THE STUDY OF FATTY ACID SYNTHESIS DURING GERMINATION OF AZOTOBACTER VINELANDII CYSTS

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ABSTRACT

THE STUDY OF FATTY ACID SYNTHESIS DURING GERMINATION OF AZOTOBACTER VINELANDII CYSTS

By

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Cysts of Azotobacter vinelandii were induced to germinate with 1% glucose in Burk's nitrogen free medium at 30 C. Cells were harvested at various times during the germination over a 12 hour period. Samples were also taken of vegetative cells during their exponential growth phase. Samples were extracted with organic solvents (methanol:chloroform:H_2O = 2:1:0.8) and the fatty acid composition in each extract was analyzed by gas-liquid chromatography. Total extractable lipids account for 10.8% and 17.5% dry weight of vegetative cells and cysts, respectively. Myristic acid (C_{14}), palmitic acid (C_{16}), palmitoleic acid ($C_{16:1}$) and octadecenoic acid ($C_{18:1}$) are four fatty acids found in vegetative cells. In addition to these, cysts also contain methylenehexadecanoic acid ($C_{17:\Delta}$), lactobacillic acid ($C_{19:\Delta}$) (these two are cyclopropane fatty acids) and octadecanoic acid (C_{18}).

Cyclopropane fatty acids are metabolically stable and $C_{17:\Delta}$ was used as an internal standard to determine the relative amounts of other fatty acids during germination. Shortly after induction, the synthesis of the four fatty acids found in vegetative cells commences. The synthesis proceeds at a relatively low rate for 8 hours and switches to a higher rate at a time which corresponds to the emergence of vegetative cells from cyst exines. In the same time the absolute amount of C_{18} and $C_{19:\Delta}$ was unchanged.

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DEDICATION

To my father and my mother

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LITERATURE REVIEW

Azotobacter vinelandii

A. vinelandii belongs to the family of *Azotobacteraceae*, genus *Azotobacter*. This family, according to *Bergey's Manual of Determinative Bacteriology* (4) consists of the genera *Azotobacter*, *Azomonas*, *Beijerinckia*, and *Derxia*. They are all heterotrophic, free-living, nitrogen-fixing bacteria which normally fix at least 10 mg of atmospheric nitrogen per gram of carbohydrate consumed. Members of the family are found in soil and water and are morphologically characterized by their large size and variable shape. While strict aerobes, *Azotobacteraceae* are also able to grow and fix nitrogen under reduced oxygen pressure. In the genus *Azotobacter*, all species have 65 ± 0.8% G + C content in their DNA. *A. chroococcum*, *A. beijerinckii* and *A. vinelandii* are the three most commonly isolated species (4, 19, 20, 36). They do not produce endospores but do form cysts (4, 36, 73). Growth occurs in a pH range of 5.5 - 8.5 with optimal growth pH at 7.0 - 7.5 (4, 14, 26, 36). The optimal growth temperature is between 20 and 30 C (4). They grow readily in nitrogen-free synthetic medium containing inorganic salts

and with an organic compound as energy source. A variety of alcohols, organic acids and sugars are utilized (36, 51, 68, 80).

A. vinelandii was first isolated by Lipman (52) from New Jersey soil in 1903. In young culture, A. vinelandii cells are gram negative, rod shaped, 2-3 x 0.8-1.0 μm in size (52,68), and highly moltile with peritrichous flagella (2,35). The cells frequently occur in pairs and they multiply by simple binary fission. In old cultures, A. vinelandii is polymorphic and as many as five forms have been described (36). Cells have a high respiration rate (51) and release a soluble pigment into media which fluoresces green-blue under ultraviolate light (37, 4, 20). A capsule composed of polyuronic acids is formed outside the cell wall. The ratio of D-mannuronic and L-guluronic acid in this structure depend on the calcium concentration in the medium (16, 31, 47).

Nitrogen Fixation

Microorganisms that are able to fix nitrogen have long been the subject of many investigations because of ecological and biochemical interest. Nitrogen fixing microorganisms are all prokayotes (58) belonging to different families. Among them are blue-green algae, symbiotic bacteria, free-living anaerobic bacteria and free-living aerobic bacteria (5). Blue-green algae which are mainly in aquatic environments are strict phototrophs

and fix gaseous nitrogen when other nitrogen sources are not readily available (25). Symbiotic bacteria form nodules on the roots of legumes and fix nitrogen (8). There are also fungi which establish symbiosis with plants and fix nitrogen (3). Free-living nitrogen-fixing bacteria have been known for a long time and are the subject of much research because they are easily grown under laboratory conditions. Azotobacter species and Clostridium pasteurianum are the most important of the aerobic and anaerobic nitrogen fixers respectively (5).

