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GRADUATE COLLEGE

LIPIDS AND MORPHOGENESIS OF *ARTHROBACTER CRYSTALLOPOIETES*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

RICHARD LEE MYERS

Norman, Oklahoma

1972

LIPIDS AND MORPHOGENESIS OF

ARTHROBACTER CRYSTALLOPOIETES

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TABLE OF CONTENTS

	Page
LIST OF TABLES	v
LIST OF FIGURES	vi
Chapter	
I. INTRODUCTION	1
II. MATERIALS AND METHODS	7
III. RESULTS	19
IV. DISCUSSION	101
V. SUMMARY	108
BIBLIOGRAPHY	110

LIST OF TABLES

Table		Page
1.	Composition of defined medium	13
2.	RQ and QO_2 values for various stages of growth of *Arthrobacter crystallopoietes* . . .	59
3.	Percent lipid in *Arthrobacter crystallopoietes* at various stages of growth	72
4.	Fatty acids found in lipid extracts of *A. crystallopoietes*	99

LIST OF FIGURES

Figure		Page
1.	Life cycle of A. crystallopoietes	10
2.	Lipid stains of A. crystallopoietes	22
3.	Nuclear and capsule stains of A. crystallopoietes	25
4.	Stage I cell.	28
5.	Stage I cell.	28
6.	Stage II cell	30
7.	Stage II cell	30
8.	Stage III cell.	32
9.	Stage III cell.	32
10.	Stage IV cell	34
11.	Stage IV cell showing the extent of elongation.	34
12.	Stage IV cell	36
13.	Stage IV cell	36
14.	Stage IV cell	38
15.	Stage IV cell	38
16.	Stage IV cell showing primary branching	40
17.	Stage IV cell showing primary branching	40
18.	Stage V cell	42

Figure Page

19. Stage V cell. 42

20. The effect of an increasing dose of x-ray to Stage II cells of A. crystallopoietes 46

21. The effect of 15,000 r of x-ray to cells of A. crystallopoietes at different stages of growth 48

22. Dry weight versus absorbance curves for A. crystallopoietes at various stages of the life cycle. 51

23. Specific activity of the malic enzyme at various stages of growth. 54

24. Specific activity of glucose-6-phosphate dehydrogenase at various stages of growth . . 56

25. Bar graph of the amount of oxygen utilized per unit of dry weight per hr for the five stages of growth of A. crystallopoietes . . . 61

26. Growth curve of A. crystallopoietes over a 96 hr period when grown in a pyruvate minimal salts (PMS) and a succinate minimal salts (SMS) medium. 64

27. Thin-layer chromatogram of the neutral lipid fraction of readily extracted lipids of A. crystallopoietes 68

28. Extraction procedure for lipid fractions extracted from A. crystallopoietes. 75

29. Gas chromatogram of crude lipids extracted from Stage IV of A. crystallopoietes. 78

30. Gas chromatogram of base hydrolyzed crude lipids extracted from Stage IV of A. crystallopoietes. 82

31. Gas chromatogram of crude lipids extracted from Stage V cells of A. crystallopoietes . . 85

32. Gas chromatogram of base hydrolyzed crude lipids extracted from Stage V of A. crystallopoietes. 87

Figure Page

33. Gas chromatogram of crude lipids extracted from Stage IV cells which were grown in SMS 90

34. Gas chromatogram of base hydrolyzed crude lipids extracted from Stage IV cells which were grown in GMS 92

35. Gas chromatogram of crude lipids extracted from Stage IV cells which were grown in GMS 95

36. Gas chromatogram of base hydrolyzed crude lipids of Stage IV cells which were grown in GMS. 97

LIPIDS AND MORPHOGENESIS OF *ARTHROBACTER CRYSTALLOPOIETES*

CHAPTER I

INTRODUCTION

Arthrobacter crystallopoietes is a soil bacterium of the family *Corynebacteraceae* and was first isolated and described by Ensign (26). This organism exhibits the phenomenon of morphogenesis. Upon inoculation into a complete growth medium, the spherical cells germinate, elongate and often branch. These elongated cells form multiple cross walls and divide into many smaller rod-shaped cells which eventually round up to form spherical cells. The process has been referred to as sphere-rod morphogenesis. Early investigators of species in the genus *Arthrobacter* used the terms "pleomorphism" and "diphtheroid" (10) and failed to recognize the defined life cycle exhibited by this genus.

Division of hyphal cells has been referred to as fragmentation in microorganisms with similar life cycles (8), and this term also has been used to describe cell division of long rods of *A. crystallopoietes* to smaller entities (27). Life

cycles of other members of Arthrobacter have been reported to be similar (13) as well as that of Nocardia corallina (75).

The synchrony of cellular morphogenesis makes this organism particularly suitable for physiological studies of regulatory mechanisms which are associated with morphogenesis. The fact that it is a procaryotic rather than a eucaryotic organism makes it an appealing experimental system. In our laboratory several phenomena related to morphogenesis have been studied. Ferdinandus (29) studied changes in specific activity of several key enzymes as a function of the life cycle. Massey (51) used this organism in hybridization experiments to study transcription and found evidence for different mRNA in rods and spheres. While studying methylation, Dalbow (17) found that the activity of the t-RNA methylases changes during the growth cycle and also found minor changes in the types of protein synthesized. Gillespie (31, 32) studied cell wall differences in A. globiformis at two different stages of growth.

Ensign and Wolfe (27) showed that morphogenesis could be controlled nutritionally. In a defined medium, the microorganism can be restricted to growth and division entirely in the coccoidal form, and the addition of inducers results in the formation of the rod stage. This fact made it possible to investigate morphogenetic changes on a biochemical basis in a system under controlled conditions.

Ferdinandus (29) suggested that A. crystallopoietes stored fats early in the life cycle and later used these as

endogenous energy for crosswall formation and division of elongated rods. Endogenous metabolites in bacteria have been discussed in a number of review articles (19, 20, 60). Endogenous respiration usually refers to the total metabolic reactions that occur within the living cell when it is held in the absence of compounds or elements which may serve as specific exogenous substrates. However, endogenous metabolism may take place in the presence of exogenous substrates. For example, glucose permease is inhibited by succinate, making it impossible for the microorganism to utilize exogenous glucose (40). Endogenous metabolism which is occurring at a time when a high energy demand must be met (fragmentation, for example) is different from endogenous metabolism occurring in an aging culture where cellular constituents are metabolized due to a starvation situation. Ensign (28) and Boylen and Ensign (6) studied starving cells of A. crystallopoietes in phosphate buffer at 30 C. Cells which were harvested both as rods and spheres were 100% viable after 30 days suggesting that endogenous metabolism was functioning. However, no mention was made of lipid as an endogenous reserve material.

O'Leary (58) suggested that there are four general ways in which bacterial lipids could function: energy storage, structural components, active transport, and involvement in biosynthetic activities. Studies on cellular components of A. crystallopoietes such as protein, RNA, DNA and a glycogen-like

polysaccharide have been conducted, but lipids have never been characterized. Bacterial lipids are very complex and diversified, and bacteria contain numerous lipids not seen in higher forms. Lipids are known to be stored in certain yeasts as shown by Mulder and co-workers (55), and microscopic studies have shown lipid (sudanophilic) deposits in certain bacteria, many of which are composed of poly-β-hydroxybutyrate (PHB).

In bacteria which contain cytoplasmic granules of PHB, this lipid has been shown to serve as a source of energy. PHB is a storage product unique to bacteria, and has been shown to accumulate in spore forming bacteria which also undergo a form of morphogenesis. In Sphaerotilus species, PHB accumulates during active growth and decreases rapidly at the end of exponential phase of growth (61) and has been studied in terms of its effect on starvation survival (68).

Mulder and Zevenhuizen (56) studied organic compounds which accumulate in the cells of certain bacteria. They showed that Arthrobacter accumulated large amounts of carbohydrate. However, not all of this carbohydrate could be shown to be a substrate for endogenous respiration. Zevenhuizen (77) found a glycogen-like structure in Arthrobacter which was used as a substrate for endogenous respiration and therefore a source of energy. Mulder and Zevenhuizen (56) further elucidated this glycogen compound; however, no mention was made of lipid storage products. Stored glycogen has been shown to

serve as a reserve food source in Aerobacter aerogenes (69) and also Escherichia coli (21).

In most bacteria, lipids are concentrated in the outer layers of the cell, especially the cytoplasmic membrane, rather than in the cytoplasm. Lipid inclusions may be formed in some genera, but membrane lipids are not usually used as energy storage. Hunter and Thirkell (34) studied the effect of age on the fatty acid composition of membrane lipid fractions from Sarcina flava. They found a variation which indicated a high turnover of fatty acids. Agate and Vishiniac (1) looked at the effect of the stage of growth on the lipids of Thiobacillus neapolitanus. Changes in lipid composition during growth is not by any means limited to bacteria. Madariaga, et al. (48) studied fatty acid distribution and composition at various stages in the development of the insect, Dacus oleae.

The question of the ability of arthrobacters to maintain themselves in soil where other microflora cannot survive has yet to be satisfactorily explained. Perhaps the arthrobacters are somewhat resistant to starvation, a condition which has been studied by Ensign (28).

In the present work an attempt was made to determine some aspects of endogenous metabolism and the role of certain lipids as a function of the life cycle of A. crystallopoietes. Lipids were studied not only from the aspect of endogenous respiration, but also to gain a broader insight into, or lead to further studies on the phenomenon of morphogenesis.

In addition to endogenous metabolism and lipid composition studies, a microscopic study of the organism was done using both light and electron microscopy. The fact that lipids are important constituents of the cell regardless of what function they serve seemed to justify the microscopic examination. Thin sections of the various stages of growth were examined with particular emphasis on areas which could be attributed to stored fat. Mesosomes and mesosomal development were carefully observed, and their role in the life cycle, especially fragmentation, was studied. The overall cytology of A. crystallopoietes was also studied with the light microscope to correlate changes in metabolism (especially that related to lipids) to morphological change.

A growth study is reported for cultural methods employed in this work. This complemented the study of endogenous metabolism and was necessary to obtain a broader insight into the morphological changes occurring in this microorganism.

A report is given in the text which describes the response of A. crystallopoietes to x-ray. This approach revealed information concerning fragmentation and division of the rods into smaller units.

CHAPTER II

MATERIALS AND METHODS

Organism

Arthrobacter crystallopoietes ATCC 15481 cultures were maintained on BBL tryptone glucose yeast extract (TGY) agar or on 8.5 g/l concentration of Bacto plate count agar. Transfers were made to slants of these media at 48 hr intervals and incubated at 30 C.

For all experiments where a complete medium was required, TGY or plate count broth was used. Broth (500 ml) was inoculated from a stock culture and incubated on a New Brunswick model VS rotary shaker at 150 rpm for 24 hr. Inoculating cultures were transferred into medium of the same composition to a reading of 0.04 absorbance at 485 nm on a Bausch and Lomb Spectronic 20. Inoculating cultures were always entirely coccoidal cells. Inoculating cultures are defined as 24 hr coccoidal cells which have completed the life cycle in broth and were prepared specifically as inoculum. Inoculum for defined media was handled in the same way.

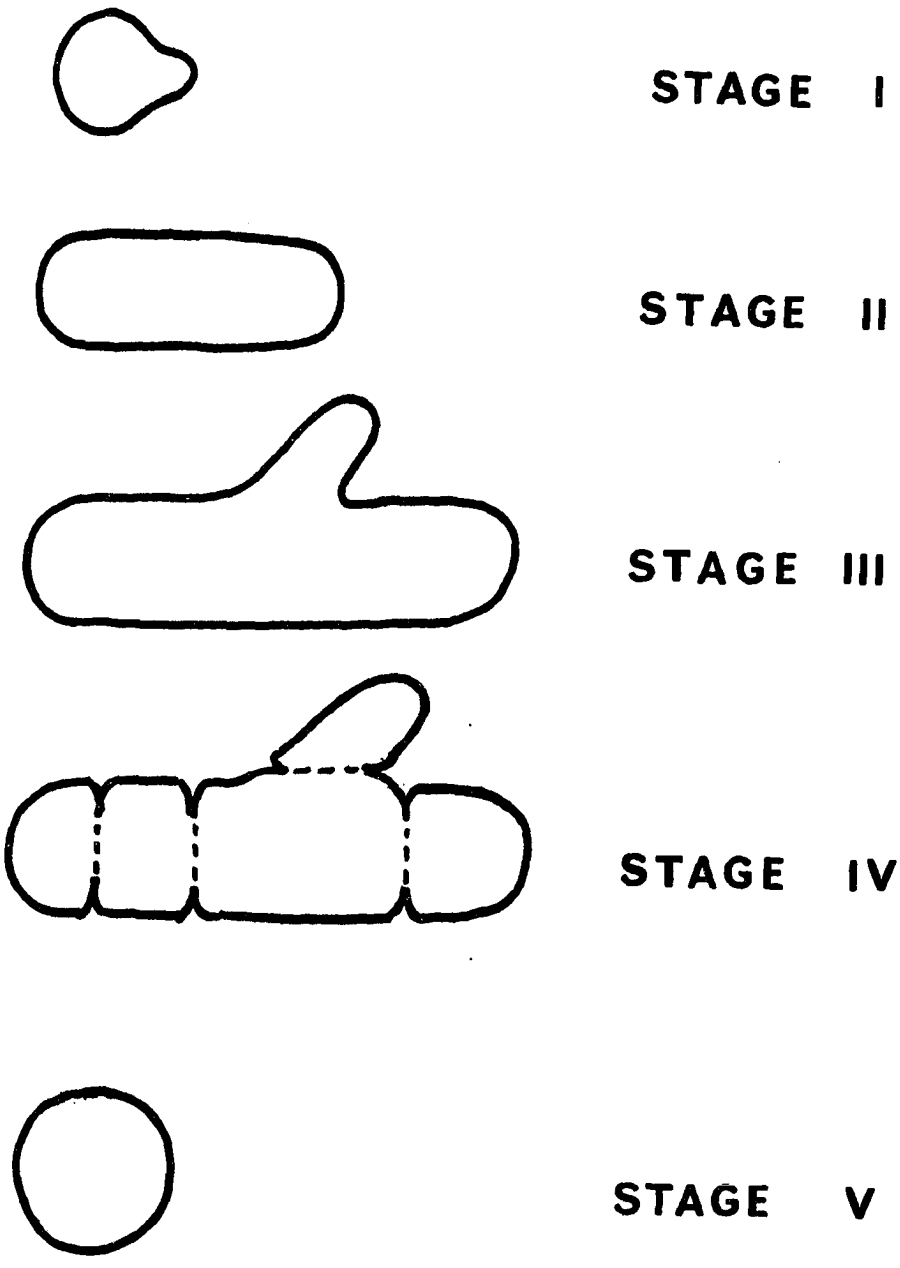
Life cycle

The life cycle of *A. crystallopoietes* has been

documented in both complete and defined media (27), but various workers have manipulated the life cycle depending upon their particular phase of work. For our study five morphological stages were chosen and designated Stage I, II, III, IV and V. Stage I represents cells which are in the process of germination and are 2-3 hr old and Stage II (5-6 hr) are short hyphal cells. Stage III (11-12 hr) are long hyphal cells showing primary branching and the beginning of cell division. Secondary branching has not been observed in these microorganisms. Stage IV (15-16 hr) is the point at which fragmentation (cell division) is actively taking place, and at Stage V (24 hr) the cells are once again coccoidal. The life cycle is depicted in Figure 1. Throughout the growth cycle the culture has an earthy odor, and although a capsule is present throughout the life cycle, after 24 hr the capsule develops to the point where it produces a very sticky consistency in the culture. There is also a strong odor of ammonia in 24 hr cultures.

Dry weight versus absorbance determinations were done with cells washed 3 times in buffer made up to a known absorbance, and known dilutions made of this. Determinations were made either of two ways: (1) by evaporation of a diluted cell suspension of known absorbance to dryness in aluminum weighing dishes or, (2) cell suspensions of known absorbance were filtered through Millipore filters (HAWP 02500 HA 0.45μ 25 mm) which was then taken to dryness. In both

Figure 1.--Life cycle of A. crystallopoietes. Stages I-V represent phases of growth used to elucidate the life cycle.


STAGE I
STAGE II
STAGE III
STAGE IV
STAGE V

cases evaporation of the water was accomplished by drying overnight at 100 C *in vacuo*. The cell dry weight was then determined.

Microscopic studies

To observe a growing culture, simple stains were made using crystal violet and observed with a light microscope. Particular stages of growth were determined in this way. Throughout the life cycle, photomicrographs were taken to record characteristics of the microorganism and also to document the various stages of growth. All photomicrographs were taken with a Leitz Orthomat camera attached to a Leitz Ortholux microscope.

For electron microscope studies, cells were fixed and stained by the method of Lancaster and Skvarla (44). Cells at various stages of the life cycle were washed twice in pH 7.6 phosphate buffer and then collected on Millipore filters (0.45μ 47 mm) and placed immediately in 2.5% glutaraldehyde diluted with Sorenson's phosphate buffer, pH 7.6. This fixation step took 24 hr. The cells were then placed in osmium tetroxide (1.0% in Sorenson's phosphate buffer with 4.5% dextrose) for 3 hr. Dehydration was accomplished in a graded series of ethyl alcohol dilutions and then acetone. The acetone dehydration was done in test tubes since the filter was soluble in acetone.

The cells were embedded in Epon Araldite and sectioned with a Porter-Blum MT-2 Ultramicrotome with a diamond knife

and picked up on 200-mesh copper grids. The sections were stained with 0.5% uranyl acetate for 20 min and then Venable's lead citrate for 3-5 min. The stained sections were observed in a Philip's EM 200 electron microscope.

Media

For certain experiments cells were needed in large quantities. Since the life cycle could be better controlled in broth, inoculum was prepared as previously described, and the bacteria grown in broth in 500 ml Erlenmeyer culture flasks to various stages on a rotary shaker and then centrifuged at 6,000 x g on a Sorvall model GLC-1 centrifuge. These were washed twice in 0.2 M phosphate buffer, pH 7.6, or 0.2 M tris (hydroxymethyl-amino-methane (Tris)-chloride) buffer, pH 7.6. Cells were then frozen in 10 ml of buffer until needed except for enzymatic studies where cells were used directly.

The composition of defined media used in growth and manometric studies is given in Table I. Either glucose with minimal salts (GMS) or succinate with minimal salts (SMS) was used with glucose and succinate serving as carbon sources. Succinate also served as an inducer of morphogenesis. Ammonium sulfate, magnesium sulfate and buffer were mixed and stored in 15 l carboys. Carbon source and trace salts were added as media were needed, and in this manner precipitation of magnesium as the insoluble phosphate was minimized. The complete medium was then filter sterilized through Millipore

TABLE I

COMPOSITION OF DEFINED MEDIUM (v/v)

1.	Trace salts solution	1%
	$CaCl_2 \cdot 2H_2O$--0.2%	
	$MnSO_4 \cdot H_2O$ --0.1%	
	$FeSO_4 \cdot 7H_2O$--0.05%	
2.	Glucose (20% solution)	0.5%
	Succinate (10% solution)	0.5%
3.	$(NH_4)_2SO_4$	0.1%
4.	$MgSO_4$	0.05%
5.	KPO_4 buffer (pH 7.0, 0.03M)	

membrane filters (0.45μ 47 mm). Five hundred ml of this medium was dispensed into 2 l Erlenmeyer culture flasks and inoculated in the same manner as TGY.

