (0.05 ng/ml) RNA synthesis (Fig. 5) was markedly delayed until outgrowth and early growth occurred. Thus corroborating that continuous RNA synthesis is required for outgrowth of clostridia (77,79).

Net protein synthesis, measured by either uptake of ^{14}C-methionine or ^{14}C-amino acid mixture, in both untreated and treated cultures started shortly after the initiation of RNA synthesis and continued to increase even during sporulation phase (Fig. 8). Labbi and Duncan have also reported the net protein synthesis during sporulation of *Clostridium perfringens* (51). Since net protein synthesis was not observed in *Bacillus subtilis* (79), it may be that continued net increase of protein synthesis during sporulation of Msp^{+} (Fig. 8, Table 3) and of *C. perfringens* is associated with the high lipid content of spores of clostridia (62).

The onset of DNA synthesis of untreated and treated cells (Fig. 6) began 20 min after activation and increased exponentially and by the

time the first cell division occurred the DNA had doubled so that most cells would have contained two genomes. In Bacillus spp., several workers have reported either a slow rate of DNA synthesis which has been attributed to be the repair type (67, 97) or have been unable to detect any early DNA synthesis during spore germination (73, 93). It is difficult to explain the rapid DNA synthesis during germination of Clostridium botulinum spores. During vegetative growth, DNA synthesis continued at a rapid rate and began to decline with the initiation of sporulation. Similarly in Bacillus subtilis, Szulmajster and Camfield (81) found that DNA synthesis ended with the onset of sporulation. This provided evidence of a close relationship between the arrest of DNA synthesis and commitment to sporulation.

The data obtained from chemical analysis (Table 3, Fig. 9) have shown that more RNA than DNA was found in vegetative cells and spores. The ratio of RNA to DNA is much lower in spores ,1.81, compared to vegetative cells ,7.0, which is in accord with the reports on Bacillus (22) and this is also consistent with the relationship found between RNA content and vegetative growth (17). Similar to Bacillus subtilis and Bacillus megaterium (98, 99), the cells of Clostridium botulinum commencing sporulation contained approximately twice as much DNA as its spores. In general, the nucleic acid status appears to be dependent on the metabolic state of free spores or vegetative cells.

In this study the retardation of outgrowth and vegetative growth, i.e. phases I and II, in rifampicin treated cultures is suspected to be caused, at least partially, by a blockage of ribosome rearrangement which is a vital step prior to initiation of protein synthesis (42)

because rifampicin has recently been shown to induce deg radation of ribosomal RNA in bacterial cells (12, 13, 100). This hypothesis is supported by the appearence of diffuse ribosomal particle in rifampicin treated cells (Hawirko et al. unpublished observation) and by continuation of DNA synthesis (Fig. 7) which requires certain specific types of RNA but not active ribosomes (48).

In the second aspect of this study, the addition of yeast-extract to trypticase peptone glucose (TPG) medium allowed spore germination followed by growth and sporulation to occur at a rapid rate (Fig. 10).