Biochemically, nitrogen fixation is an "anaerobic" process of electron transfer and reduction. Gaseous nitrogen is converted to NH_3 by a sequence of enzyme catalyzed reactions (83). An intracellular "conflict" occurs in obligate aerobes because nitrogenase is sensitive to O_2 and therefore higher oxygen partial pressures inhibit their growth. This, of course, takes place during growth in nitrogen free medium but not in cultures grown on ammonium ion (17, 18). Two possible protective mechanisms for nitrogenase in Azotobacter are postulated (65, 83). In one, respiratory protection, Azotobacter cells increase their rate of respiration via a non-phosphorylating NADPH dehydrogenase pathway to reduce the p O_2 at the expense of energy yield (1, 17). The second, the conformational protection hypothesis suggests that nitrogenase undergoes a conformational change which makes it oxygen

tolerant (5, 17). In crude extracts the Azotobacter nitrogenase is oxygen tolerant (10, 11), but when it is purified and dissociated, it becomes oxygen sensitive (14, 43).

A. vinelandii possesses an extensive internal membrane network (63) whose appearance is correlated with the induction of nitrogenase (61, 23). Nitrogenase is composed of two metal containing proteins (5, 13), Fe-protein and Mo-Fe protein. These two proteins can be separated by protamine sulfate precipitation or on a column of DEAE cellulose or Sephadex (13, 44, 15). The Fe-protein is cold labile, sensitive to oxygen and has a molecular weight around 50,000 Daltons (65). The Mo-Fe protein has a molecular weight of 270,000 Daltons and contains 34-38 Fe (15). Both proteins are required for the reduction of molecular nitrogen (45) and a ratio of 2:1 Fe:Mo-Fe protein gives the highest in vitro activity (79). Ammonia inhibits the synthesis of both proteins.

The reactions of biological nitrogen fixation are endothermic (9) and ATP is required. Using purified nitrogenase, about 12 to 15 moles ATP are needed per mole of N_2 fixed in vitro (22, 13, 81), but the amount needed for the same reaction in vivo is unknown. During nitrogen fixation, electrons pass through a series of carriers to the nitrogenase and reduce molecular nitrogen. Electrons are generated during oxidative catabolism in Azotobacter and NADPH is the electron donor in nitrogen fixation in A.

vinelandii (16). Substrates such as pyruvate (46), β-hydroxybutyrate (75), and molecular hydrogen serve as electron donors in nitrogen fixation (5). Hydrosulfite is a non-biological reductant which functions in substrate amounts and couples directly to nitrogenase independent of the electron transfer agent in Azotobacter (12). Nitrogenase also reduces substrates other than molecular nitrogen. N_2O was the first substrate other than N_2 shown to be reduced by nitrogenase (59, 28). Cyanide (30), azide and acetylene (71, 21) are also reduced and the reduction of the latter compound to ethylene offers a very useful qualitative and quantitative test for nitrogen fixation (46, 75, 27). Magnesium is the preferred ion for nitrogenase activity (29) and the optimal pH for Azotobacter nitrogenase is above 7 (11).

The carriers that transfer electrons from a reductant to nitrogenase are of the ferredoxin and flavodoxin types. They have been found in bacteria, blue-green algae and plants and have many functions in cellular metabolism in addition to nitrogen fixation (78, 5). All of them are low redox potential, reversible oxidation and reduction compounds with no catalytic activity of their own (5). In 1969 Yoch et al. (85) isolated Azotobacter ferredoxin from A. vinelandii by DEAE-cellulose chromatography. Ferredoxin has a molecular weight of 14,500, contains eight atoms each of iron and sulfide residue, and is

re-oxidized in air in two stages. A flavoprotein-like electron carrier isolated by Benemann et al. (6) from A. vinelandii is called Azotoflavin. It has a molecular weight 23,000, contains one FMN molecule per molecule of protein, but is devoid of metals and does not contain labile sulfide (76). Flavodoxin accepts two electrons each at a different oxidation level (57). The reduced ferredoxin from Azotobacter can transfer electrons directly to nitrogenase (84). Yate and Johns (83) suggest that ferredoxin and flavodoxin are alternative electron carriers to nitrogenase in Azotobacter as in other bacteria, but Benemann et al. (7) suggest both proteins are required for acetylene reduction in Azotobacter extracts. This question needs further study.

Lipids and Membrane

The protoplasm of cells is surrounded by a membrane which has multiple functions that include active transport, selective permeability and respiration and energy production. The membrane also contains sites of localization of enzymes involved in macromolecular synthesis. Membrane consists of protein and lipid which in bacteria is primarily phospholipid (54, 67). The fatty acids in the bacterial membrane vary according to the growth temperature, growth stage and also to the composition of the growth medium (56, 72, 39, 77, 42, 24).