The succinate was prepared in the following manner. A 10% solution of succinic acid in water was prepared and since crystalline succinic acid is only slightly soluble in water, concentrated ammonium hydroxide was added dropwise until the pH of the solution reached 7.0. At this point all the succinic acid was in solution. The additional nitrogen from ammonia had no detectable effect on the life cycle.

Cell enumeration

During certain phases of this work, it was necessary to determine numbers of bacteria present in a suspension. Dilution plating on spread plates was the method chosen for the enumeration of cells. Cells were diluted using buffer as blanks and plated on TGY agar and were counted at the end of 2.5 days incubation. Triplicate plates showed good agreement at each stage of the life cycle. For comparison, direct microscopic counts were made using a Petroff-Hausser counting chamber which showed very good agreement with the plate counts. Microscopic counts showed minimal clumping; therefore, it was not considered a factor in plate count results.

X-ray studies

Studies using x-ray were conducted using a 250 maximum KV unit with up to 15 MA. Cells to be irradiated were

placed in a glass vessel with agitation, and depth of the cell suspension kept at a minimum so that all cells had an equal chance to receive an identical dose. For most studies the cells were placed at a distance of 5.5 to 6.0 cm from the window of the x-ray unit in order to obtain the specified dose. Dosimetry was measured with a Victoreen roentgen rate meter.

Enzyme studies

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was determined by measuring the reduction of NADP by glucose-6-phosphate at 340 nm (42) and the malic enzyme (EC 1.1.1.40) activity was determined by measuring the reduction of NADP by malate at 340 nm (57). Enzyme assays were measured on a Gilford model 2000 recording spectrophotometer at 25 C.

Cell lysis

Cells were grown to the appropriate stage, collected, and diluted with 0.1 M Tris buffer, pH 7.6. Cell walls were digested with lysozyme (EC 3.2.1.17) at a concentration of 200 μg per ml for suspensions containing 30 mg wet weight bacteria per ml. This was incubated in a 37 C water bath for 45 min to effect complete lysis. At this point, the mixture was very viscous, and microscopic examination revealed greater than 98% lysis of cells. The lysate was centrifuged in a Sorvall RC2-B Superspeed refrigerated centrifuge at 2,000 x g for 5 min to remove cells that had not been lysed.

Deoxyribonuclease (EC 3.1.4.5) was added to the thick, gel-like lysate (30 µg/ml) to reduce the viscosity. Treatment was effected for 30 min at 37 C and resulted in a marked reduction in viscosity. The membranes were then deposited by centrifugation at 20,000 x g in a refrigerated centrifuge at 4 C, separated from the lysate, and both used for lipid determination. Lipids were taken from cell sap by extraction with petroleum ether in a separatory funnel and then treated as described previously.

Thin-layer and gas chromatography

Separation of lipid classes on plates covered with silicic acid was accomplished by virtue of their differences in polarity which depends primarily on the type and number of functional groups in the molecules. Separation of the substances and the reproducibility of the results depended upon the degree of saturation of the chamber. Best results were obtained when porous paper board was placed in the chamber and the board and chamber supersaturated. TLC plates were spotted in different concentrations (10-60 µl) using µl pipettes and a Research Specialties model 1260 sample applicator. For preparative plates as much as 2.0 mg of crude lipid were spotted. Thin-layer chromatograms were developed in solvent, removed from the chromatographic tanks and air dried for 30 min at room temperature.

Fatty acids were analyzed in a gas chromatograph as methyl esters with methyl esters of standards and unknown

fatty acids prepared by the method of Metcalfe and Schmitz (52). The free fatty acid is not required for esterification to take place since the methylating agent will react directly with most complex lipids, but lipids were subjected to preliminary saponification in some cases to free all fatty acids. Esterification was accomplished by heating the acids in a boron trifluoride-methanol reagent on a steam bath followed by extraction with petroleum ether. The extract was concentrated by evaporation at 60 C in a rotary film evaporator.

The residue was taken up in n-hexane and 1 μl of this injected into a Packard model 409 gas chromatograph. A Hamilton 10 microliter syringe was used for injection. The gas chromatograph was equipped with flame ionization detectors and either glass (0.2 mm inside diameter by 6 ft length) or stainless steel (0.15 mm inside diameter by 6 ft length) columns were used. Glass columns were packed with Chromosorb W 80/100 mesh (AW-DMCS) coated with 10% DEGS (Supelco, Inc.), and stainless steel columns were packed with Chromosorb W 100/120 mesh (AW-DMCS) coated with 10% SP-1000 (Supelco, Inc.)

The instrument was operated isothermally at 190 C in most instances, but to separate low molecular weight fatty acids, 150 C was occasionally used. Volatilization loss of short chain fatty acids methyl esters may have occurred. The temperature was programmed in some cases for a linear increase of 20 C per minute from 100 to 190 C. The detector temperature was 260 C; the injector temperature was 270 C; the

electrometer attenuation varied depending upon the concentration of lipids in the samples. Nitrogen was used as the carrier gas at a flow rate of 50 cc per minute. A recorder input signal of 1 mV was used with a chart speed of 1 inch per min. Tentative identification was made by comparing retention times of unknown compounds with those of known standards.

Techniques

Protein was determined by the method of Lowry et al. (46) using bovine serum albumin as standard, and manometric studies were determined using the manometric techniques of Umbreit et al. (70). Buffers were prepared according to need using different formulae (18, 33).

Protein was detected on thin-layer chromatograms with 0.2% ninhydrin in n-butanol and by the method of Stein-Moore (54). Phospholipids were detected on thin-layer chromatograms with molybdenum spray by the method of Dittmer and Lester (22), and alpha glycols were detected by the periodate-Schiff reagents of Shaw (64).

Chemicals and enzymes

Standary methyl ester fatty acids were obtained from Applied Science Labs, Inc. DPNH used for enzyme studies and lysozyme for membrane extractions were obtained from Sigma. DNase was obtained from Worthington Biochemical Corp.

CHAPTER III

RESULTS

Since acetate is a precursor of lipids, various concentrations of acetate were added to complex growth media to determine if the presence of acetate would promote formation of fat bodies. No difference was noted, nor did it affect the life cycle to any significant degree. Excess glucose was added to TGY media for the same purpose, but no increase in sudanophilic inclusions was noted in either medium. However, with excess glucose, hyphal growth was delayed with a concurrent increase in absorbance which indicated increase in numbers of coccoidal cells. Ensign and Wolfe (27) have shown that a defined medium with glucose as the only carbon source will not support the typical developmental cycle, and succinate will trigger the morphogenetic cycle in Arthrobacter. Recently, Luscombe and Gray (47) declared that morphology of an Arthrobacter isolate is related to specific growth rate, and that the change from cocci to rods does not require a specific inducer. However, morphogenesis can be triggered in other microorganisms simply by the addition of one or more

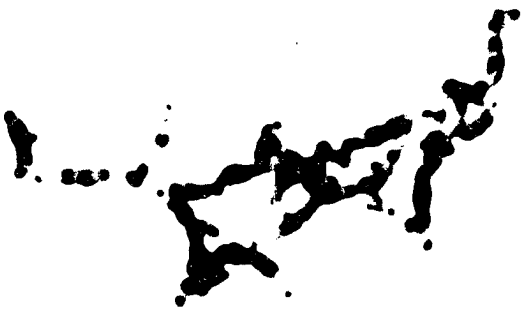
substrates. For example, Divordin and Sadler (25) showed that morphogenesis in Myxococcus xanthus could be induced by the addition of glycerol.

I. Cytological Study

The problem of substrates for endogenous metabolism in A. crystallopoietes was studied first by considering morphology of the microorganism by means of differential staining techniques. Photomicrographs were taken of cells stained with Burdon's fat stain (16) in order to show fat bodies, if present. Figure 2 shows fat stains of this microorganism prior to cross-wall formation. Ferdinandus (29) reported increases in stainable fat material prior to fragmentation in A. crystallopoietes and evidently the darker areas in Figure 2 are the areas he considered. Excessive branching is seen at this stage, and therefore this is the point at which considerable energy is needed by the microorganism for fragmentation. Clark and Aldridge (14) reported the appearance of fat inclusions in N. corallina, a microorganism that undergoes morphogenesis much like that of Arthrobacter. It must be kept in mind, however, that microscopic observations like those reported here are not based on precise cytochemistry.

In order to elucidate further the morphology of the microorganism, capsule stains were prepared by the method of Anthony (16). Arthrobacter has a capsule throughout the life cycle, but after 24 hr incubation a very prominent capsule is formed around the coccoidal cells, and the entire culture

Figure 2.--Lipid stains of A. crystallopoietes. Lipophilic areas are darkly stained. Note excessive branching (arrows). x 5,000.







takes on a slimy consistency. Photomicrographs of capsule stains of Stage III cells are shown in Figure 4. A metachromatic granule stain was prepared by the method of Ljubinsky and also with methylene blue (16), and it was determined that the inclusions were not metachromatic granules.

Nuclear stains of A. crystallopoietes were prepared by the method of Chance (11), and were done to watch the development of nuclei prior to cross-wall formation and fragmentation. Figure 3 shows nuclear stains of the first 3 stages in the life cycle. The cells go from a coccus with a single nucleus to a rod-shaped microorganism with from 2-8 nuclei per cell just prior to fragmentation.

II. Ultrastructure Study

Cells representing the various stages of the growth cycle were fixed and stained, sectioned and examined by electron microscopy. There has been no report on the internal organization of A. crystallopoietes, but Stevenson (67) studied the fine structure of A. pascens. Few attempts have been made to study morphogenesis by studying development of this microorganism with thin sections. This was done to show fine structures which might be implicated in the morphogenetic process.

Stage I cells demonstrate coccoidal enlargement as seen in Figures 4 and 5. Internal structures are seen in the form of developing mesosomes. Figures 6 and 7 show Stage II cells which are elongated with the rod stage evident. This is

Figure 3.--Nuclear stains and also a capsule stain of A. crystallopoietes. Darker areas are the nuclear regions (N). (C) capsule is labelled. x 5,000.

C

the only stage where areas were seen on the photomicrographs of thin sections that could actually be attributed to lipids. Stage III and Stage IV cells are shown in Figures 8-19. At these stages branching is seen and is specifically shown in Figures 16 and 17. There is considerable variation in the morphology of rods which is also seen in the fat stains of Figure 2. Figures 18 and 19 show Stage V cells to be coccoidal once again.

Mesosomes appear as early as Stage I and are convoluted and tubular in nature. The cell wall is also a discrete entity with a plasma membrane in close proximity. The cytoplasm appears as a dense particulate region.

The mesosomes continue to occur more frequently as the cells begin elongation, and at Stage III they are prominent structures. At Stage III and IV the beginning of cross septa are seen indicating that A. crystallopoietes divides through centripetal growth of cross-walls. These can be seen in various stages of completion in Figures 10 and 11. The actively dividing cells occasionally show a second or third septa before the first division is complete.

In stationary cells (Figures 18 and 19), there are few mesosomes present, and when seen, are only rudimentary membranous structures. Upon inoculation into fresh media they become more numerous and differentiate into well-defined, organized structures. During Stages III and IV these mesosomes seem to be closely associated with areas of new cell wall

Figure 4.--Stage I cell. Developing mesosomes (M) are seen. The cytoplasm appears as a densely stained area. x 95,500.

Figure 5.--Stage I cell. Developing mesosomes (M) are evident. x 87,000.

M

M

Figure 6.--Stage II cell. Lipid (L) makes up a large percentage of the inclusions at this stage. x 95,500.

Figure 7.--Stage II cell. The cell wall (CW) and plasma membrane (PM) are shown in this figure. Lipid (L) is also present. x 88,200.


L

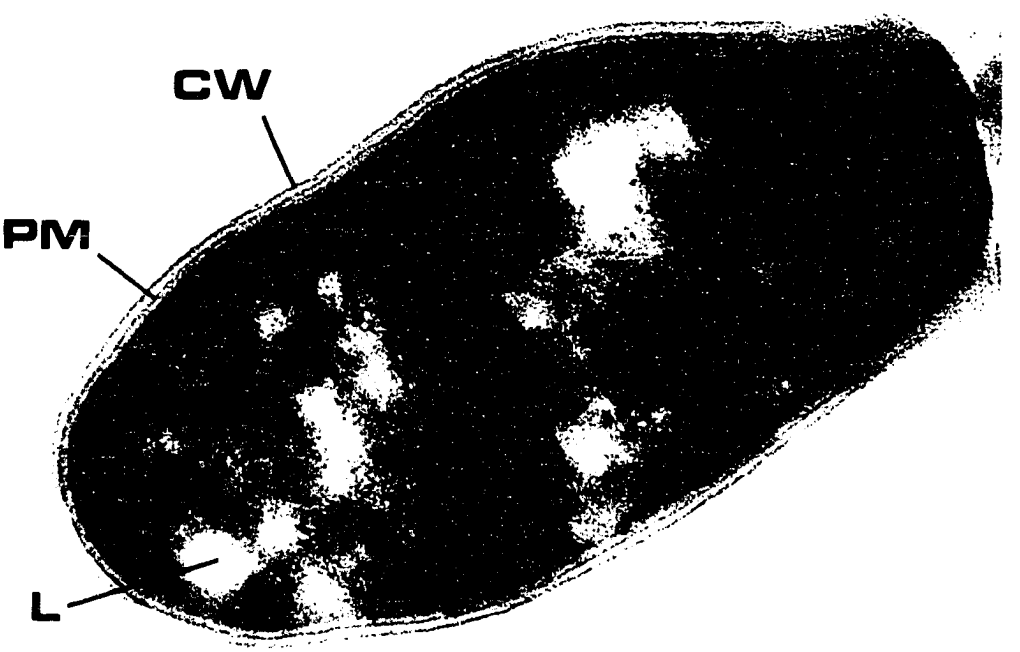

CW
PM
L

Figure 8.--Stage III cell. Elongation and division of the cell have begun. x 51,000.

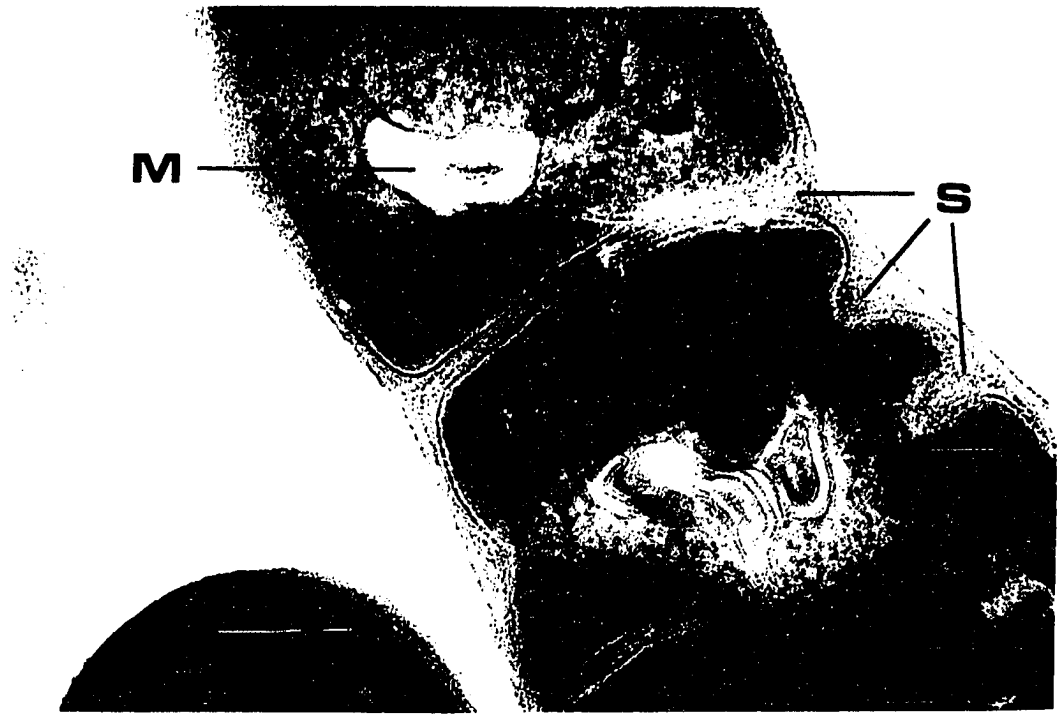
Figure 9.--Stage III cell. Numerous mesosomes (M) are seen as the elongated cell begins to divide. As many as 5 cells may result from the division of this cell as determined by the septa (S) being formed. x 50,400.

N
L

S
M

Figure 10.--Stage IV cell. These are long hyphal cells that have begun to fragment. Numerous mesosomes (M) are seen, and invaginations have begun at several points as evidenced by septa (S). x 129,000.

Figure 11.--Stage IV cell showing the extent of elongation. Fragmentation is evident. A nuclear region (N) is labelled. x 27,000.


M
S


N

Figure 12.--Stage IV cell. The mesosome (M) is closely associated with the septum (S). x 97,000.

Figure 13.--Stage IV cell. The cell wall (CW) and plasma membrane (PM) are clearly defined. A mesosome (M) appears near the area of septum formation.