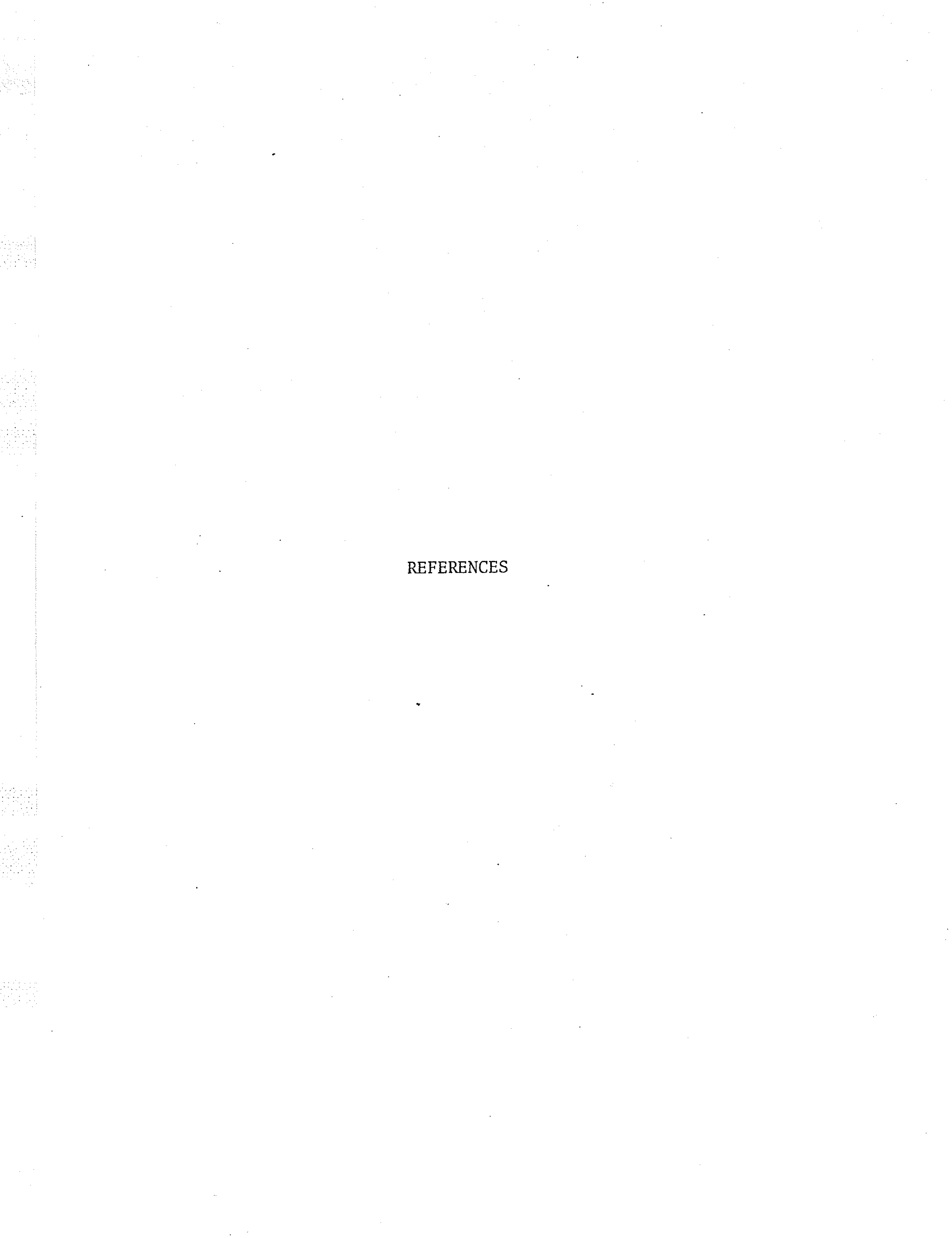
Sodium thioglycollate which was routinely added as a reducing agent has also been reported to function as chelating agent stimulating germination of spores of Clostridial spores (55). However in some cases germination of spores of C.botulinum 62-A (86) and C.bifermentans (27) was inhibited by the addition of sodium thioglycollate to hydrolysates of casein.

Glucose was essential and, for the most part, required for outgrowth and the developmental phases of Msp^+ in TPGY (Fig. 11). The effect of glucose on sporulation was shown to be concentration dependent with an upper limit of 1% (55). During the phases of vegetative growth and sporulation (Fig . 11, 13), the decrease in pH was probably due to rapid oxidation of glucose to pyruvate and acetate (15, 91) and other acids which accumulated in the medium.

In repeated experiments (Fig . 3,7,8,10,11,13), using heat-activated spores as inocula, it was noted that growth, measured at A_{600}, continued to increase after endospores were observed by phase microscopy. The growth curves did not show a distinct stationary growth phase even when

100% endospores were observed. However, when log-phase cells of Msp^+ were used as the inoculum (Fig. 12), a distinct stationary growth phase occurred and endospores were not observed until 6 h after the end of logarithemic growth.

Sporulation is, normally, initiated until after the completion of growth and the exhaustion of glucose is considered to be a necessary prerequsite for sporulation (91). The utilization of glucose during the developmental phases of a spore inoculum was compared with that of vegetative cells (Fig. 12, 13). The data showed that the presence of glucose was required for continuous vegetative growth but its absence was not essential for sporulation. Contrary to the present day concept it would appear that, under certain conditions, both vegetative growth and sporulation can occur at the same time. Studies are needed to determine whether factors such as structural residues or enzymes which are released during outgrowth of germinating spore play a role in initiating spore formation during mid-log phase.



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