In 1962, Kaneshiro and Marr (40) found that in A. vinelandii grown in Burk's nitrogen free medium, 10.4% of dry weight was lipid, of which 90-94% was phospholipid. The principle extractable lipid is phosphotidyl ethanolamine. The fatty acid components of phospholipids are myristic (7%), palmitic (35%), palmitoleic (41%) and octadecenoic (17%) acids (40, 55). After alkaline hydrolysis of the residue, another 2.6% of bound lipid can be extracted with diethyl ether. Approximately half of the bound lipids are hydroxy acids, principally 3-hydroxydecanoic, 3-hydroxydodecanic and 2-hydroxydodecanic acids (41). A. vinelandii also contains an extensive internal membrane network (66, 63). Oppenheim and Marcus (61) and Hill et al. (32) found that the internal membrane was present in cells grown on N_2 but was suppressed by ammonia and suggested the major function of the internal membrane was to protect netrogenase from O_2 inactivation in vivo (83) and to maintain an Eh within the cell (60, 17) that enables the bacteria to fix nitrogen under aerobic conditions (5, 83). Pate et al. (64) found internal membranes in cells grown on N_2, NH_4+ or NO_3 and suggested that the extent of internal membrane was controlled by O_2 concentration in the medium. These authors think it unlikely that membranes have a sole function of protecting nitrogenase from O_2 inactivation. The real function of internal membrane is unknown, but Marcus and Kaneshiro

(55) found that cells possessing internal membranes contained 30% more total phospholipid, 50% more coenzyme Q, 80% fewer neutral lipids and 50% fewer anionic phospholipids. The increased phospholipid and coenzyme Q content correlates with greater respiratory activity.

Jurtshuk and Schlech (38) isolated "R_3" electron transport particles from resting cells of N_2-grown A. vinelandii. An analysis of the particles showed that certain phospholipids, which were present in whole cells, were absent from this membrane fraction. On this basis the investigators suggested the possibility that other types of membranous subcellular organelles may exist in A. vinelandii which are not associated with electron transport function.

Encystment and Germination

Encystment of A. vinelandii has been studied along with sporulation of Bacillus as a model of cell differentiation (69). Cysts of A. vinelandii are resting cells that are quite distinct from vegetative cells. Morphologically, cysts are round cells, refractile under phase-contrast microscopy and consist of two layers, the exine and intine, and a protoplast, the central body (82, 73). Chemically, cysts contain more carbohydrate and almost twice as much lipid as corresponding vegetative cells (50). Cyst exines are multilayered structures (49) which consist of 32% carbohydrate, 28% protein and 28% lipid (50). Intine is an

intermediate electron transparent layer between the exine and the central body (82). Upon the rupture of cysts with ethylene diamine tetraacetic acid (EDTA), the intine extrudes from exine as a viscosous gel-like polymer (49) and can be washed away from the exine and central body by mild agitation. The intine consists of 44% carbohydrate, 9.1% protein and 36% lipid (50). Uronic acids account for 31.7% of the intine and 13% of the exine dry weight (62). Only mannuronic acid and guluronic acid are present in these fractions. The exine has a higher content of polyguluronic acid whereas the intine has higher polymannuronic acid content (62). Polymannuronic acid is changed into polyguluronic acid by the enzyme L-mannuronic C-5-epimerase whose activity is dependent on the concentration of calcium ion (31). Thus the ratio of guluronic acid to mannuronic acid is to a large extent dependent on the concentration of calcium ion in the medium. Calcium ion is a structural element of cysts by virtue of binding with guluronic acid in the cyst coats (62). The central body is a miniature cell surrounded by cell wall and cell membrane. Polyribosomes and strands of nuclear material are readily observed by electron microscopy. Large amounts of polyhydroxybutyrate granules are also present inside the central body (82, 33).

Encystment can be induced by replacing the glucose as substrate with crotonate or β-hydroxybutyrate in the

growth medium (48). The presence of glucose plus β-hydroxybutyrate in the medium or the elimination of calcium ion from the medium will cause abortive encystment (48, 62). This results in the release of coat material, mainly uronic acid from the developing cyst into the growth medium and a sharp increase in the viscosity of growth medium.

At the induction of encystment, cells start a final cell division without new initiation of DNA synthesis. DNA content drops from 15×10^{-14} gram per cell in exponential growth cultures to 3.4×10^{-14} gram per encysting cell. Poly-β-hydroxybutyrate granules accumulate inside and cells become bright under phase-contrast microscopy (70). Nitrogen fixation and glucose-6-phosphate dehydrogenase activity decrease immediately to very low levels and these events are followed by an increase in the specific activities of BHB dehydrogenase and isocitrate dehydrogenase as well as enzymes involved in the glyoxylate shunt and gluconeogenesis (34). The specific activities of the various enzymes of encysting A. vinelandii thus far examined seem to cycle with an 18 hour period. For example, fructose-1,6-diphosphate aldolase reaches a maximum specific activity at 6 hour then decreases to a low value at 18 hour and increases again to a maximum at 24 hour. Fructose diphosphatase reaches its highest activity at the 9th hour, then decreases but increases to

another peak at 27 hour. These two enzymes seem to have a temporal relationship during the encystment (34).