S

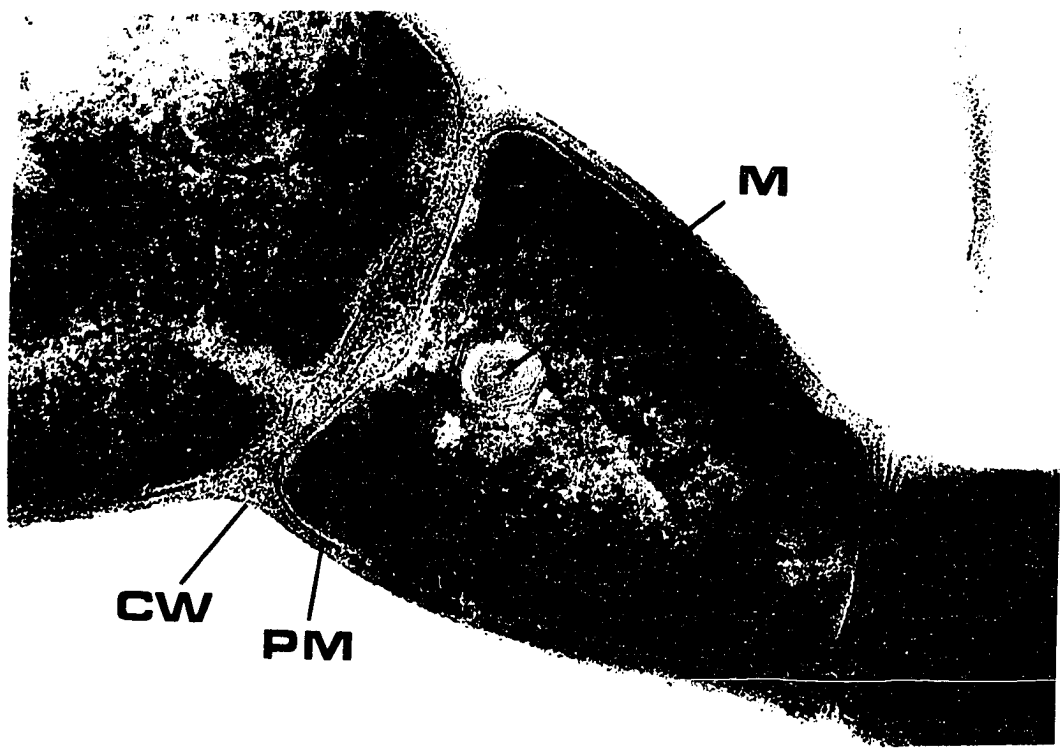
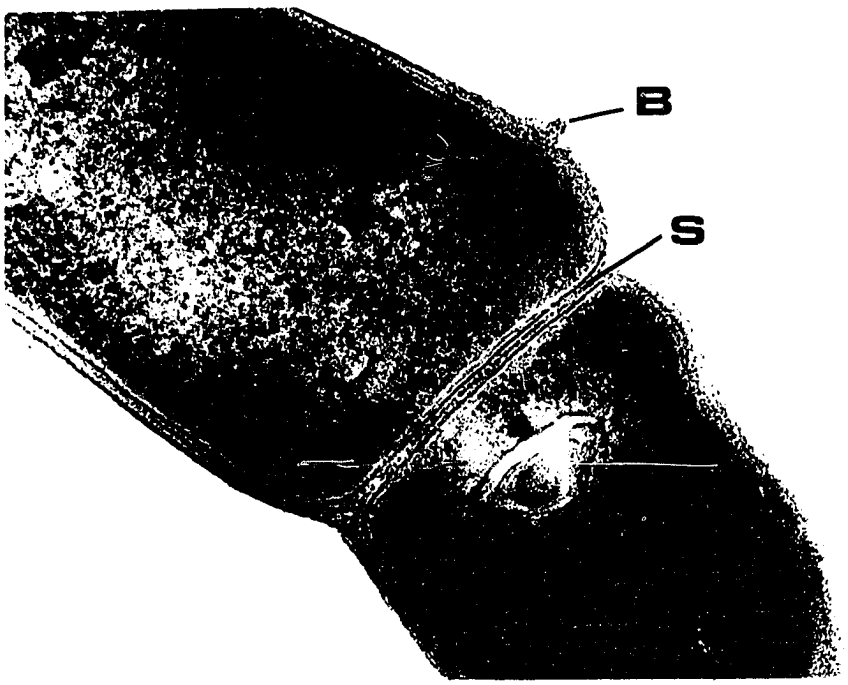

M
CW
PM

Figure 14.--Stage IV cell. Cell division in the final stages. The "burr" (B) is seen as snapping division progresses. x 77,910.

Figure 15.--Stage IV cell. Invagination and formation of the cross-wall in cell division. x 73,500.


B
S

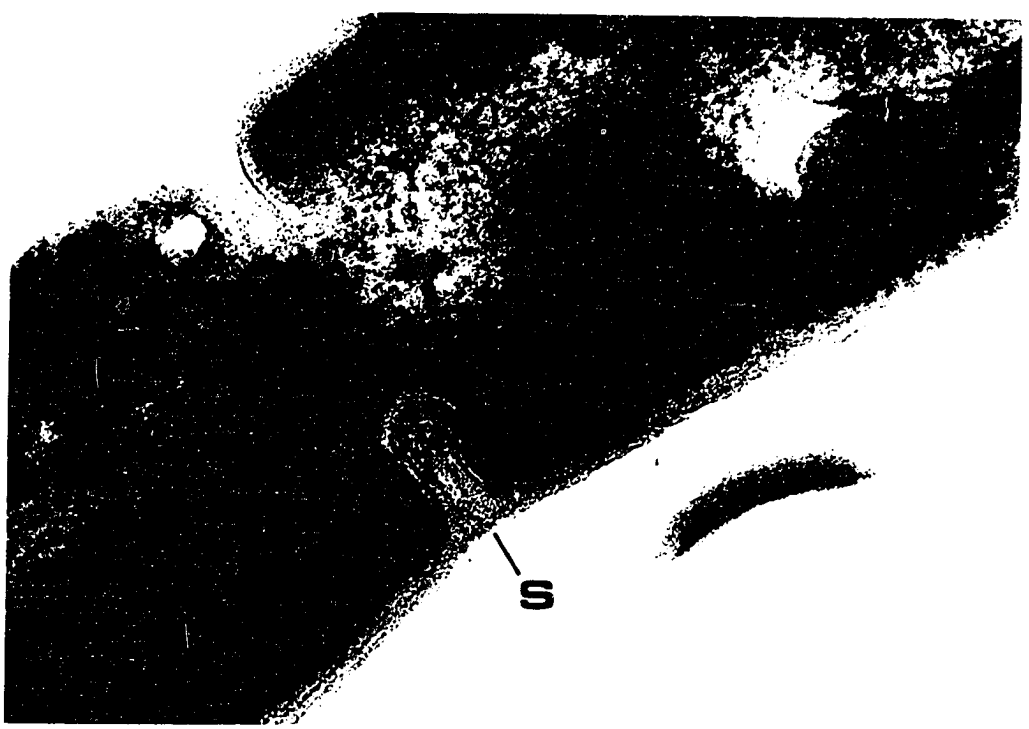

S

Figure 16.--Stage IV cell showing primary branching (arrow) as well as fragmentation. x 19,000.

Figure 17.--Stage IV cell showing primary branching (arrow). x 42,500.



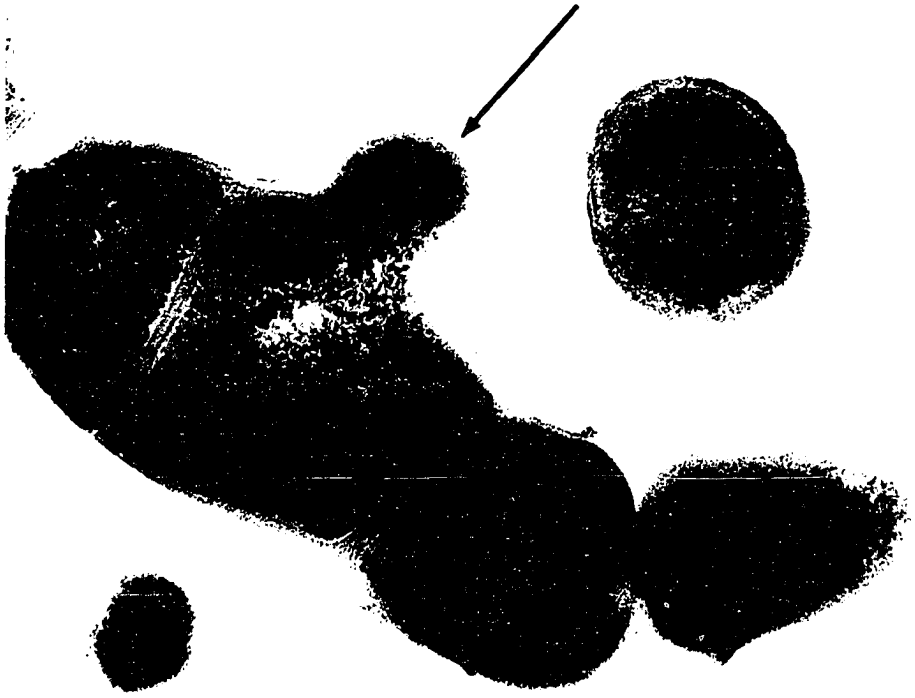
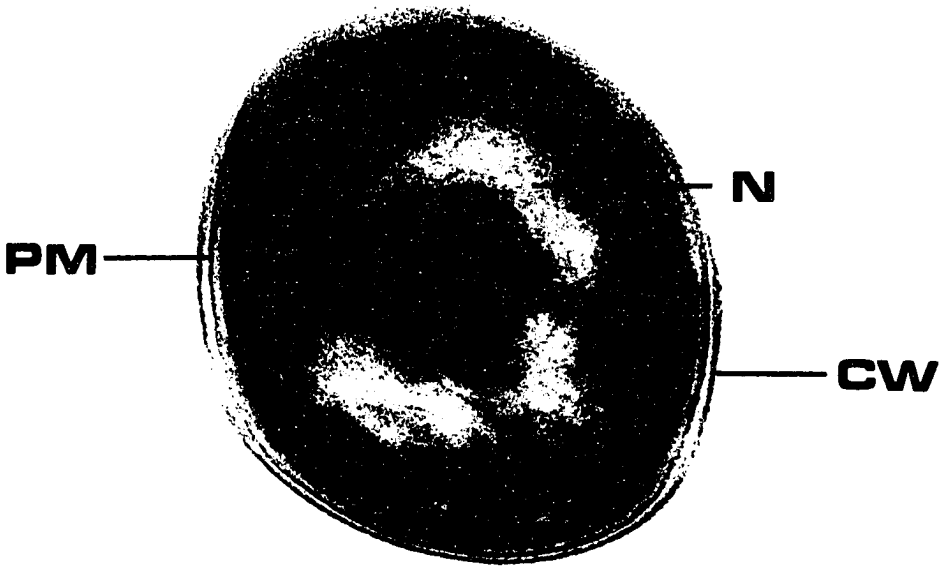


Figure 18.--Stage V cell. At this stage the microorganism has completed the life cycle and is once again coccoidal. x 73,500.

Figure 19.--Stage V cell. No mesomal structures are obvious at this stage, but a well defined nuclear region (N) is seen as well as a differential between the cell wall (CW) and plasma membrane (PM). x 73,500.




N
PM
CW

synthesis. It has been suggested that in some microorganisms the mature mesosomes have a role in cross-wall formation as well as regulatory functions in the cell. The actual roles the mesosome plays is still unresolved, but due to its association with areas of cross-wall formation, it seems plausible to assign to it a role in cross-wall formation.

Mesosomes have been the topic of study in other microorganisms such as Chondrococcus columnaris (59). Here the mesosomes appeared as highly organized structures produced by the invagination and proliferation of the plasma membrane. There is no reason to believe that Arthrobacter varies from this model.

Snapping division is seen at Stage IV and appears as a V-shaped apposition that occurs when dividing cells remain attached only by the newly formed septum. This process is seen in Figure 14. A "burr" is seen which represents areas where the outer cell wall has broken apart. Kurlwich and Pate (41) have investigated the ultrastructure of dividing rods and their study revealed two layers with separation of the outer layer being responsible for the burr.

It can be seen from a study of photomicrographs that at only one stage of the life cycle (Stage II) did areas appear that could definitely be called lipid deposits. They appear as light areas in a densely stained cytoplasm, and at subsequent stages no lipid deposits per se were detected. Mesosomes are so numerous at certain stages of the growth

cycle that they might possibly appear to be inclusions under the light microscope when the cells have been treated with lipophilic dyes since membranes have long been known to be partially composed of lipids. In many cases what appear to be lipid inclusions are actually accretions of non-lipid substances covered with a thin veneer of lipid, and it is this lipid veneer that is stained with lipophilic dyes (43). Perhaps the lipid stains which have been done on A. crystallopoietes, particularly Stages III and IV, stain the lipid-rich mesosomes and cause these to appear as large fat accumulations.

III. Radiation Studies

Radiation studies were done on A. crystallopoietes to determine viability of the microorganism when exposed to x-ray. Figure 20 shows the result of increasing dose upon cells which had just begun elongation. A 99% inactivation was obtained by a dose of 27,500 r, and the curve is not a typical inactivation curve expected for single cell microorganisms or microorganisms with a single nucleus, but on the contrary, it is the type curve expected of cells with more than one nucleus. Webb (74) found this also to be the case for N. corallina.

Cells at various stages were exposed to x-ray at 200 KV, 15 MA and a dose rate of 500 r per min. Each stage was exposed for 30 min for a total of 15,000 r and were compared to an unirradiated control. These results expressed as percent survivors are given in Figure 21. Stage I cells were

Figure 20.--The effect of an increasing dose of x-ray to Stage II cells of A. crystallopoietes.

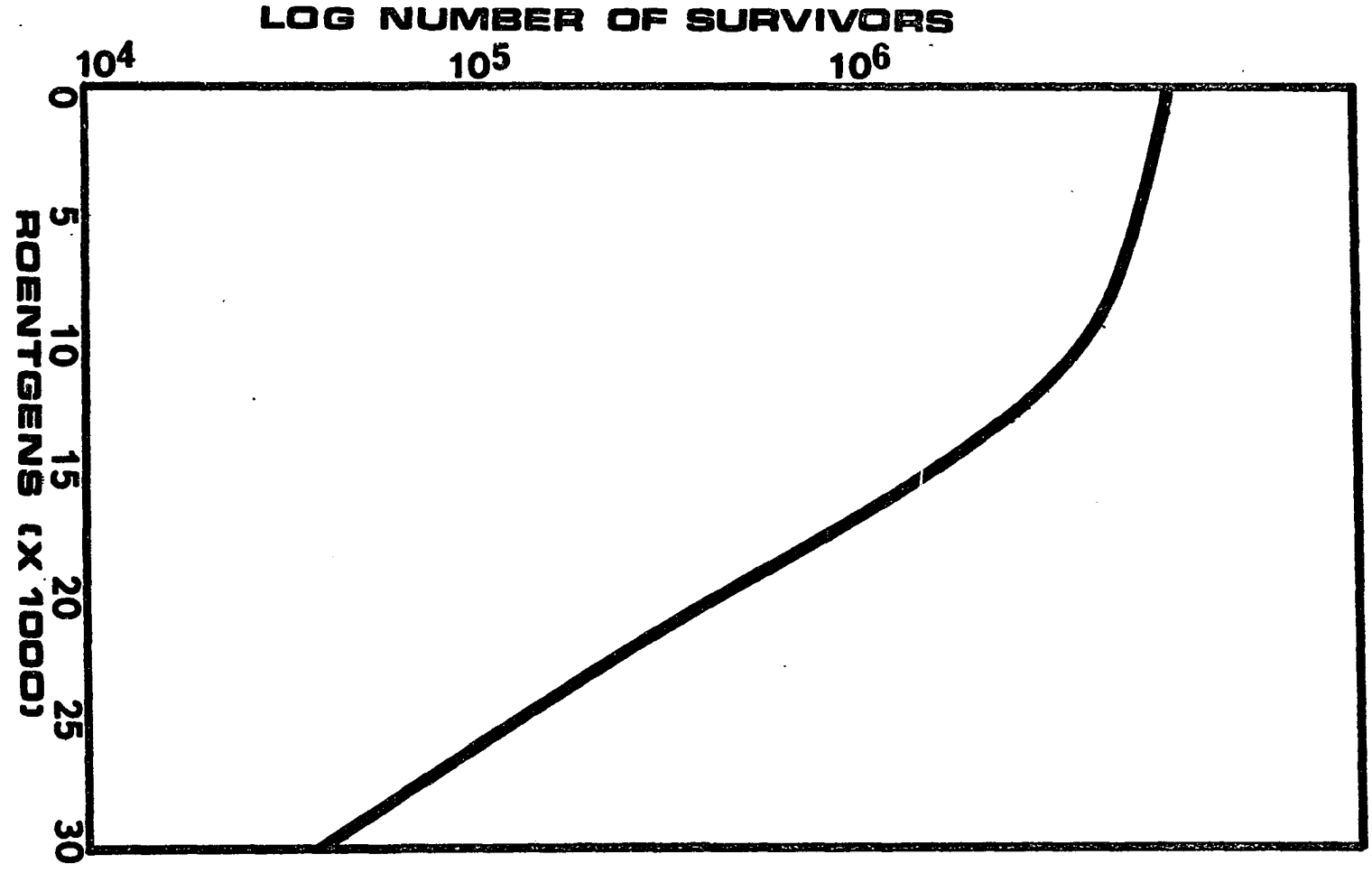
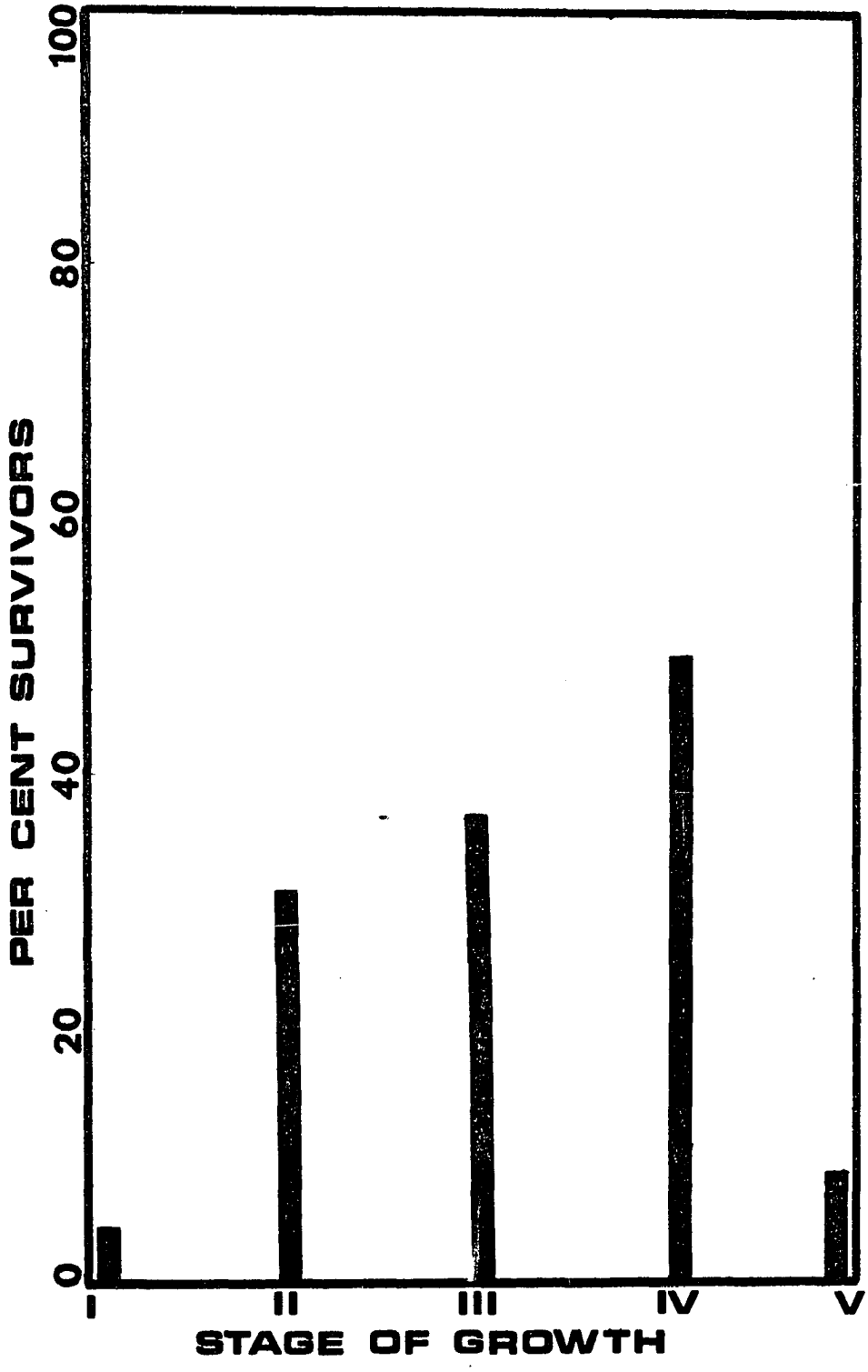

LOG NUMBER OF SURVIVORS
10^4
10^5
10^6
0
5
10
15
20
25
30
ROENTGENS (X 1000)

Figure 21.--The effect of 15,000 r of x-ray to cells of A. crystallopoietes at different stages of growth.