Upon induction of encystment, RNA synthesis continues for 12 hours but protein synthesis takes place throughout the 36 hour encystment period at about one third rate of that occurring in vegetative cells (34).

Cysts are more resistant to various harmful agents than vegetative cells (74). Cysts require twice as much as ultraviolet radiation to achieve 90% kill than do vegetative cells. The difference is even greater in the case of gamma radiation and sonic treatment but cysts are only slightly more tolerant to heat than vegetative cells. Cysts withstand moderate desiccation for 2 weeks without a detectable drop in viability but most vegetative cells die within one day under the same condition (74).

When cysts are placed in a suitable environment with an exogenous carbon source, they germinate and reinitiate the vegetative phase of their life cycle (53). Germination at 30 C with glucose as carbon source can be divided into two phases: initiation and outgrowth. These processes proceed at a low rate for about 3-4 hours, then after a sharp increase of respiration rate, DNA synthesis and nitrogen fixation begin. RNA and protein synthesis proceed at high rate until the emergence of vegetative cells from cysts coat. During germination cysts gradually lose their refractility and their resistance to various physical and chemical agents (82, 74).

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FATTY ACIDS SYNTHESIS DURING THE GERMINATION OF *AZOTOBACTER VINELANDII* CYSTS

Introduction

Cysts of *Azotobacter vinelandii* are resting cells formed from vegetative cells. They are distinct from their progenitors in morphology (24) and in their resistance to deleterious physical and chemical agents (25, 28). Encystment can be induced in cultures of exponential phase cells by replacing the glucose in growth medium with β-hydroxybutyrate or crotonate (13). The sequence of certain morphological and biochemical events which occur during encystment has been described (22, 6, 7). Once formed, cysts will germinate under optimal conditions of temperature, pH, and exogenous carbon source to regenerate vegetative cells (28, 15). This process occurs over an eight-hour period at 30 C if 1% glucose is used as the exogenous carbon source in Burk's N-free medium (15). Upon germination, cysts lose their resistance gradually (25) and initiate RNA and protein synthesis (15). Midway in germination between 4 and 5 hours, there occurs a sharp increase in the respiration rate and the rate of macromolecular synthesis at which time both DNA synthesis and

nitrogen fixation are reinitiated. These latter two events involve membrane associated enzymes (4, 19, 20, 21, 29). Certain of the unsaturated acyl components of cellular phospholipids undergo conversion during encystment to their corresponding cyclopropane fatty acids (22). It was of interest therefore to determine the fatty acid composition of membranes during the germination and outgrowth of cysts and to ascertain whether it could be correlated with certain macromolecular syntheses in the developing cells.

Materials and Methods

Vegetative Cells.--A. vinelandii ATCC 12837 was used throughout this study. Cells were grown in Burk's N-free buffer (27) with 1% glucose as the carbon source. Incubation was at 30 C until desired cell densities were obtained and then the cells were harvested by centrifugation.

Cyst Preparation.--Cysts were prepared in the two step procedure described by Lin and Sadoff (13). Cells of A. vinelandii were grown in 3 liters of Burk's buffer with 1% glucose as the carbon source, incubated at 30 C until late exponential growth (1 x 10^8 cells/ml), harvested by centrifugation, washed once with Burk's buffer and resuspended in 3 liters of Burk's buffer with 0.2% BHB (β-hydroxybutyrate). These cells were then incubated at 30 C

for another 72 hours. Mature cysts were washed once with Burk's buffer and stored at -20 C.

Germination.--A sufficient quantity of cysts were suspended in 400 ml of Burk's buffer to obtain a turbidity corresponding to an optical density of 0.50 (6.3×10^7 cells/ml). The suspension was incubated at 30 C and a 40 ml sample was taken at 0 time. Sufficient 50% glucose solution (7.2 ml) was added to the remaining cysts so that a final concentration of 1% glucose was achieved in the suspension. This initiated germination and samples were taken at intervals over a 12 hour period.

Lipid Extraction.--Cells or germinating cysts in 40 ml samples were harvested and resuspended in 3.2 ml of water. These were sonicated (Measuring Scientific Equipment, Ltd.) repeatedly for 15 sec and cooled in ice for 30 sec until a total of 2 min treatment had occurred. This was done to enhance the lipid extraction (12). The lipid was extracted by the monophasic method described by Bligh and Dyer (2, 1). Methanol, 8 ml, and 4 ml of chloroform was added to cell material, mixed, and incubated at 4 C overnight. An additional 4 ml of chloroform plus 4 ml of H_2O was added, mixed and separation into two layers occurred. The chloroform layer (lower layer including the interphase) was removed, washed once with 1.5 ml of 0.72% NaCl and rinsed with 1.5 ml of water to remove the

impurities (3). Nitrogen gas was bubbled through the lipid solution to remove the chloroform, then 1 ml of benzene, absolute ethanol mixture (4:1 v/v) was added and removed by bubbling N_2 in order to dry the lipid.

Methanolysis.--Mild alkaline methanolysis was carried out on the membrane lipids by a modification of the method of White (26). To each 5 mg of extract, dissolved in 0.5 ml of absolute methanol plus 0.5 ml of toluene, was added 1 ml of 0.2 N KOH in absolute methanol solution. The reaction mixture was left at room temperature for 1 hour and then neutralized with the weakly acidic resin Biorex 70 (Bio-Rad Laboratory, Richmond, Calif.). The methylesters were extracted twice with 1 ml of chloroform, dried with N_2 at room temperature, and redissolved in 0.5 ml ethylacetate in preparation for gas-liquid chromatography.

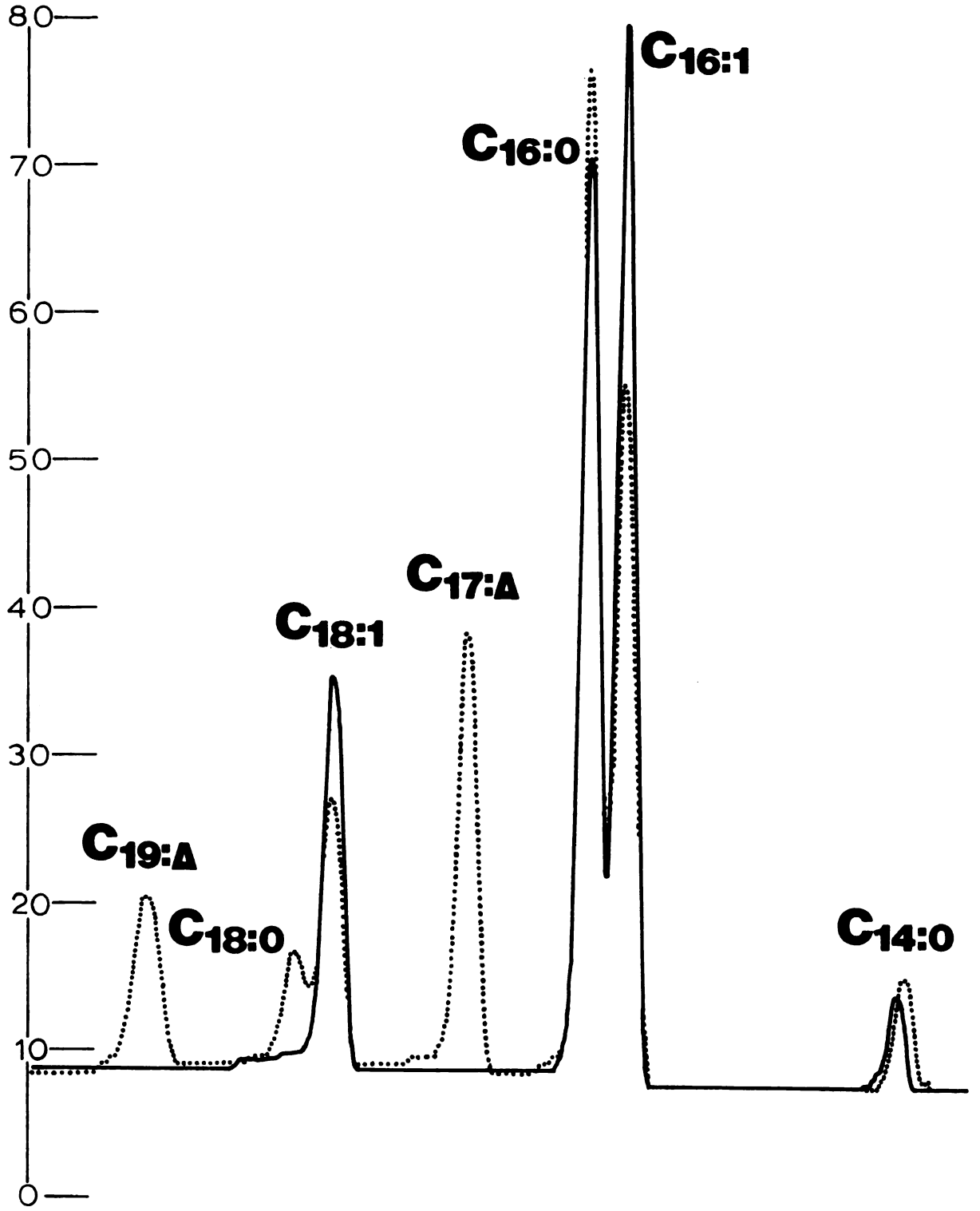
Gas-Liquid Chromatography.--This was carried out with a Varian Aerograph 1400 (Varian Instrument, Palo Alto, Calif.) equipped with a 6 ft column of 1.5% SE-30 and a H_2 flame ionization detector. The instrument was programmed to heat at 10 C/min over the range 120 C to 220 C and after reaching the higher temperature to operate isothermally for 5 min. The sample injection temperature was 250 C, the detection temperature 275 C, helium was used as the carrier gas with a flow rate of 25 ml/min and

and 0.5 to 2 µl of sample was used. The instrument was calibrated with known commercial methylesters purchased from Sigma Chemical Co. Unknowns were identified by comparison of retention time and the elution temperature of standards and the results in the literature (11, 22). The percentage of each fatty acid in the mixture was determined from the area of its individual peak compared to the total peak area.