PER CENT SURVIVORS
0
20
40
60
80
100
I
II
III
IV
V
STAGE OF GROWTH

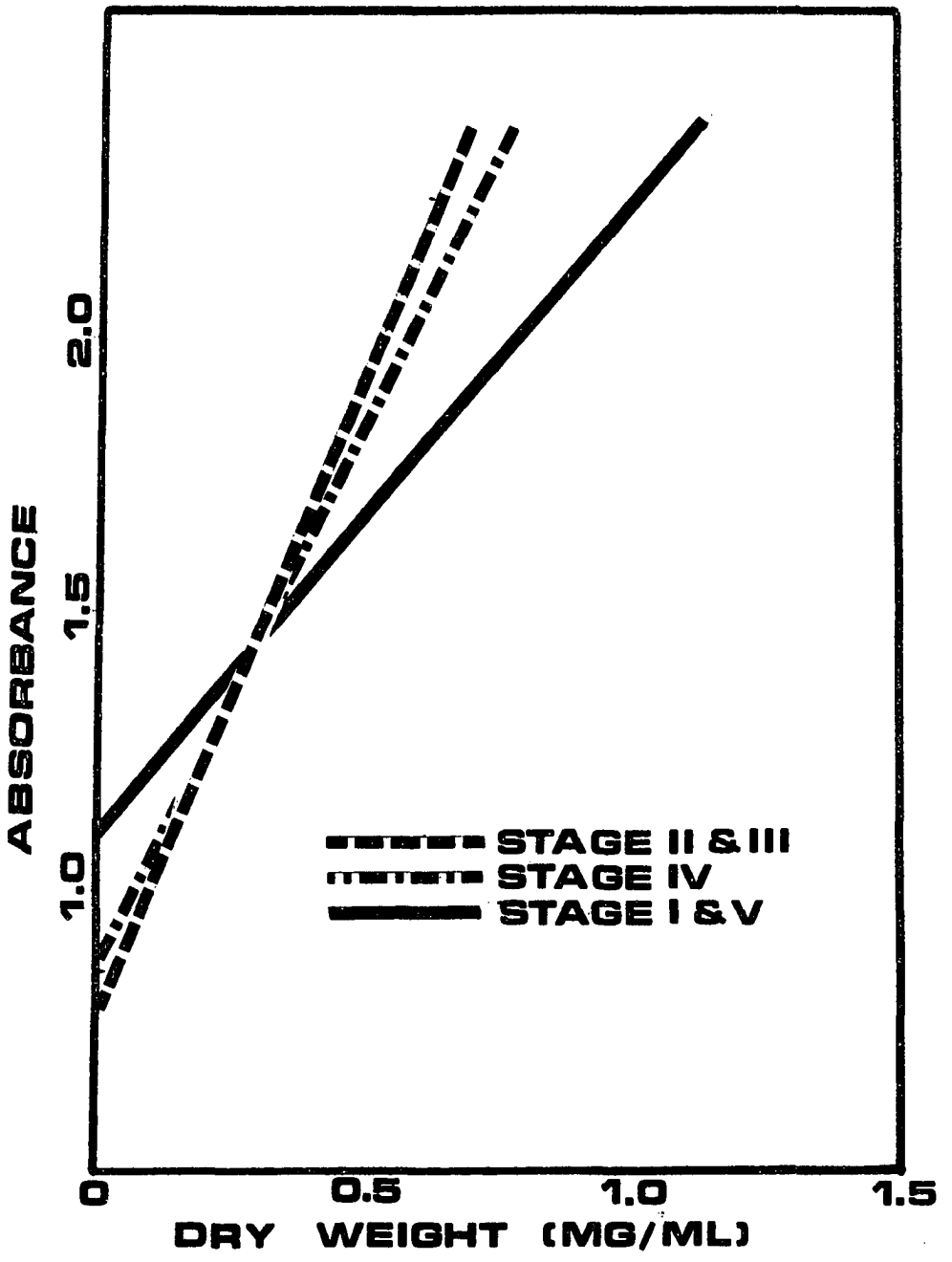
very susceptible to x-ray with a very small percent of the population surviving 15,000 r. As the cells progressed through the life cycle, resistance increased to the point of fragmentation, and then decreased.

By exposing various stages of the life cycle to x-ray, we were able to determine an accurate time at which nuclear division supplies the hyphal cells with more than one functional nucleus. This was the point at which the fragmentation stage was nearing completion and therefore external energy sources depleted. An increase in survival rate was seen. As daughter cells became viable as independent units, this was evident on the growth curve by a rise in numbers, but at the same time, percent inactivation increased. Experiments with x-ray were used to mark specific stages in the life cycle.

IV. Enzymatic and Growth Study

A. crystallopoietes was assayed by two different methods to determine if the sudanophilic inclusions were possibly PHB. Readings obtained were compared to dry weight determinations which were made at various stages of growth and an attempt was made to correlate the amount of PHB, if present, to the dry weight of cells. Dry weight versus absorbance determinations are given in Figure 22. These results are important for several experiments run on A. crystallopoietes, because cell mass changes drastically per unit cell because of

Figure 22.--Dry weight versus absorbance (485 nm) curves for A. crystallopoietes at various stages of the life cycle.


ABSORBANCE
2.0
1.5
1.0
0
0.5
1.0
1.5
DRY WEIGHT (MG/ML)
STAGE II & III
STAGE IV
STAGE I & V

cell differentiation during morphogenesis, and dry weight gives a parameter to which a comparison can be made.

No PHB was detectable in cells of any age of A. crystallopoietes. PHB, therefore, is not the constituent of the inclusions in this microorganism which have been attributed to fat. Boylen and Ensign (6) also recently reported that PHB was not found. Sobek et al. (66) showed that Azotobacter agilis had lower PHB levels in cells from succinate-grown cultures as opposed to cells from glucose-grown cultures.

Specific activity of two key enzymes that have been implicated in the metabolism of fats was studied. The malic enzyme and glucose-6-phosphate dehydrogenase have been associated with lipogenesis, and Ferdinandus (29) has suggested that rod-formation is closely associated with increased lipogenesis. The activities of these enzymes have been reported for A. crystallopoietes by Ferdinandus, and are discussed here as a function of the life cycle as seen using growth conditions as described previously.

Specific activities of the enzymes in question are given in Figures 23 and 24 for cells grown in SMS, the medium which allows A. crystallopoietes to go through the life cycle. There was an increase of both enzymes to hyphal growth and thereafter a decrease. This decrease began long before Stage IV, the time at which lipogenesis should reach a peak if, in fact, lipids are stored for use during fragmentation. If lipogenesis is occurring for the purpose of fat storage, then

Figure 23.--Specific activity of the malic enzyme at various stages of growth.

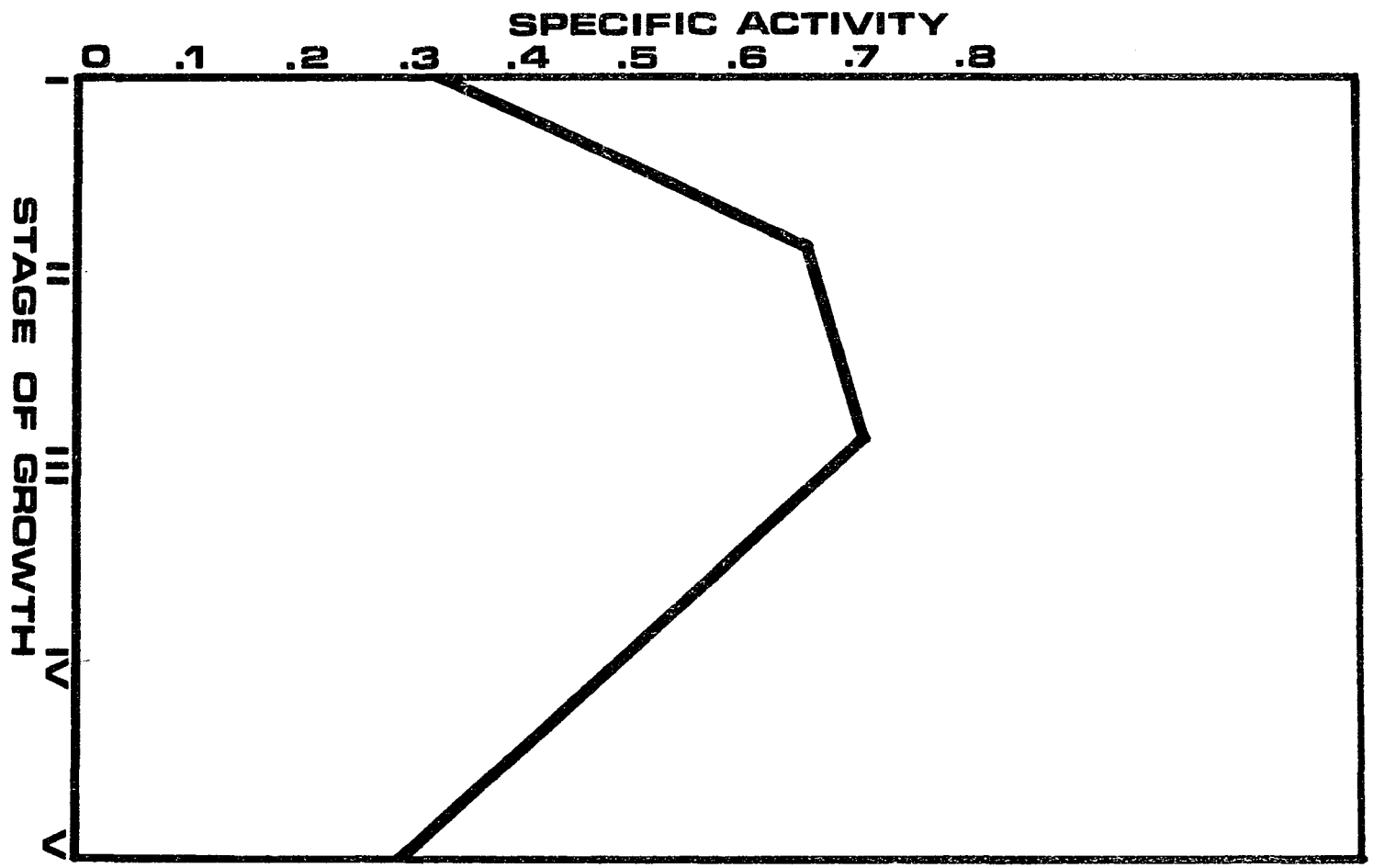
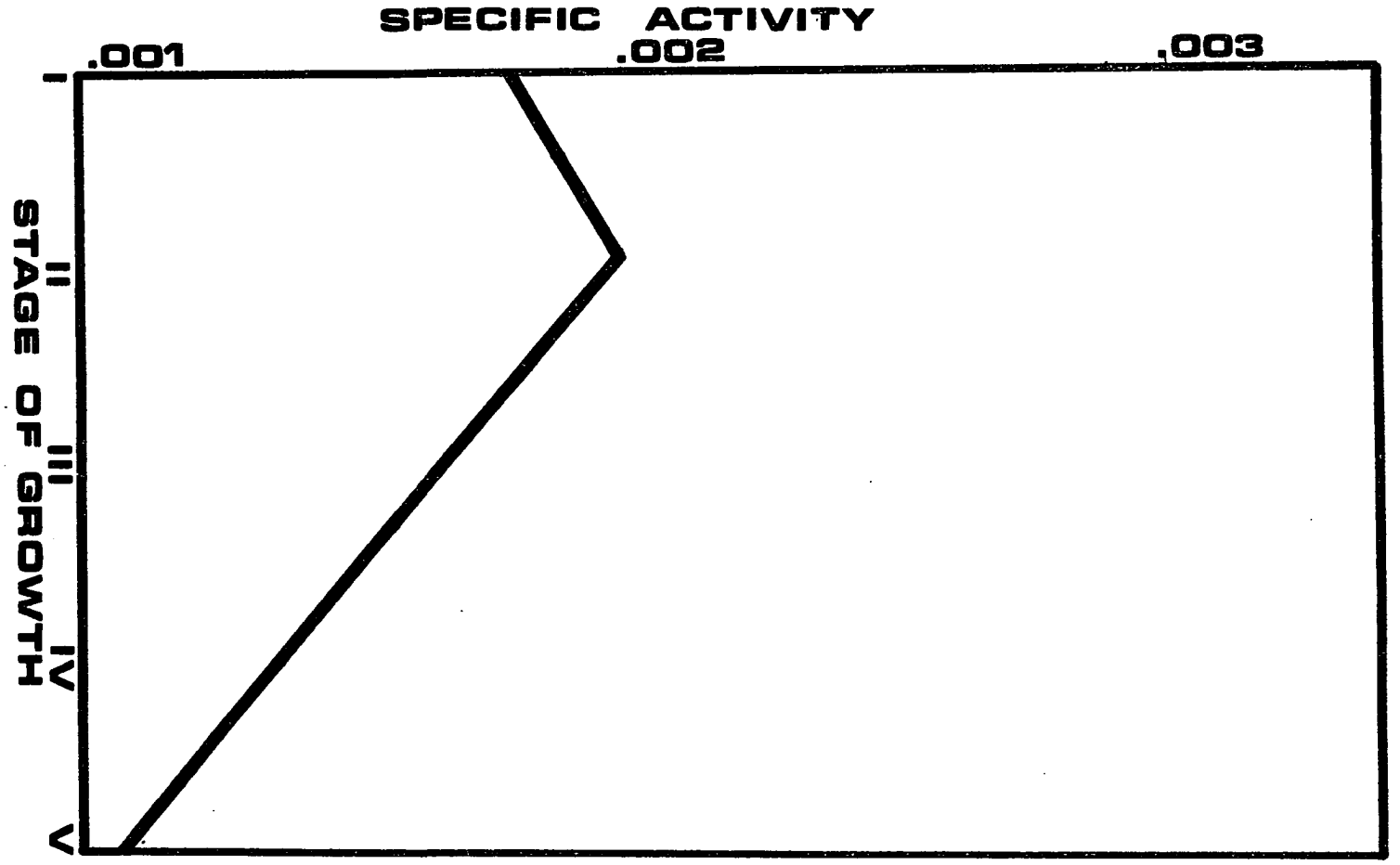

SPECIFIC ACTIVITY
0
.1
.2
.3
.4
.5
.6
.7
.8
STAGE OF GROWTH
I
II
III
IV
V

Figure 24.--Specific activity of glucose-6-phosphate dehydrogenase at various stages of growth.


SPECIFIC ACTIVITY
.001
.002
.003
STAGE OF GROWTH
I
II
III
IV
V

this storage is complete long before fats should be used for energy. The peak enzyme activity coincided with the disappearance of fat after Stage II as seen in photomicrographs.

In GMS, which kept growth in the coccoidal state, the malic enzyme was not present, and the age of the culture had no effect on specific activity of glucose-6-phosphate dehydrogenase. This supports the results reported by Ferdinandus. Bland et al. (5) recently showed that Myxococcus xanthus synthesized malate synthase de novo as the organism converts to microcysts. This conversion also is considered to be cellular morphogenesis.

A study of endogenous respiration was initiated in order to give some idea of substrates utilized. Warburg constant volume respirometers were used to determine oxygen uptake and carbon dioxide production. A respiratory quotient (RQ) was determined and served to indicate the nature of the metabolism since the RQ is an index of the processes occurring in endogenous respiration.

At the various stages of cell differentiation, cells were centrifuged out of suspension, washed 3 times in potassium phosphate or tris buffer (pH 7.6), and the amount of cells added to the respirometer determined by absorbance readings on a Bausch and Lomb Spectronic 20. Standard curves based on dry weight had previously been prepared. A 10% solution of KOH was used to saturate fluted filter paper in the center well. Washed cells (0.2 ml) and 0.2 M phosphate buffer

(0.3 ml) were added to the vessel. RQ and QO_2 values for the five stages are given in Table II. Microliters of oxygen taken up per mg dry weight per hr at 30 C is given in Figure 25. This represents endogenous respiration.

From Table II it can be seen that the RQ values for Stages II, III and IV suggest that these cells utilized carbohydrates for endogenous respiration rather than lipids, and Stages I and V, which are entirely coccoidal cells, more likely utilize lipids. When 72 hr old cells were tested for endogenous QO_2 values, it remained approximately unaltered from the Stage V value. Perhaps this has some relationship to secondary growth that is seen in old cultures of Arthrobacter which will be discussed later. Figure 25 shows that endogenous respiration was highest at Stage V. During the time of most active metabolism (Stage II-IV), the endogenous respiration was almost constant. Cell suspensions of N. corallina have been studied manometrically to determine endogenous respiration (53).

Different concentrations of succinate were put into SMS to determine what effect, if any, the concentration of succinate had on the life cycle and lipid inclusions. All growth flasks were inoculated with Stage V cells grown on SMS medium, and results showed concentrations of from 0.005% succinate to 0.5% succinate made little difference on the amount of lipid over a period of 48 hr.