Results

The profile of fatty acids in vegetative cells and cysts are shown in Fig. 1. The principal fatty acids in vegetative cells were palmitic acid, palmitoleic acid and octadecenoic acid. Total extractable lipid accounts for 10.8% of the dry weight of whole cell. Our results correspond to those published by Kaneshiro and Marr (11). The principal fatty acids in cysts were palmitic acid, palmitoleic acid, methylenehexadecanoic acid ($C_{17:\Delta}$) and octadecenoic acid, the minor fatty acids in cysts were myristic acid, octadecanoic acid and lactobacillic acid ($C_{19:\Delta}$) as shown by Sadoff et al. (22). Methylenehexadecanoic acid, octadecanoic acid and lactobacillic acid are three fatty acids only present in cysts. Total extractable lipids account for 17.5% of the dry weight of cysts which is close to the result of Lin and Sadoff (14). The percentage of various fatty acids in vegetative cells at different optical densities (620_{nm}) and in cysts

Fig. 1.--The profile of fatty acids in vegetative cells (solid line) and cysts (dotted line).


80
70
60
50
40
30
20
10
0
C19:A
C18:0
C18:1
C17:A
C16:0
C16:1
C14:0

at various times during the course of germination are presented in Table 1. The membrane's content of $C_{16:1}$, C_{16} and $C_{18:1}$ fatty acids increased steadily during germination with a corresponding decrease in percentage of $C_{17:\Delta}$, C_{18} and $C_{19:\Delta}$ fatty acids.

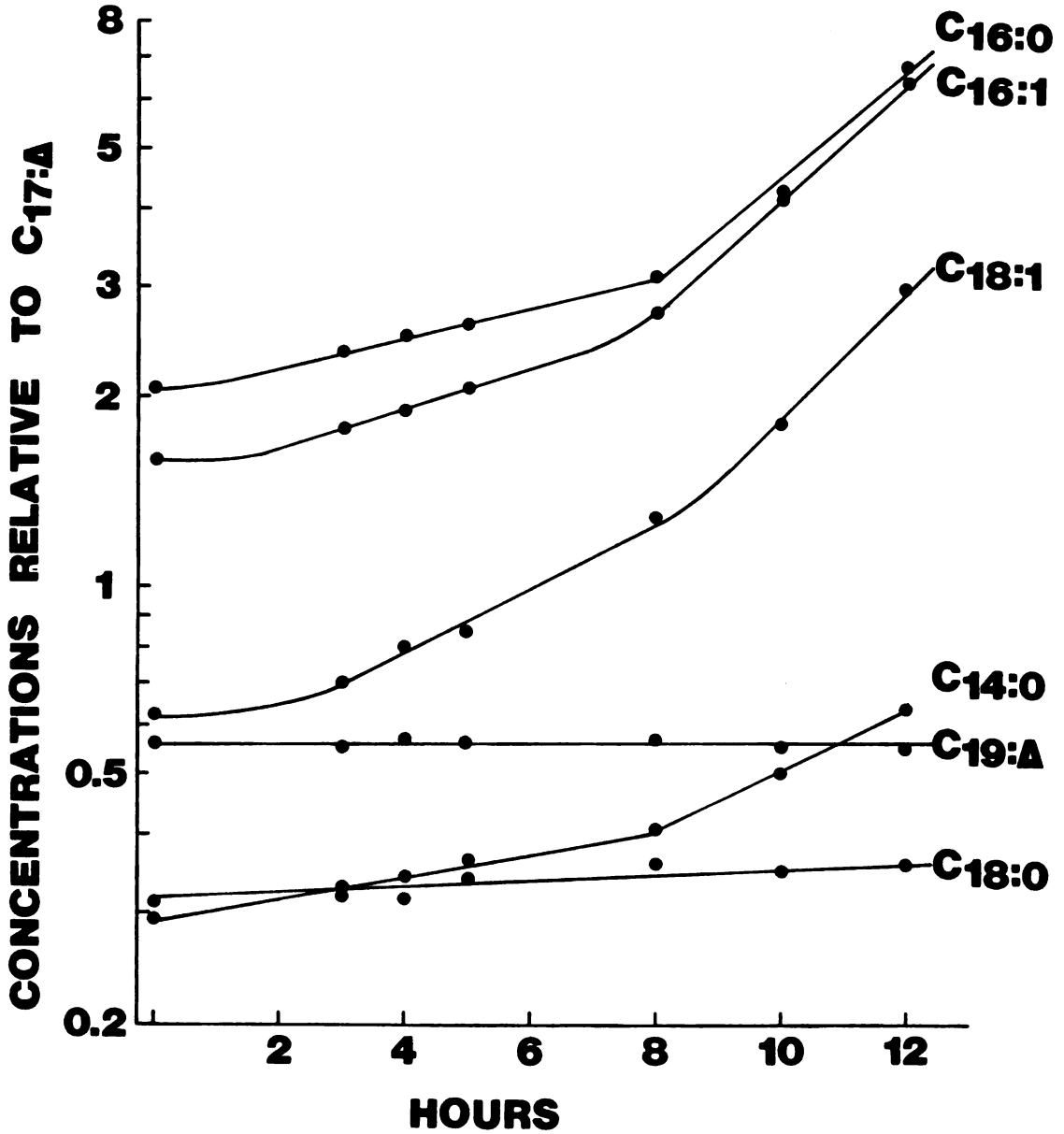
The total percentage of $C_{16:1}$ plus $C_{17:\Delta}$ and $C_{18:1}$ plus $C_{19:\Delta}$ in all samples was relatively constant. $C_{17:\Delta}$ is not a constituent of vegetative cells but it does occur in young vegetative cells derived from cysts (sample 6, 7). The cyclopropane fatty acids are metabolically stable (18) and the amount of $C_{17:\Delta}$ present in the membrane lipids was used as an internal standard in calculating the rate of synthesis of other membrane fatty acids during germination (Fig. 2). The invariability of the amount of $C_{19:\Delta}$ present in the membrane lipid (relative to $C_{17:\Delta}$) lends credence to this approach. The increase of those four fatty acids, C_{14}, C_{16}, $C_{16:1}$ and $C_{18:1}$ found in vegetative cells correlate with the cell size increase during the germination of cysts. From 0 to 8 h, differences in the rate of increase (synthesis) of the fatty acids were noted but after 8 h, cells began their exponential growth and the rates of increase of the three major fatty acids were identical. Cells newly derived from cysts contain $C_{17:\Delta}$, $C_{19:\Delta}$ and C_{18} fatty acids in their membranes. These are presumably lost by dilution as the culture progresses into exponential growth.