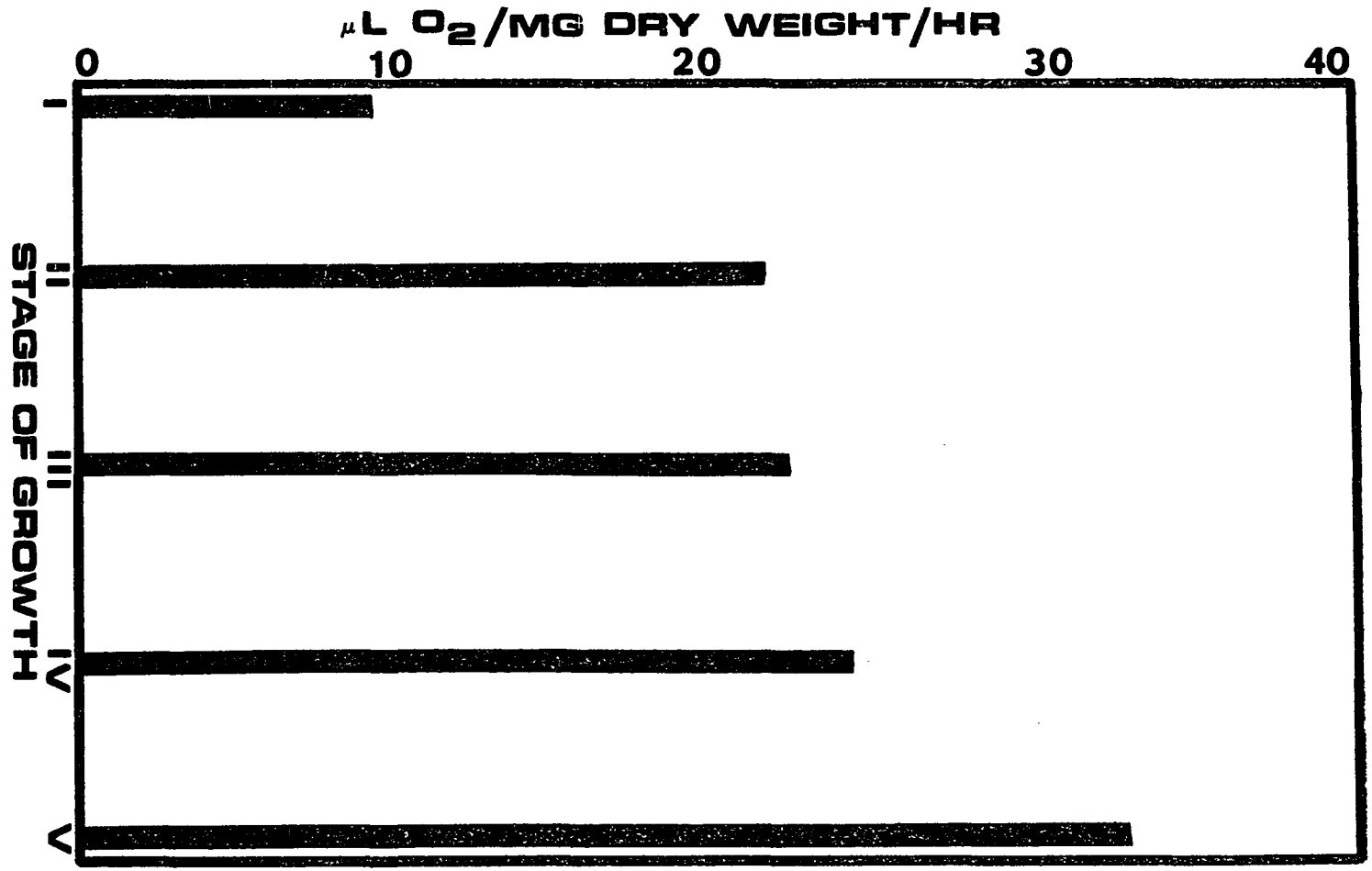
Growth curves of cells grown in SMS and pyruvate minimal salts (PMS) for extended periods of time were

TABLE II

RQ AND QO_2 VALUES FOR VARIOUS STAGES OF GROWTH OF *ARTHROBACTER CRYSTALLOPOIETES*

	Stages of Growth				
	I	II	III	IV	V
RQ	0.93	1.04	1.03	1.02	0.825
QO_2	7.24	20.8	19.4	23.4	34.0

Figure 25.--Bar graph of the amount of oxygen utilized per unit of dry weight per hour for the five stages of growth of A. crystallopoietes. This represents endogenous respiration.


μL O2/MG DRY WEIGHT/HR
0
10
20
30
40
STAGE OF GROWTH
I
II
III
IV
V

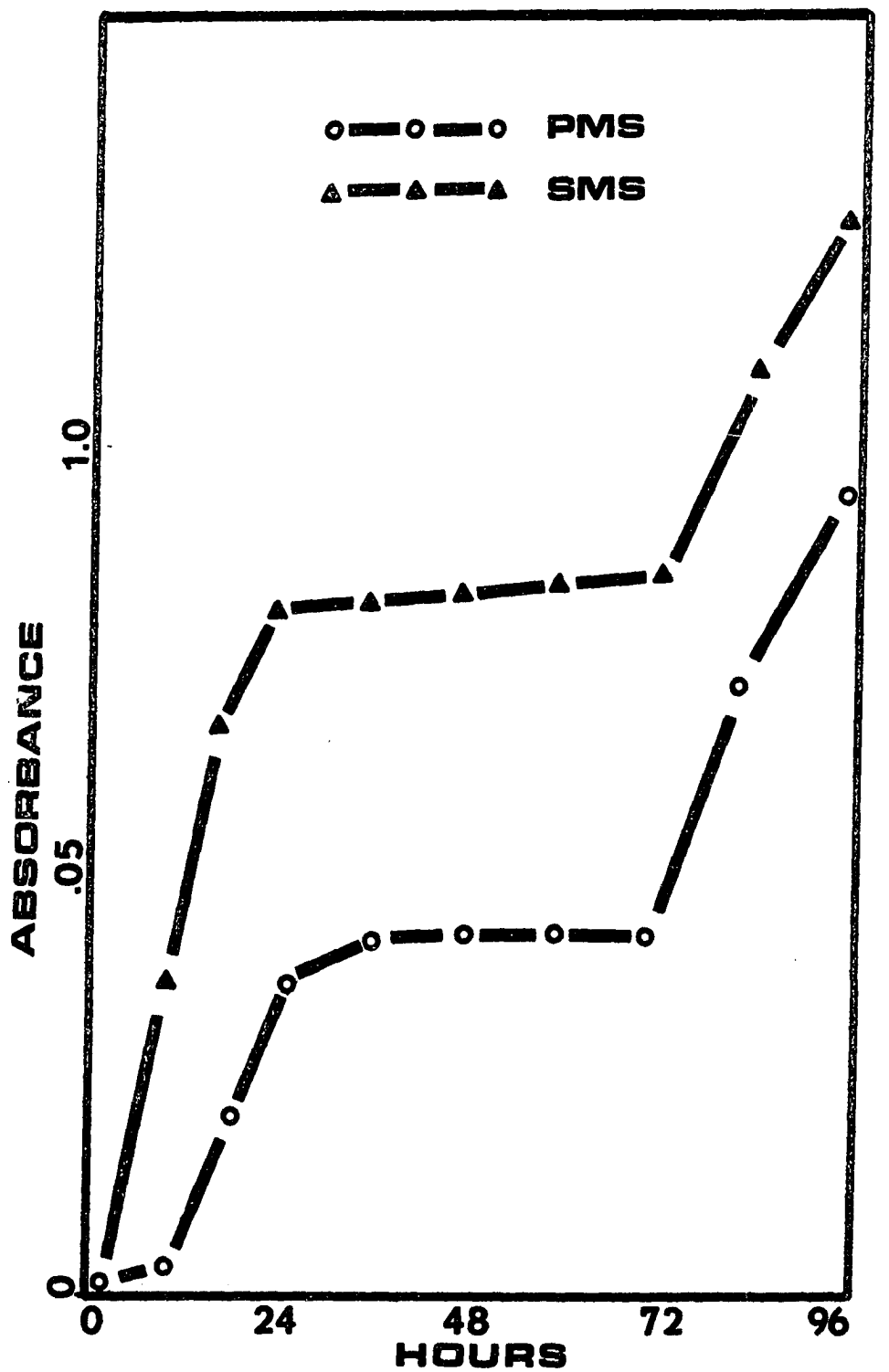
determined from absorbance readings and are given in Figure 26. A maximum stationary phase was reached in both media at approximately 34 hr. Exponential growth began sooner in SMS and a greater number of cells were present at the beginning of the stationary phase in SMS. At 72 hr a secondary growth occurred in both SMS and PMS which could not be explained due to nutrients released from lysed cells since microscopic examination excluded this possibility. Ensign (28) suggested that cryptic growth was not responsible for long-term survival of A. crystallopoietes in buffer. However, the metabolic functions of bacteria in a nutrient medium and in a buffer might be entirely different.

V. Lipid Study

1. Lipid Extraction

A series of lipid extractions were done on the various stages of growth of A. crystallopoietes to determine what lipids were present as cell differentiation occurred. In most bacteria the lipid composition is markedly dependent on the growth conditions, and therefore growth conditions were carefully controlled for the work reported here. The lipids extracted would be an indication of lipids which could make up or at least be a part of inclusions that are involved in the life cycle of A. crystallopoietes, perhaps from an endogenous respiration standpoint. Fatty acid studies have been done on many bacteria with such precision that the fatty acid composition of bacteria has been used to determine the

Figure 26.--Growth curve of A. crystallopoietes over a 96 hr period when grown in a pyruvate minimal salts (PMS) and a succinate minimal salts (SMS) medium.


PMS
SMS
ABSORBANCE
0
.05
1.0
0
24
48
72
96
HOURS

taxonomic position of some species as has been done with Neisseria (7).

In the first attempt to obtain crude lipids from A. crystallopoietes, lipids were extracted in a Soxhlet apparatus, but the yield of lipids was found not to be acceptable. Therefore, a chloroform-methanol lipid extraction (2:1 v/v) of wet whole cells was performed (65). A magnetic stirrer was used for agitation of cells and solvent for periods up to 24 hr at room temperature. The cells were easily lysed in the solvent and this method removed almost all the lipid. Some lipid remained as determined by ether extraction. The lipids were then extracted from the solvents for chromatographic analysis and dry weight determinations as well as for gas chromatography (50). Tightly bound lipids were probably not extracted by this procedure. During all phases of the lipid work, nitrogen was used as a gas phase when possible, and all lipids and lipid classes were stored under nitrogen at 4 C.

2. Thin-Layer Chromatography

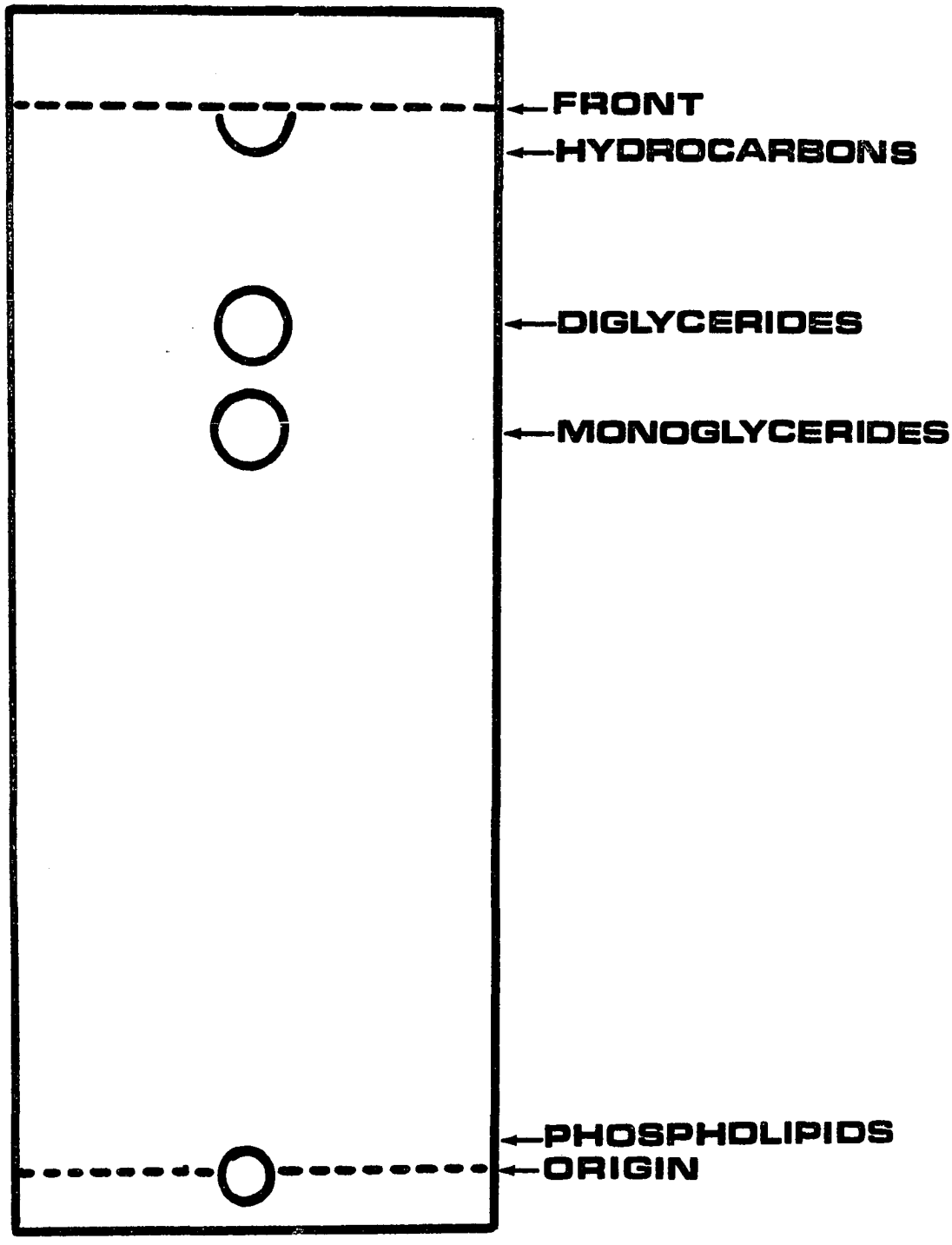
In order to separate the individual classes of lipids for indentification, thin-layer chromatographic separation (TLC) and colorimetric analysis were performed. The separation was accomplished by ascending chromatography on glass fiber paper (Type SG, Gelman Instrument Co.) using appropriate standards and detection methods. Complete structural analysis of fatty acids has been accomplished using only thin-layer

chromatography (3). The solvent system for TLC finally chosen was that of Malkins and Mangold (49) which was composed of 90 volumes of petroleum ether (B.P. 38-54 C), 10 volumes of diethyl ether and 1 volume of acetic acid.

Using the petroleum ether, diethyl ether, acetic acid solvent system, the crude lipid was separated into 4 broad fractions of compounds, and the location of these was determined by short wave ultraviolet light. No indicator was necessary, because each lipid class showed a small amount of fluorescence. The 4 fractions were; (1) phospholipids, (2) mono- and di- glycerides, (3) triglycerides (if present), and (4) hydrocarbons (72). Phospholipids did not migrate in the solvent system mentioned, and the Rf of the other classes increased respectively as shown in Figure 27. Known standards were run and compared for verification. Triglycerides were not detected in this microorganism and were not considered further. However, triglycerides which have accumulated in Blastomyces dermatitidus have been reported to be readily metabolized in the dying phase of the growth curve (23).

Phospholipids are a major component and an important constituent in bacteria (24) and yeast (45), and the bacterial membrane is especially rich in phospholipids. Phospholipids were therefore considered in A. crystallopoietes, and an attempt was made to separate the phospholipid components. They were eluted with chloroform-methanol (2:1 v/v) from the chromatogram and respotted on TLC plates of the same composition.

Figure 27.--Thin-layer chromatogram of the neutral lipid fraction of readily extracted lipids of A. crystallopoietes.


FRONT
HYDROCARBONS
DIGLYCERIDES
MONOGLYCERIDES
PHOSPHOLIPIDS
ORIGIN

The solvent system used was chloroform-methanol-acetic acid-water (125:37:10:2 v/v). No differences in the composition or concentration of phospholipids were seen for any particular stage with the preparative techniques used. However, Bertsch et al. (4) studied the abundance of phospholipids in B. megaterium as they related to the several stages of sporulation which they termed morphogenesis. No attempt was made to identify any of the phospholipid components in A. crystallopoietes.

There was little difference between the total readily extracted lipids at any stage; however, there was a difference in neutral lipids* at Stage III and IV. The diglyceride in Figure 28 is the neutral lipid fraction that was involved. The possibility therefore existed that a particular lipid in this large class of neutral lipids might be one involved in morphogenesis. Because of this, further work on phospholipids was discontinued, and a concentrated effort was made to characterize and identify the neutral lipid component. The particular lipid in question appeared to build up to a maximum concentration before and then disappear after fragmentation. This could be the accumulated fat that Ferdinandus (29) suggested could be utilized as an internal energy source.

*Neutral (simple) lipids are defined as those that contain only carbon, hydrogen and oxygen and no hydrolyzable covalent bonds except fatty acyl ester linkages. Polar (complex) lipids include all lipids that have elements other than carbon, hydrogen and oxygen and hydrolyzable bonds other than fatty acyl esters.

This compound might also be the precursor needed for cross-septum and coccoidal wall formation, and would not necessarily have to be stored as a lipid deposit to serve in this capacity.

In order to avoid contamination of lipid extracts with nonlipid material, the crude lipid in a chloroform-methanol-water (60:30:4.5 v/v) system was passed through a column of G-25 Sephadex (76). The column used was 0.6 cm i.d. x 40 cm long with a teflon stopcock plugged with glass wool. This purification procedure was used in several experiments, but it was later determined that the contaminants (which appeared to be protein or protein complexes) did not interfere with subsequent analytical methods employed, and the procedure was discontinued.

N. corallina, another microorganism that also undergoes cell differentiation, was studied in conjunction with A. crystallopoietes to determine if there was some similarity between the lipids, and particularly the neutral lipids, of N. corallina and A. crystallopoietes. N. corallina was grown on TGY agar which had been enriched with 1% fructose (74), and lipids were extracted as had been done for A. crystallopoietes. The pigment of N. corallina was extracted with all solvents and was used as a marker in TLC analysis. The same general pattern emerged for N. corallina as had been seen with A. crystallopoietes except that the neutral lipid component in question was never seen in N. corallina.

There was a variation in the total extractable lipids at various stages of growth of A. crystallopoietes. This was determined by a chloroform-methanol extraction (2:1 v/v) for 24 hr at room temperature followed by ether extraction for 1 hr. The extractions were combined and dried in vacuo. Percent lipid was determined and based on dry weight of the cells. The results are given in Table III which show that the amount of lipid increased to a high point at Stage II and at fragmentation reached a low. This low corresponds to the time when the unknown neutral lipid had disappeared, and the high at a time when the enzymes linked to lipogenesis show greatest activity. The high is also the point at which fat bodies are seen in photomicrographs. N. corallina cells equivalent to Stage III in A. crystallopoietes had 14.4% lipid, a figure that is considerably higher than that of Arthrobacter. In the sporulation process, B. subtilis synthesizes fatty acids and phospholipids well beyond the end of log phase, and this synthesis reaches a maximum just before the appearance of refractile sporangia. There is a shift in the fatty acid ratios during sporulation (63).

Using a 20 x 20 cm thin-layer plate for TLC, the distance from origin to solvent front in all runs was 13.5 cm and the Rf for the unknown component was 0.70. This spot could be detected with 2,7 dichlorofluorescein, rhodamine B and also 6G, bromthymol blue, 1% iodine in methanol, sulfuric acid and short wave ultraviolet light.

TABLE III

PERCENT LIPID IN *ARTHROBACTER CRYSTALLOPOIETES* AT VARIOUS STAGES OF GROWTH

Stage	Percent Lipid
I	7.0
II	10.8
III	8.45
IV	4.2
V	7.3

3. Silicic Acid Chromatography

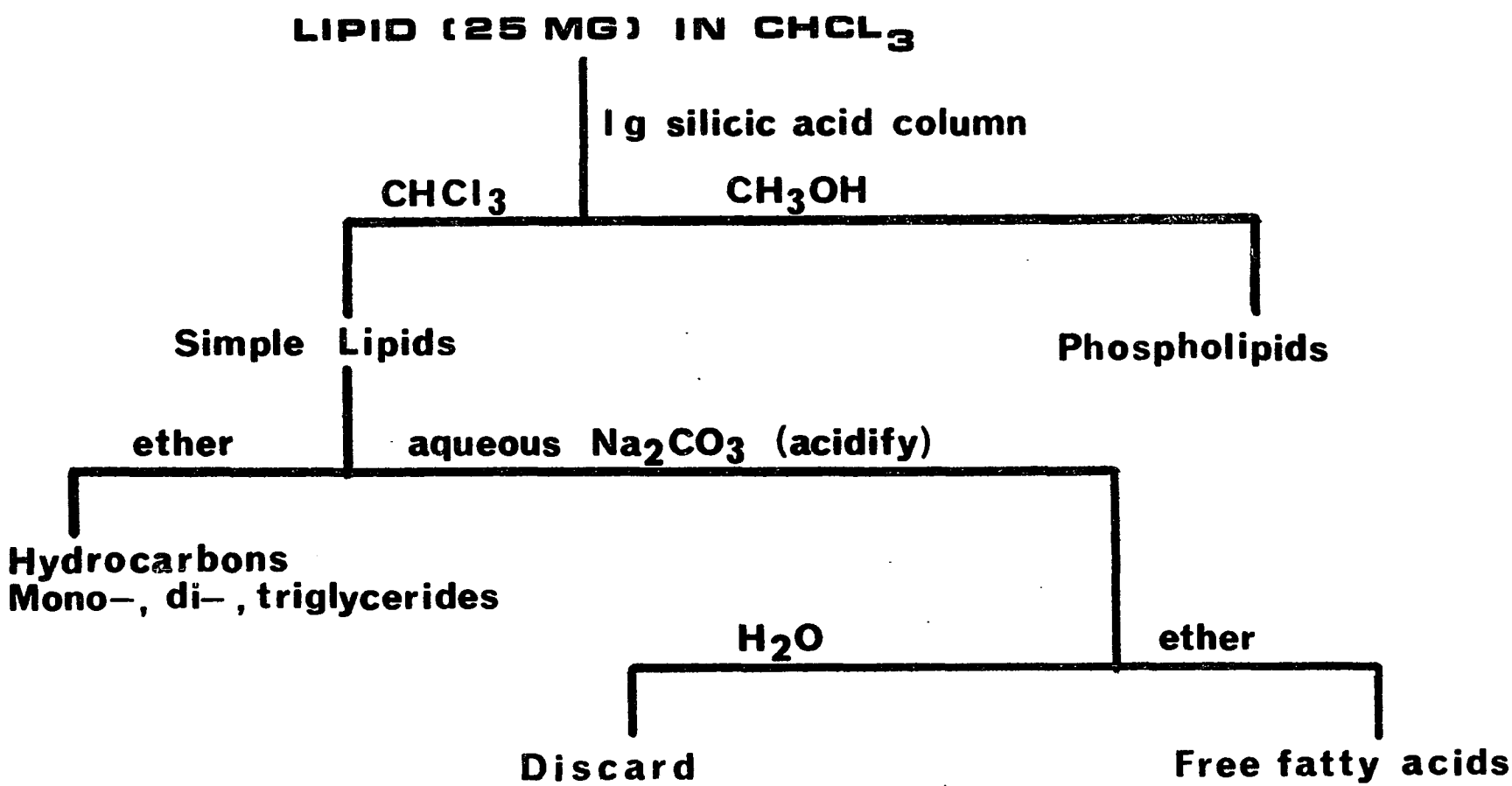
It became necessary to separate simple and complex lipids on a larger scale than could be done using TLC and this was accomplished with silicic acid column chromatography. Silicic acid (100 mesh, Mallinckrodt, AR) was dried overnight at 110 C, prepared in chloroform, and the column (0.8 cm i.d.) made so that 20-25 mg of total lipids in chloroform was added per g column weight. Simple lipids were collected by passing anhydrous chloroform through the column until TLC monitoring showed no other neutral lipids on the column. This fraction contained the unknown lipid compound. Complex lipids were eluted by passing methanol through the column as shown in Figure 28.

From the neutral lipid fraction, free fatty acids were separated by solvent extraction according to Figure 28. These were chromatographed by TLC and none had an Rf corresponding to the unknown. At this point it had been determined that the unknown lipid was a neutral lipid, possibly a mono or diglyceride. The unknown was chromatographed with known standards using the solvent system as before, and it was seen that the unknown migrated with the diglycerides. The Rf of the unknown was exactly that of a dipalmitin standard.

4. Gas Chromatography

Much progress has been made in the field of analysis of free fatty acids (FFA) since James and Martin (35) first described the gas chromatographic separation of fatty acids.

Figure 28.--Extraction procedure for lipid fractions extracted from A. crystallopoietes.

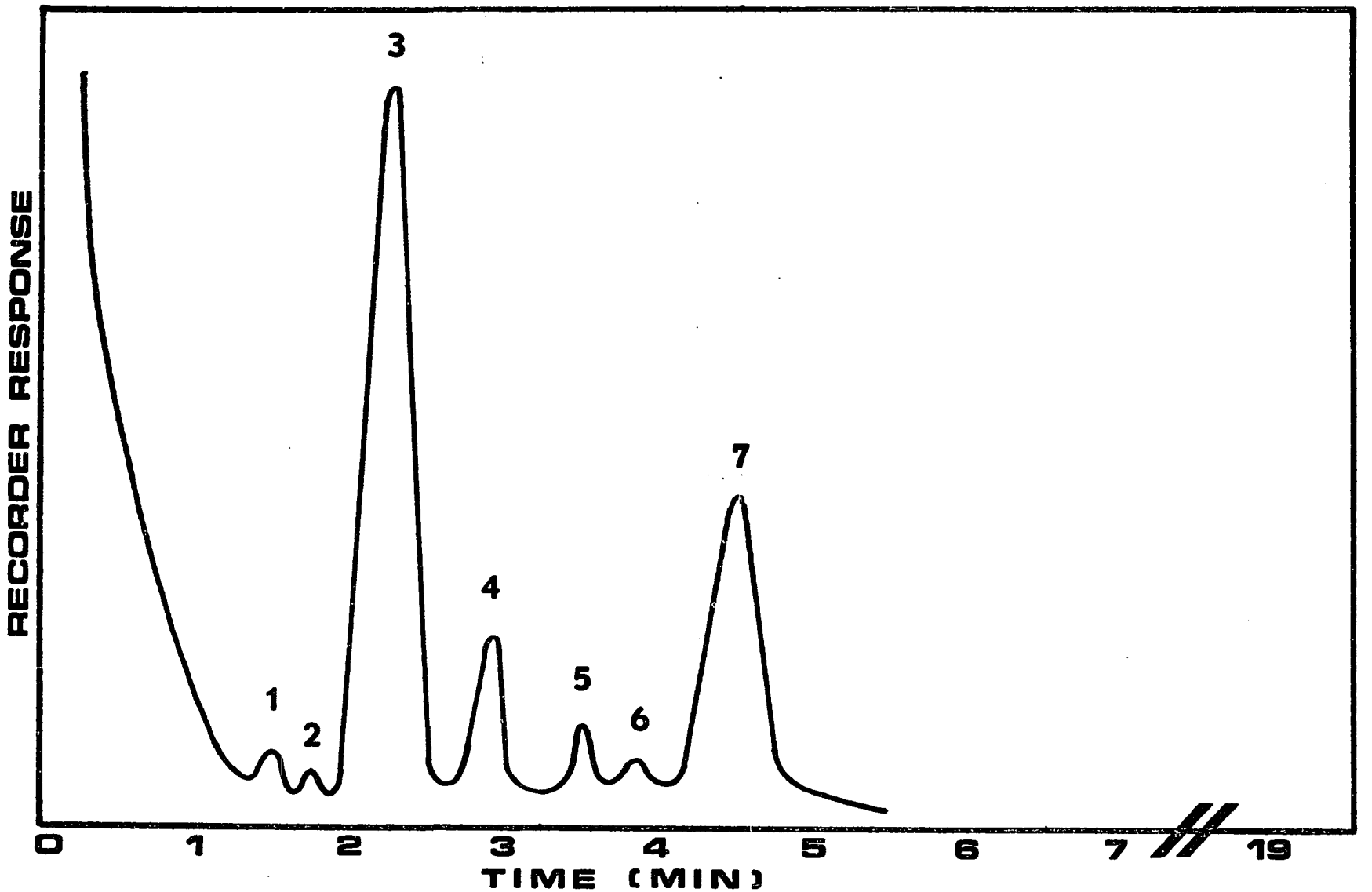

LIPID (25 MG) IN $CHCL_3$
1 g silicic acid column
$CHCl_3$
CH_3OH
Simple Lipids
Phospholipids
ether
aqueous Na_2CO_3 (acidify)
Hydrocarbons
Mono–, di–, triglycerides
H_2O
ether
Discard
Free fatty acids

The fatty acids of A. crystallopoietes have never been elucidated and were therefore analyzed by gas chromatography. Ferdinandus and Clark (30) have shown the fatty acids of Arthrobacter to be important in that they have a feedback regulatory effect on selected enzyme systems in this microorganism. Methyl ester fatty acid standards and reference fatty acids were made up to known concentrations and retention times on a Packard model 409 gas chromatograph determined. Chromatography on a 10% DEGS column gave single symmetrical peaks as an indication of purity of the fatty acid standards.

A. crystallopoietes cells were harvested just prior to fragmentation (Stage IV), the point at which the unknown lipid was found to be most prominent. Special care was used in the washing procedure to make certain that no chemical contamination was introduced.

The crude lipid extract was methylated to prepare volatile esters and to reduce the polarity of the compounds, and 1 μl of the methylated lipids in n-hexane was injected into the gas chromatograph. The distinguishable peaks obtained from methylated crude lipid are shown in Figure 29. These are numbered in order of increasing retention times as peak 1, 2, etc., and represent FFA found prior to fragmentation. At this point seven individual fatty acids were seen, the most predominant of these being 12-methyl tetradecanoic acid (a-C15), peak 3, with 14-methyl hexadecancic acid (a-C17), peak 7, being next. Both of these major acids are

Figure 29.--Gas chromatogram of crude lipids extracted from Stage IV of A. crystallopoietes.


RECORDER RESPONSE
1
2
3
4
5
6
7
0
1
2
3
4
5
6
7
19
TIME (MIN)

branched chain fatty acids and are not uncommon to bacteria. Both iso* and anteiso fatty acids have been reported for A. globiformis (70) and B. subtilis (36). The other acids found in order of abundance were: isopalmitic acid (i-C16), peak 4, palmitic acid (n-C16), peak 5, isomyristic acid (i-C14), peak 1, myristic acid (n-C14), peak 2, and palmitoleic acid (C16), peak 6. Palmitoleic acid has never been reported for Arthrobacter. Palmitoleic acid was the only unsaturated fatty acid found at this point in the fatty acid assay.

The same crude lipid sample was alkaline hydrolyzed to break down the complex lipids into individual fatty acids to determine what fatty acids of A. crystallopoietes had not been detected. Perhaps the diglyceride unknown would be hydrolyzed and another fatty acid peak would be seen. For the alkaline hydrolysis, lipid was placed in a screw cap tube with 2 ml of 2 N KOH in ethanol:water (2:1). This was heated in a boiling water bath for 2 hr, cooled, and 1 ml of water added. The non-saponifiable lipid was removed by extracting 2 times with 3 ml of petroleum ether. The hydrolysate was acidified with sulfuric acid, and the fatty acids were extracted with three, 3 ml portions of petroleum ether. The petroleum ether extracts were combined and washed 3 times with 2 ml portions of water. The fatty acids thus obtained were

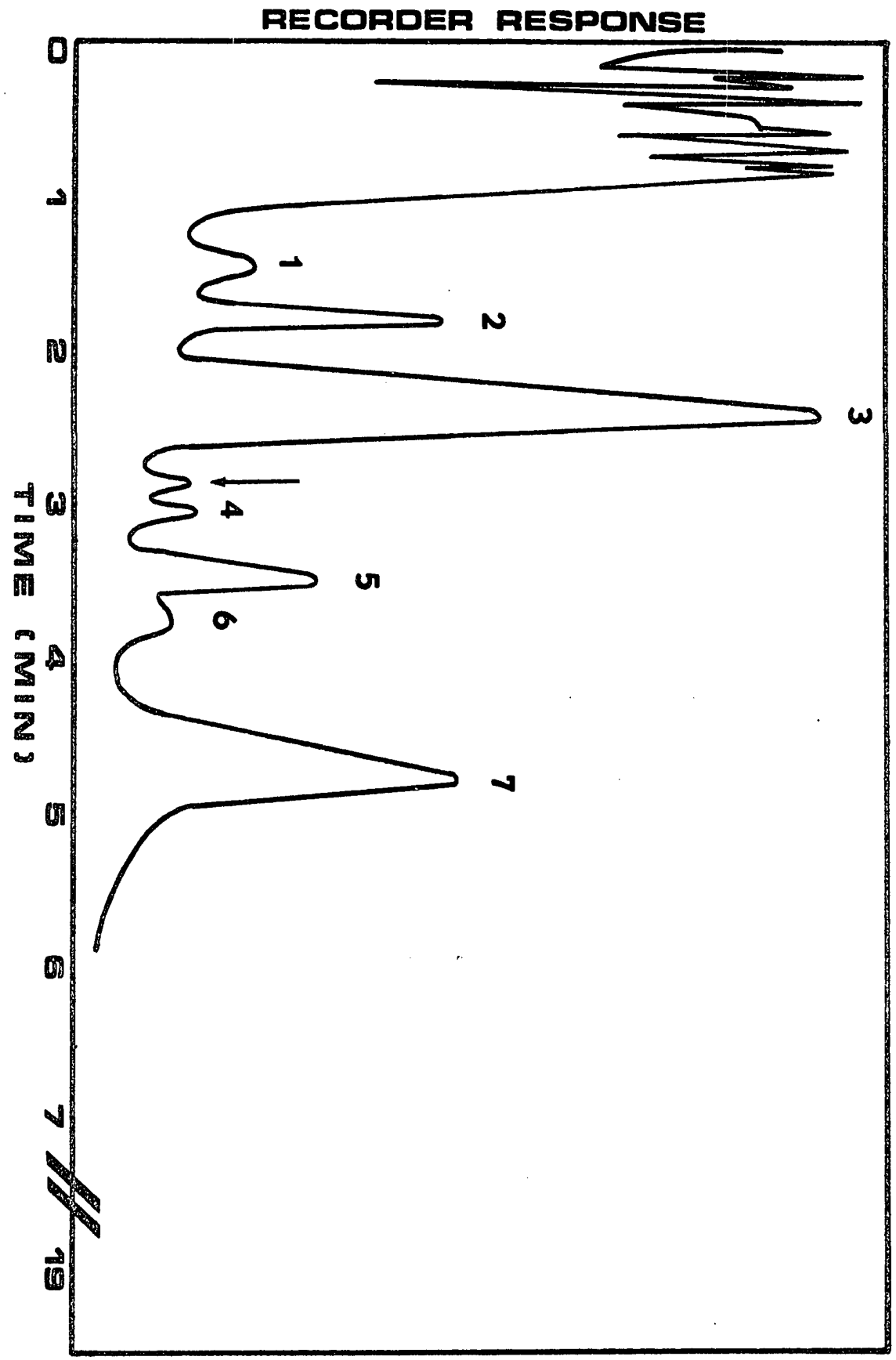
*Terms used are iso (i-) for a methyl side chain in the penultimate position, and anteiso (a-) for a methyl side chain in the antepenultimate position.

methylated and injected into the gas chromatograph, and the results are given in Figure 30.

A great deal of difference was noted in the hydrolyzed sample as opposed to the non-hydrolyzed sample. There were many low molecular weight fatty acids that appeared which were identified as fatty acids with less than 13 carbons. These were present in the non-hydrolyzed sample but in very small quantities. Much more myristic acid was present in the hydrolyzed sample as well as palmitic acid. There appeared to be less isopalmitic acid. A peak appeared between a-C15 and i-C16 which had not been present before hydrolysis. This unique fatty acid was not found in subsequent analysis of lipids from cells grown in any other medium except TGY and was not found at any stage other than Stage IV. The reason that it was not found in lipid extracts from SMS grown cells can be explained by the fact that the life cycle was much shorter in time and not as pronounced in SMS; therefore, the concentration was below detectable limits. This peak which is shown in Figure 30 was identified as pentadecanoic acid (C15). The peak is indicated by an arrow.

The oven temperature was lowered to 150 C to obtain separation of low molecular weight fatty acids which were present before and which had been freed upon hydrolysis. Excellent separation was obtained, and again the acids were identified as those with carbon numbers less than 13 but since subsequent findings did not show variations in them, they were not considered important in morphogenetic control.

Figure 30.--Gas chromatogram of base hydrolyzed crude lipids extracted from Stage IV of A. crystallopoietes. Arrow refers to pentadecanoic acid (C15).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The hydrolyzed crude lipid was purified using a silicic acid column. The neutral lipids were collected, methylated, and run on the gas chromatograph in order to compare the crude lipid with partially purified lipid. Also, fatty acids obtained by solvent extraction procedure shown in Figure 28 were methylated and run.

Three of the lower molecular weight fatty acid peaks disappeared during the purification of the neutral lipids. Either they were retained on the silicic acid column or they were in such small amounts that after purification they were not detectable. It is possible also that these low molecular weight fatty acids were lost by volatilization. All other peaks were present. There was, however, an increase in palmitic acid and a decrease in myristic acid in the purified lipid sample.