Table 1.--Percentage of various fatty acids at various time during the germination of Azotobacter vinelandii cysts and vegetative cells at different OD.

Sample Number	1	2	3	4	5	6	7	8	9
Components / Incubation time (hour)[a]	0	3	4	5	8	10	12	Vegetative OD=0.4	Vegetative OD=0.7
% of $C_{14:0}$	4.58	4.56	4.64	4.67	4.27	3.97	3.39	3.35	4.29
% of $C_{16:1}$	24.63	25.28	25.53	26.50	28.85	32.73	34.22	39.64	40.38
% of $C_{16:0}$	31.98	33.44	33.63	33.50	33.01	33.84	36.21	35.22	36.71
% of $C_{17:\Delta}$	15.52	14.28	13.47	12.84	10.62	7.92	5.34	0	0
% of $C_{18:1}$	9.68	9.87	10.75	10.89	13.34	14.40	15.96	21.79	18.62
% of $C_{18:0}$	4.88	4.71	4.26	4.36	3.84	2.76	1.92	?	?
% of $C_{19:\Delta}$	8.73	7.86	7.72	7.24	6.07	4.38	2.96	0	0
$C_{16:1} + C_{17:\Delta}$	40.15	39.56	39.00	39.34	39.47	40.65	39.56	39.64	40.38
$C_{18:1} + C_{19:\Delta}$	18.41	17.75	18.47	18.13	19.41	18.78	18.92	21.79	18.62

[a]The times listed are those post germination initiation (except in the cases of vegetative cells).

Fig. 2.--The relative amount of various fatty acids compared to $C_{17:\Delta}$ at various times during the germination and outgrowth of Azotobacter vinelandii cysts.


8
5
3
2
1
0.5
0.2
CONCENTRATIONS RELATIVE TO C17:A
2
4
6
8
10
12
HOURS
C16:0
C16:1
C18:1
C14:0
C19:A
C18:0

Discussion

The germination of A. vinelandii cysts has been studied and compared to the germination of Bacillus spores (15, 28). Loperfido and Sadoff (15) divide the germination of cysts into two stages: germination and outgrowth by applying the parameter that the initiation of DNA synthesis signals the beginning of outgrowth (23). The germination process yields cells whose membranes contain fatty acids characteristic of vegetative cells.