Stage IV hydrolyzed and non-hydrolyzed lipids were compared to Stage V hydrolyzed and non-hydrolyzed lipids. The results of this assay can be seen in Figures 29, 30, 31, and 32. It was at this point in the lipid analysis that stearic acid and an octadecenoic acid were detected. Stage V cells had these fatty acids present in large amounts and when Stage IV cells were checked again, it was discovered that they too had these fatty acids. Stearic and octadecenoic acids were seen in both hydrolyzed and non-hydrolyzed samples. These fatty acids have not been reported for the genus Arthrobacter. It was not determined whether the unsaturated fatty acid was

Figure 31.--Gas chromatogram of crude lipids extracted from Stage V cells of A. crystallopoietes. Stearic acid and an octadecenoic acid are peaks 8 and 9, respectively.

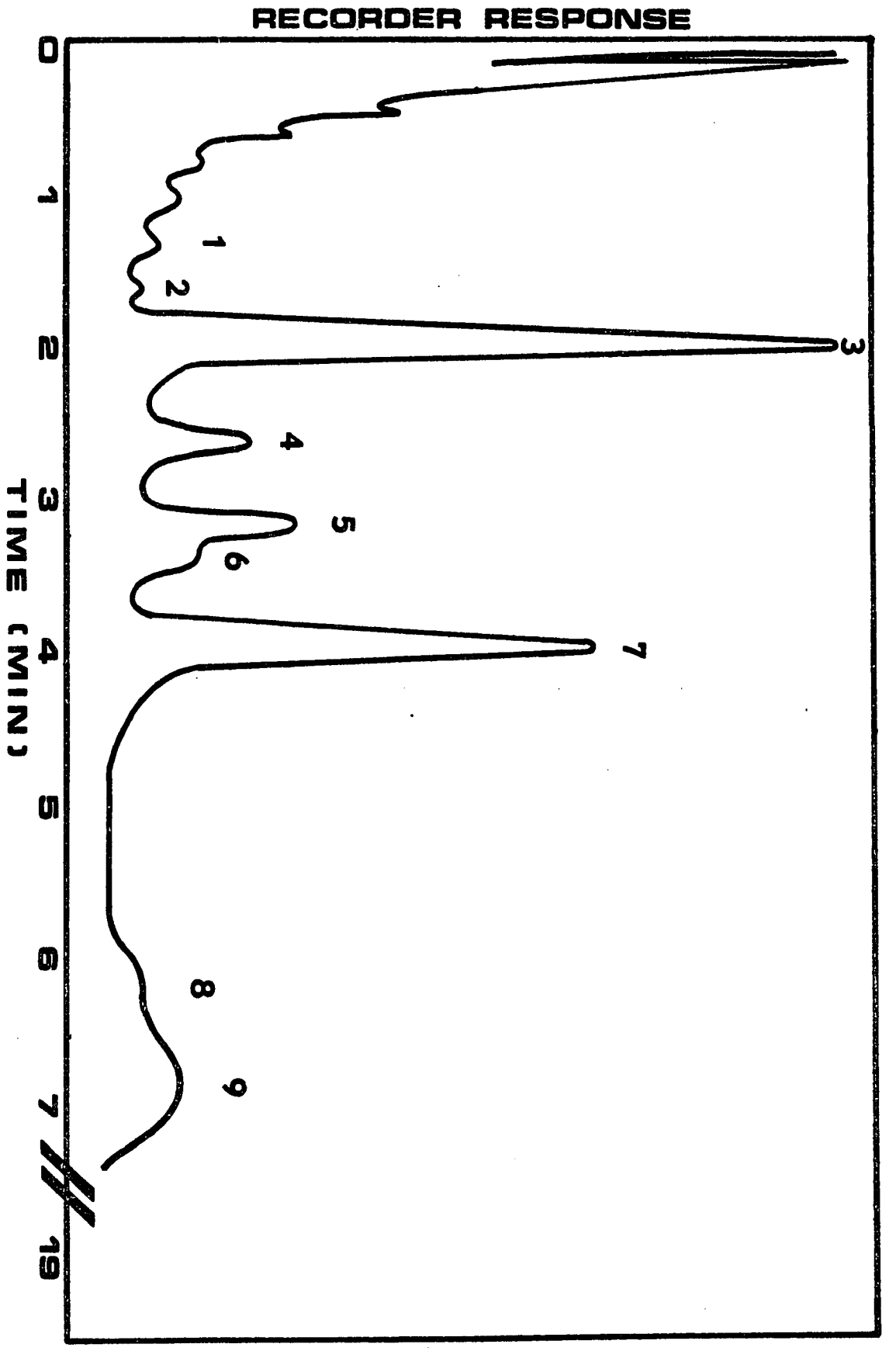
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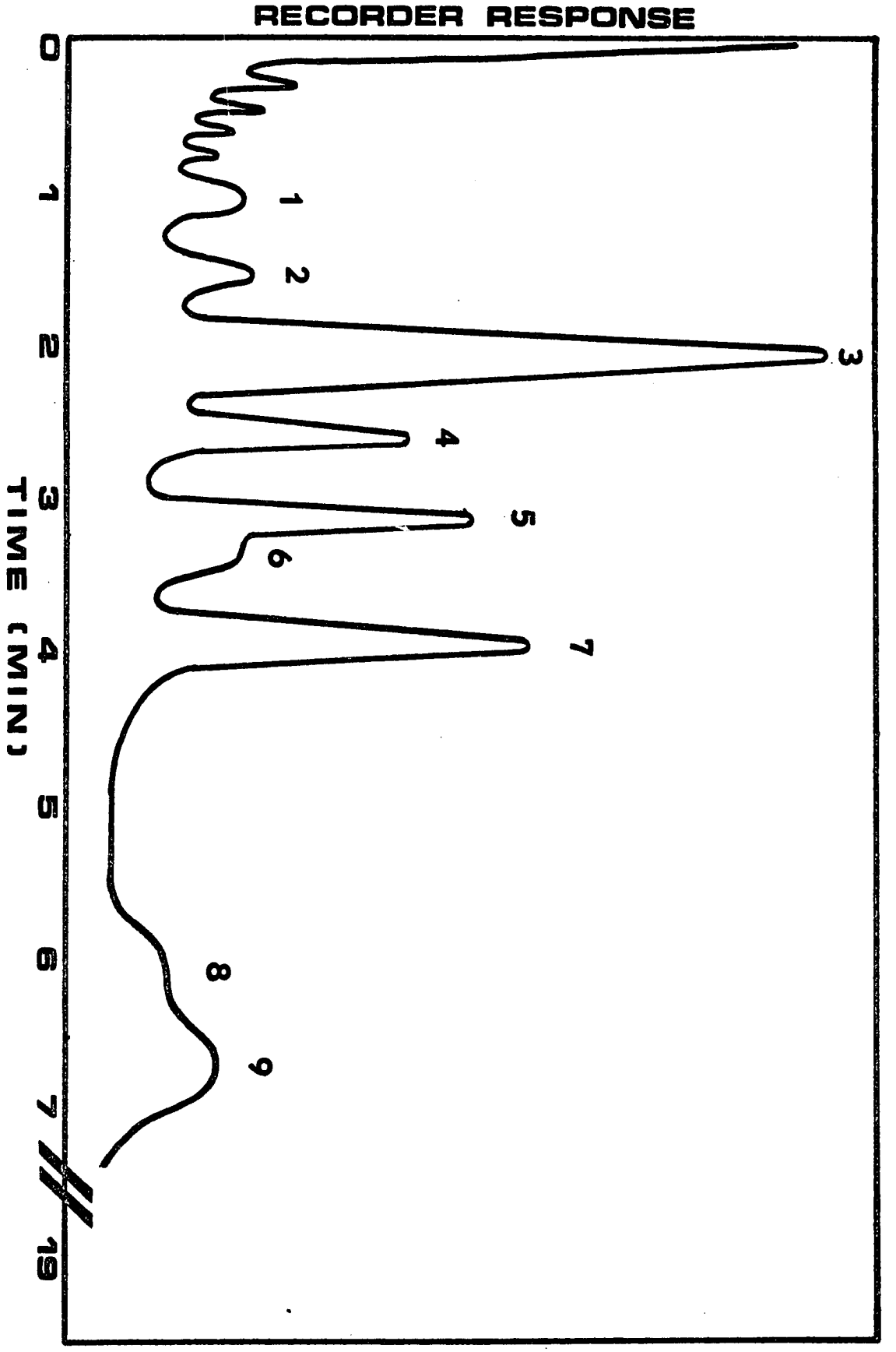
Figure 32.--Gas chromatogram of base hydrolyzed crude lipids extracted from Stage V of A. crystallopoietes.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oleic or cis-vaccenic acid. They are indicated in Figure 31 by peaks 8 and 9.

The fatty acids of Stage IV hydrolyzed and non-hydrolyzed lipids have been discussed. It has been seen that slight variations occurred in concentrations of the fatty acids but not to a significant degree. The same is true for Stage V lipids. A major difference between the Stage IV and V lipids is that they are low molecular weight fatty acids present in the non-hydrolyzed sample in concentrations as large as the hydrolyzed. Six low molecular weight fatty acid peaks showed up which were also present in Stage V hydrolyzed cells. This may be explained by the fact that complex lipids have been broken down at Stage IV and V cells, and fatty acids that were liberated are detected. However, Stage V lipids, either non-hydrolyzed or hydrolyzed, did not contain pentadecanoic acid as seen in the hydrolyzed sample of Stage IV lipids.

In order to determine if there was any difference in the lipids of cells grown in TGY as compared to cells grown in SMS, lipids were extracted from Stage IV cells grown in SMS and chromatographed. The results of SMS grown cells are shown in Figures 33 and 34 and are compared to Figures 29 and 30.

Again, when the lipid was hydrolyzed, many low molecular weight fatty acids were seen. The common fatty acids were in the same proportions in both TGY and SMS. From this, it was concluded that succinate as the only carbon source or as an inducer of morphogenesis does not cause a considerable

Figure 33.--Gas chromatogram of crude lipids extracted from Stage IV cells which were grown in SMS. Peak 10 indicates behenic acid (C22).

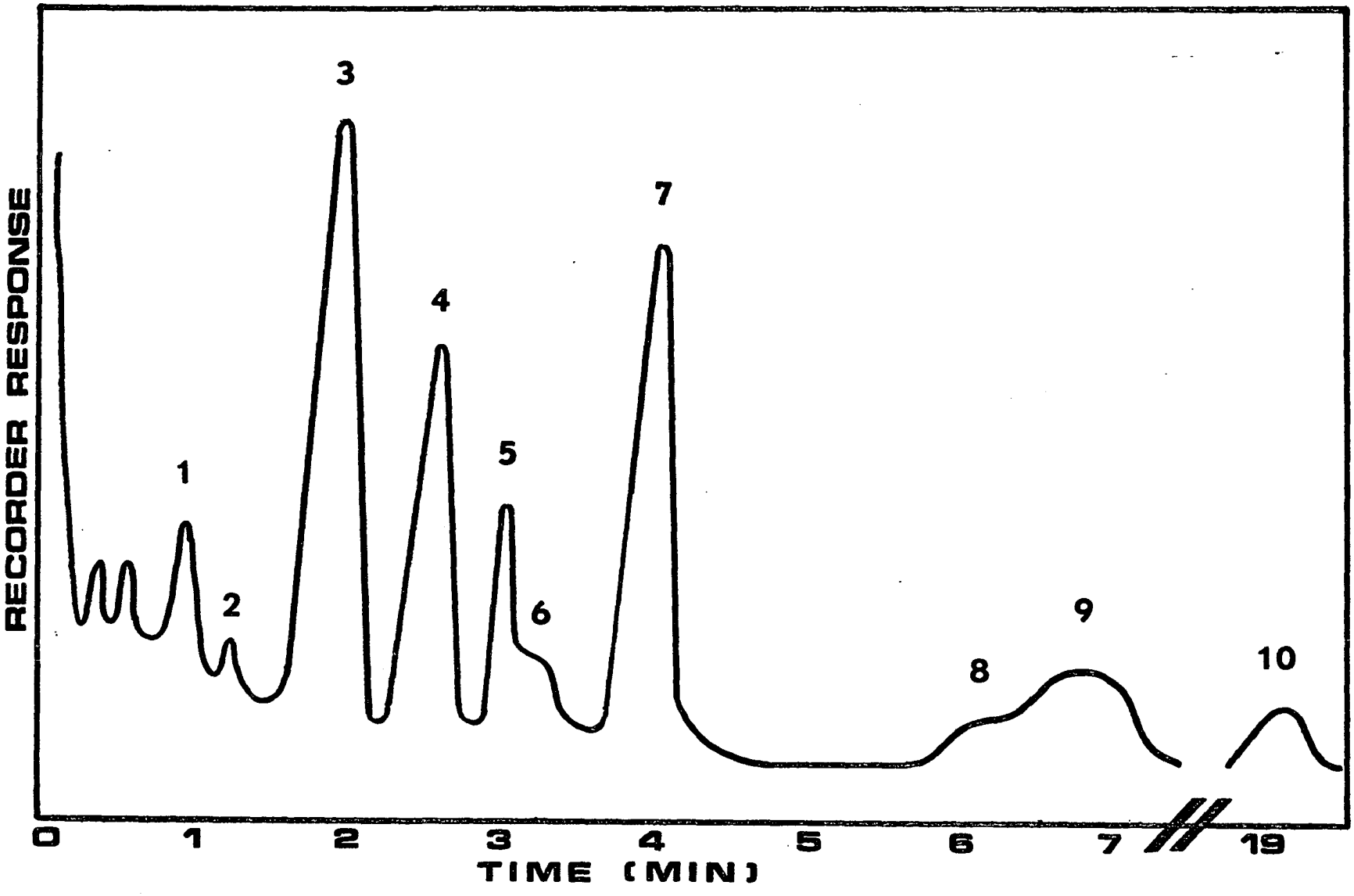
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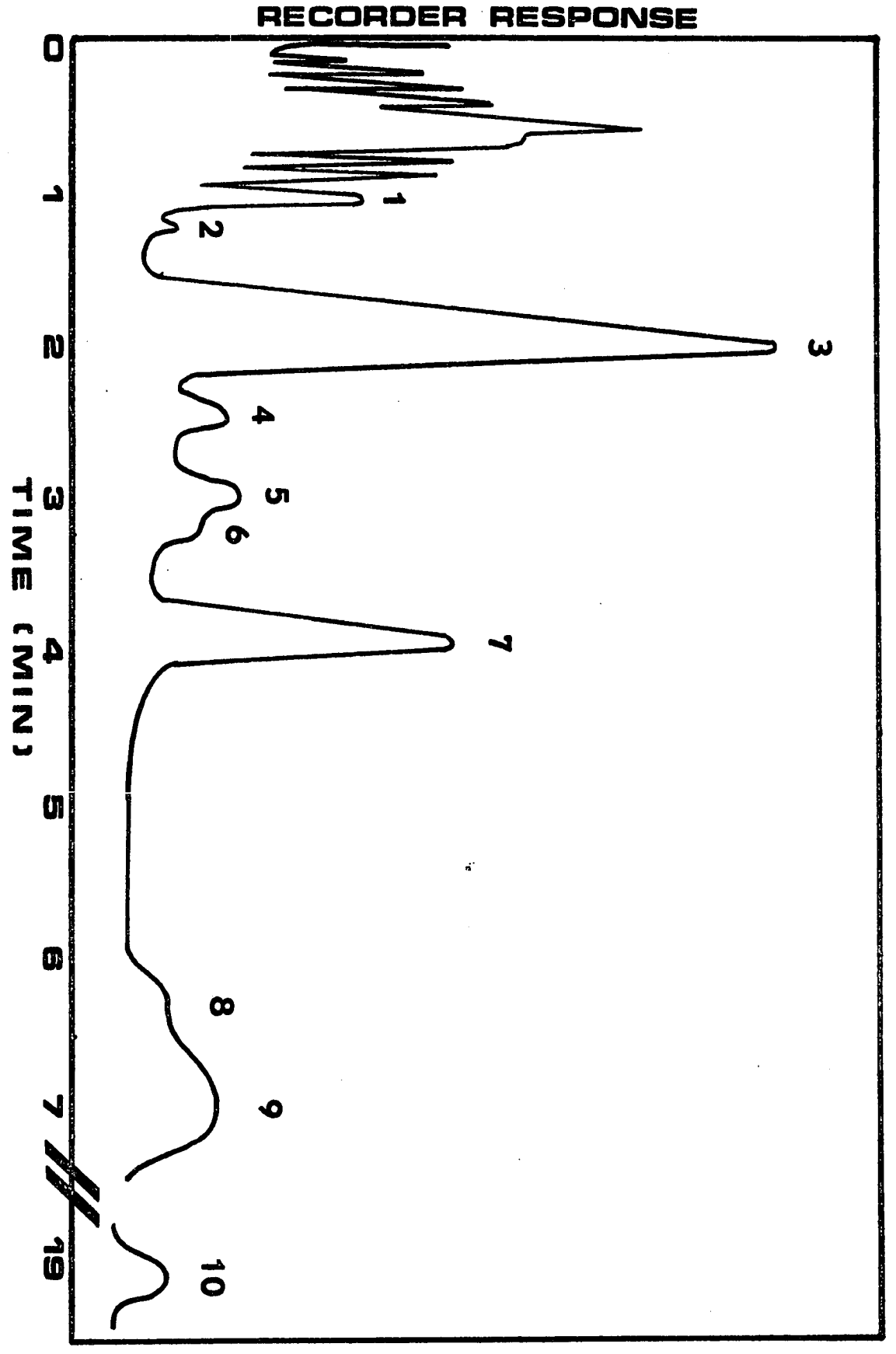
Figure 34.--Gas chromatogram of base hydrolyzed crude lipids extracted from Stage IV cells which were grown in SMS.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change in fatty acid content of Stage IV lipids. However, the C15 fatty acid seen in TGY grown cells was not detected in SMS grown cells. One other fatty acid appeared at this point of our work when the sample was allowed to run for an extended period of time. This fatty acid is shown in both Figures 33 and 34 and is numbered peak 10. The peak corresponded to behenic acid, a 22 carbon fatty acid. This acid has never been reported for Arthrobacter. It was also seen in lipids from cells grown in TGY and was not dependent on substrate.

Stage IV cells grown in SMS were compared to spheres which were in the exponential phase of growth in GMS. Both hydrolyzed and non-hydrolyzed lipids were run. The results are shown in Figures 35 and 36. Again, there appeared to be no difference in fatty acids present or in quantitation of either hydrolyzed or non-hydrolyzed fatty acids in the lipids extracted from cells grown in SMS with the exception that these lipids appeared to have more oleic and stearic acid than lipids from cells grown in TGY or GMS.

Cells grown in GMS showed little difference between hydrolyzed and non-hydrolyzed cells. However, two fatty acids appeared in very small amounts that had not been seen either in SMS or in TGY. These were identified as linolelaidate (C18) and arachidate (C20) or cis-5-eicosenoate (C20). Linolelaidate and cis-5-eicosenoate are unsaturated fatty acids. These are not shown in Figures 35 or 36. Table IV

Figure 35.--Gas chromatogram of crude lipids extracted from Stage IV cells which were grown in GMS.

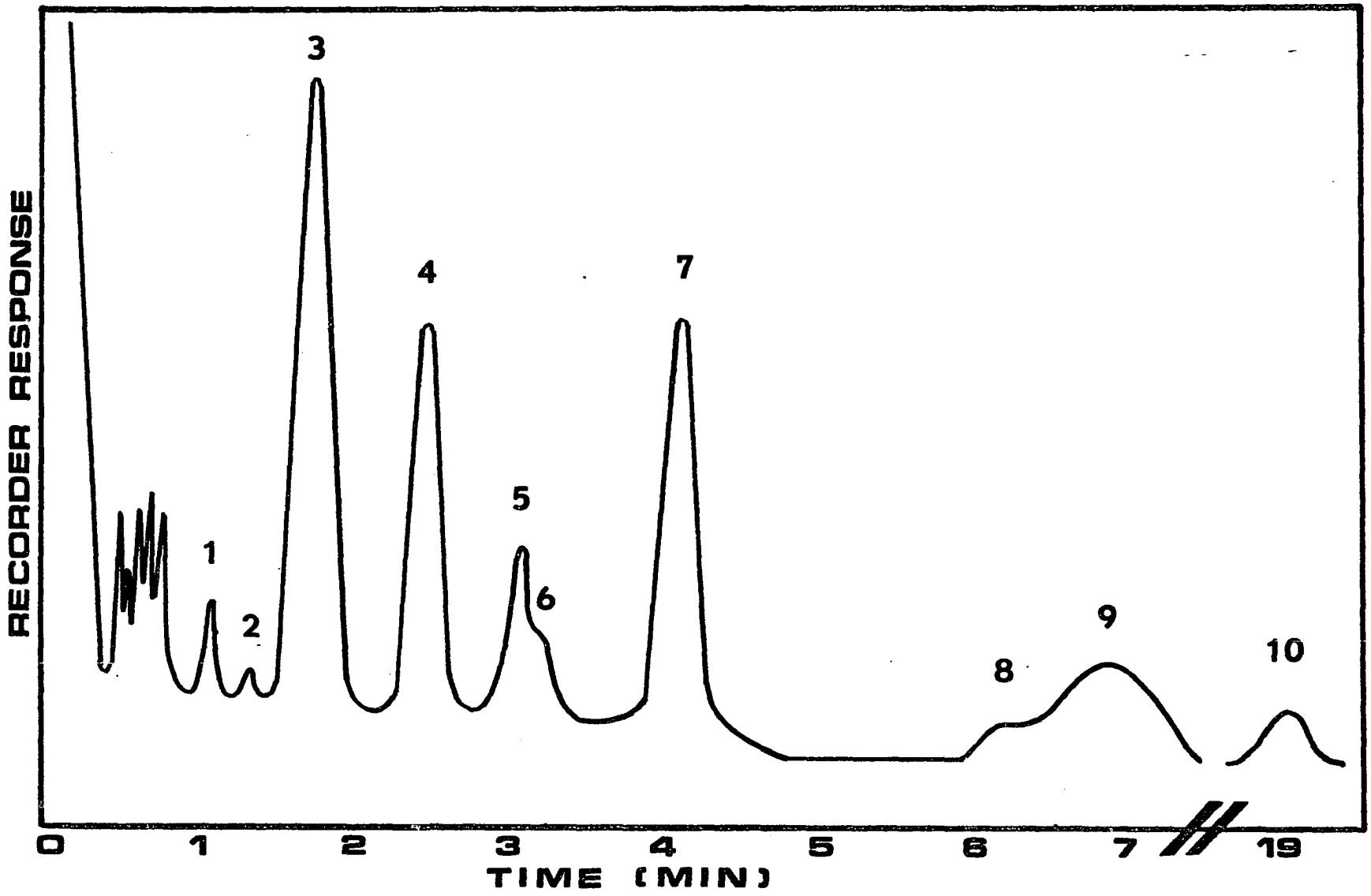
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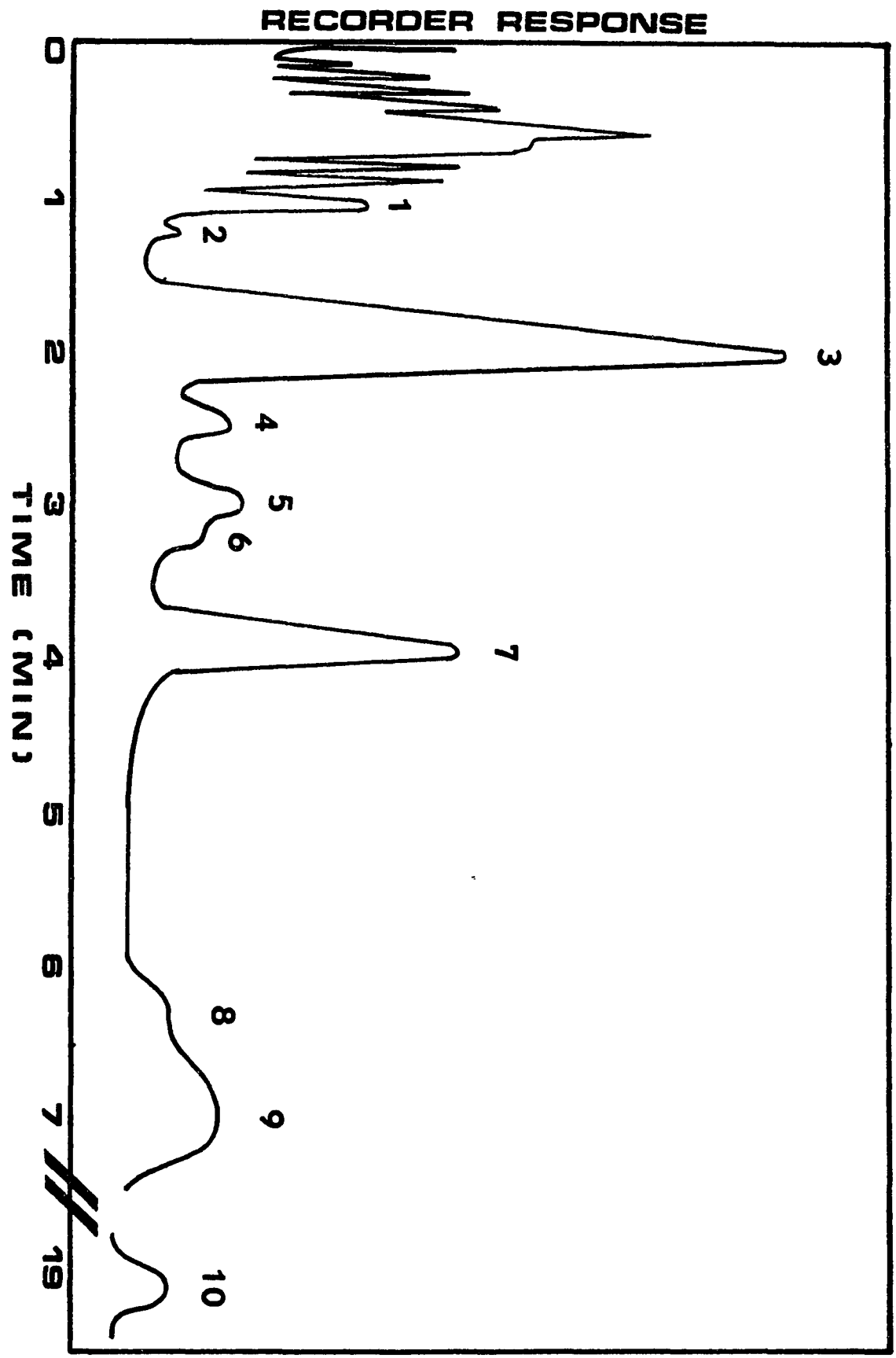
Figure 36.--Gas chromatogram of base hydrolyzed crude lipids of Stage IV cells which were grown in GMS.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shows the major fatty acids found under all growth situations and are listed in order of relative abundance.

VI. Membrane Isolation

The question arose as to the location of the fatty acids consistantly seen in whole cell crude lipid extracts. If in the cytoplasm, then perhaps they were storage for endogenous energy for the fragmentation process. Perhaps these acids constitute part of the lipid inclusions seen in Figures 9, 10 and 11. If in the membrane fraction, perhaps they are structural entities that are easily extracted. The possibility also might exist that they are simply in close proximity to or loosely associated with the membrane. Perhaps they are the results of various syntheses occurring in these areas.

To answer these questions crude lipids that were extracted from isolated membranes and cell sap were run on TLC in order to determine lipid classes present. The membrane fraction gave a spot indicative of the diglyceride fraction.

Gas chromatograms showed that all fatty acids reported previously were present in both fractions. However, there was a difference in quantitation of these fatty acids. More C14 was present in membrane lipids and considerably less anteiso C15 was seen in cell sap. Low molecular weight fatty acids were in equal proportions in each fraction.

These results indicate free fatty acids in the cell sap which are probably loosely associated with the cytoplasmic membrane. This would not appear to be an unusual situation in

TABLE IV

FATTY ACIDS FOUND IN LIPID EXTRACTS OF *A. CRYSTALLOPOIETES*

(In order of relative abundance)

	Name	Peak Number
1.	12-methyl tetradecanoic acid. .	3
2.	14-methyl hexadecanoic acid . .	7
3.	octadecenoic acid	9
4.	isopalmitic acid	4
5.	palmitic acid	5
6.	isomyristic acid.	1
7.	palmitoleic acid.	6
8.	myristic acid	2
9.	pentadecanoic acid.	(arrow)
10.	stearic acid.	8
11.	behenic acid.	10

view of the fact that bacterial lipids are usually concentrated in the outer layers of the cell, especially the cytoplasmic membrane.

CHAPTER IV

DISCUSSION

The investigations of the lipids of A. crystallopoietes revealed through photomicrographs that there are lipids present at Stage II in the form of lipid inclusions that disappear early in the rod stage. Total lipid determinations as well as glucose-6-phosphate dehydrogenase and malic enzyme assays showed lipid to be highest and lipogenesis to be more pronounced at Stage II. The sudanophilic areas seen in later stages under the light microscope and reported to be lipid inclusions are probably mesosomes or membranous areas that are rich in adhering lipid and only appear as fat bodies. Total extractable lipids declined in amount after Stage II and finally reached a low at the completion of fragmentation. The present work suggests that lipids are not substrate for endogenous respiration at Stages II-IV but rather a polysaccharide is functioning in this regard.

In the majority of bacteria, lipids do not serve as an energy source and when present as stored lipids are often composed of fatty acid polymers (58). Respiratory quotient

values at various stages also indicated that A. crystallopoietes did not utilize fats for energy. It was at this point in our work, therefore, that it seemed plausible to assign the role of lipids in Arthrobacter to functions other than that of an endogenous energy source.

Lipids did fluctuate in total amount over the life cycle but did not appear to be a result of aging of the culture. This was shown by holding the microorganism indefinitely in the rod stage by continuous addition of succinate. In microorganisms that do store lipids, substrate is important to the amount of product stored. For example, Sobek, et al. (66) found that Azotobacter agilis cells grown on succinate had lower PHB levels than cells grown on glucose. However, succinate grown cells were more effective in their use of PHB reserves. We have shown that PHB is not a reserve material in A. crystallopoietes, and also that substrate such as acetate, a precursor of lipids, has no effect on total amount of lipid.

Inducers of morphogenesis are important, but actual mechanisms of these compounds has not been determined. For example, succinic acid might be an important precursor of lipids of A. crystallopoietes. The addition of related compounds such as propionate or valerate to the culture medium of B. subtilis caused the formation of two new odd numbered fatty acids (37) which are found in Arthrobacter as well.

Inducers are important in other microorganisms. It has been suggested that the inducers of microcyst formation

(a form of morphogenesis) in Myxococcus xanthus may alter a membrane-DNA relationship, initiating the read-off of that part of the genome associated with microcyst formation (62). DNA is associated with cell membrane either structurally or metabolically.

A. crystallopoietes has a very detailed membrane structure as seen in electron photomicrographs. Mesosomes become more prominent at the time of fragmentation and are in close proximity to areas of cross-wall formation, and due to this fact, it appears that they contribute significantly to cross-septation and cell wall formation. Membranous structures are notorious for their lipid content as either lipid loosely associated with the membrane or structurally related. The majority of the total lipids of most bacteria are located in the plasma membrane (58), and this relationship could have a role in cross wall formation in Arthrobacter as has been shown for certain bacteria by Anderson, et al. (2). They showed that lipid acts catalytically as a carrier of phospho-disaccharide pentapeptide units for polymer synthesis in cell wall of Staphylococcus aureus and Micrococcus lysodeikticus.

The role of lipids cannot be strictly assigned to membrane lipids. Total content of the cell is highest at Stage II whereas membranous structures are more prominent at Stage IV. Lipid inclusions seen in photomicrographs are only present at Stage II and therefore, may have other functions in the cell which could possibly be kinetic. Lipid inclusions

of Stage II could be precursors of more complex membrane lipids.

Extraction and identification of lipids of Arthrobacter has revealed that significant differences occur as the microorganism undergoes morphogenesis. The major difference was seen just prior to fragmentation and was shown by TLC to be a build-up of a neutral lipid fraction which chromatographed with a diglyceride standard. Diglyceride moieties have been isolated from other bacterial lipids with glycosyl diglycerides isolated from A. globiformis by Walker and Bastl (72). These glycolipids are important in the respect that they could be related to the diglyceride found in the neutral lipid fraction of A. crystallopoietes.

In the synthesis of glycosyl diglycerides, biosynthesis appears to proceed in a stepwise manner involving the transfer of a glycosyl residue from the appropriate sugar nucleotide to the hydroxyl group of a diglyceride. This may be followed by addition of a second hexose residue. Certainly more of the diglyceride moiety would be needed at fragmentation if this is the case in A. crystallopoietes. Cohen and Panos (15) found that levels of diglucosyldiglyceride increased in membrane lipids of an L form of Streptococcus pyogenes as compared to a much lower level for the parent strain. These workers postulated that this compound could be involved in cell wall synthesis. The inability of the L form to synthesize a cell wall resulted in elevated levels of this compound.

In A. crystallopoietes it could not be determined if the diglyceride extracted was exclusively associated with the plasma membrane or found in the cell sap. The fact remained however, that the diglyceride could be one of the precursors for a glycolipid component. Diglycerides might also serve as precursors of phosphatidic acid which could in turn be a precursor of other phospholipids (58). Either of the above would require a pool of metabolically active diglyceride that could easily be detected at the point of highest concentration. Since phospholipids are a major component of membranes, it follows that the highest concentration of diglyceride would be at fragmentation when membrane synthesis is the highest. The idea of a diglyceride has been considered in other microorganisms, but Chang and Kennedy (12) found no pool of diglycerides in Escherichia coli. The enzyme diglyceride kinase might give an indication of a diglyceride pool since diglyceride would be substrate for this enzyme.

Fatty acids in bacteria have been characterized and acids ranging from 8 to 32 carbon atoms in length have been repeatedly reported. The principle fatty acids found in most bacteria are palmitic, myristic and stearic acid in order of abundance and the cornynebacteria and mycobacteria contain very long chain acids with 32 or more carbons. The fatty acid composition of A. crystallopoietes proved to be quite different from most bacteria in that high concentrations of branched chain fatty acids were found to be the predominant acids.

Pentadecanoic acid (C15) is the only fatty acid which could be uniquely associated with the diglyceride in question. This fatty acid was present only after hydrolysis of a lipid mixture which contained the diglyceride.

The fatty acids and fatty acid changes found in A. crystallopoietes could be part of a fatty acid pool. Changes in a fatty acid pool might directly affect the diglyceride concentration making it easy to speculate that the lipid deposits seen at Stage II are fatty acid precursors of the diglyceride found at the later stages. This would explain the disappearance of the lipid inclusions and also the appearance of the diglyceride. This would also account for RQ values obtained in that the lipids were not used for endogenous respiration but rather as a pool of acids for synthesis rather than energy. The idea of a fatty acid pool has been discussed in relation to fatty acids used in sporulation of B. megaterium (63). Fatty acids of A. crystallopoietes might be those responsible for enzyme inhibition which was reported by Ferdinandus and Clark (30).

The differences in cell wall of the two morphological types of A. crystallopoietes arise from the alteration of cell wall synthesis during morphogenesis. Krulwich and Ensign (38) studied composition of the cell wall. If endogenous respiration plays some role in morphogenesis (although the substrate is not lipid), the cell wall and its chemical nature are determined for the most part by the product stored for

endogenous respiration. Products which can be easily converted to acetate would be important in this respect. Brown and Reda (9) showed that the inability of N. corallina to utilize glucose was due to a loss of permeability rather than a lack of a specific enzyme. Ferdinandus (29) and Krulwich and Ensign (40) demonstrated by means of pulsing experiments with radioactive glucose that A. crystallopoietes was relatively impermeable to exogenous glucose during both rod formation and rod fragmentation. This suggests that permeability to glucose may be associated in some way to morphogenesis and of course, the cell membrane would be involved. Increased lipid would be important either as a precursor or as a constituent of the cell membrane.

CHAPTER V

SUMMARY

Arthrobacter crystallopoietes is a bacterium which undergoes sphere-rod morphogenesis, a process which has gained considerable interest in the past few years. This microorganism has a well defined life cycle in a complete and minimal salts medium and is easily suited to studies concerning both morphological and physiological changes which occur during the morphogenetic cycle.

A cytological study revealed lipid deposits in the early rod stage of growth, which were not seen thereafter. Specific activities of the malic enzyme and of glucose-6-phosphate dehydrogenase (enzymes involved in lipogenesis) were highest at this point and percent total extractable lipid was also highest at the early rod stage.

A QO_2 determination indicated that endogenous respiration is not the critical metabolic process at fragmentation since QO_2 values do not change significantly. Respiratory quotient values show that a polysaccharide is functioning as substrate for the endogenous respiration that is occurring. Lipid is likely endogenous substrate in aging cells.

Lipid studies showed a diglyceride fraction to be present just prior to fragmentation and a fatty acid analysis indicated that the diglyceride could be composed of pentadecanoic acid. Fatty acids are reported for A. crystallopoietes that have not been reported for this genus previously.

Photomicrographs revealed changes in the extent of membranous structures over the life cycle. As the microorganism progressed through the life cycle, mesosomes became more prominent but became only rudimentary structures after fragmentation. Lipid differences are probably associated with changes in membranous structures throughout the life cycle.

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