Fatty acid synthesis during the germination of cysts does not follow the pattern of total protein and RNA synthesis during germination (15). It occurs at a low exponential rate and lacks the critical transition in rate between 4 and 5 h (15). All four fatty acids found in membranes of vegetative cells of A. vinelandii are synthesized very soon after the initiation of germination and maintain their distinctive synthetic rates throughout the germination and outgrowth. The resumption of DNA synthesis and nitrogen fixation and the sharp increase in respiration rate which occur at the beginning of outgrowth are membrane related functions (4, 19, 20, 21, 29) but do not appear necessarily to be the result of changes in rate of membrane fatty acid synthesis. Fralick and Lark (4) have suggested a role for unsaturated fatty acids in the initiation of DNA synthesis. If this is so, our results are consistent with the view that a threshold level of unsaturated fatty

acids must be achieved or a unique membrane site containing unsaturated fatty acids must be generated.

Cyclopropane fatty acids which were found in cysts have also been found in a variety of bacteria and higher plants (5). Metabolically, these acids are stable (18) and thus we used them in this study as internal standards. The cyclopropane acids are synthesized from the corresponding unsaturated fatty acids by their accepting a methyl group from S-adenosylmethionine to form the ring structure (17, 30). In all samples the total amount of $C_{16:1}$ plus $C_{17:\Delta}$ and $C_{18:1}$ plus $C_{19:\Delta}$ was relatively constant. It is of interest that the cyclopropane fatty acids occur in cysts of A. vinelandii but not in vegetative cells. Their role in the encysting cell membrane is unknown. Hofmann et al. (9) have found $C_{19:\Delta}$ is capable of substituting metabolically for $C_{18:1}$ in Lactobacillus arabinosus and L. casei. Haest et al. (6) suggest that cyclopropane fatty acids are more stable forms of unsaturated fatty acids with the same physicochemical properties in the membrane.

Marr and Ingraham (16) found that the stationary phase cells of E. coli contained large amount of cyclopropane fatty acids compared to cells in the exponential phase. They further noted that ammonium ion in the medium could decrease the amount of cyclopropane and increase palmitic acid level and thus suggested that the formation

of cyclopropane fatty acids could be due to nitrogen limitation. A. vinelandii cells cease their nitrogen fixation immediately after the induction of encystment (8) resulting in nitrogen starvation of cells which could induce the formation of cyclopropane fatty acids.

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APPENDIX

THE CHANGE IN C-5-EPIMERASE ACTIVITY AND THE CALCIUM UPTAKE DURING THE ENCYSTMENT OF *AZOTOBACTER VINELANDII*

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THE CHANGE IN C-5-EPIMERASE ACTIVITY AND THE CALCIUM UPTAKE DURING THE ENCYSTMENT OF AZOTOBACTER VINELANDII

Introduction

A. vinelandii under most cultural conditions possesses a capsule that is mainly composed of polysaccharide. During growth, some of the capsular component is released into the medium as a slime. In 1964 Cohen and Johnstone (2) purified the extracellular polysaccharides from the growth medium and subjected them to chemical analysis. They found that the extracellular polysaccharides from three different strains of A. vinelandii were carboxylic acid hetropolysaccharides of apparent high molecular weight. Various analytical methods indicated that the polymers contained in common galacturonic acid, α-D-glucose and rhamnose in a ratio of 43:2:1 as well as a hexuronic acid lactone, probably mannurono-lactone. Minor differences existed between different strains. In 1967 Gorin and Spencer (4) found that the extracellular polysaccharides of A. vinelandii has a composition similar to alginic acid consisting of partly acetylated polyuronic

acids, mainly of D-mannuronic acid but also a small portion of L-guluronic acid. Larsen and Haug's (10) results were similar and they found that the addition of acetate to the growth medium increased the extracellular polysaccharide production greatly, especially when ammonium acetate was used. The proportion of D-mannuronic acid to L-guluronic acid in the extracellular polysaccharides was greatly influenced by the calcium concentration in the medium (10). Subsequently, they found that an enzyme, 5-epimerase, was present in the growth medium (7) and was capable of epimerizing D-mannuronic to L-guluronic acid in the polymer chain.

Polymannuronic acid 5-epimerase was found in A. vinelandii as well as in some marine algae (14, 6). It was partially purified by ammonium sulfate precipitation. The optimal pH of this enzyme is about 7 and calcium ions are essential for its activity, stability and possibly participate in the epimerization. Strontium ions can replace calcium in the reaction but are less effective. Both guluronic-mannuronic (GM) and guluronic-guluronic (GG) blocks are the end products of this enzyme's activity (7).

D-mannuronic acid (M) and L-guluronic acid (G) are the only uronic acids present in the exine and intine of the cyst of A. vinelandii. The ratio of G/M is higher in the exine than the intine which resembles the vegetative cell capsule in block composition and G/M ratio.

Polymannuronic acid 5-epimerase is present in central bodies and culture fluid of the mature cysts. When calcium ion or magnesium ion is omitted in the encystment medium, abortive encystment occurs and causes the release of cell materials into the medium. This results in an increase in the viscosity of the medium. In the case of abortive encystment due to calcium or magnesium omission, the enzymatic activity of 5-epimerase is lower than normal but the enzyme activity can be restored to 60% of the normal level if calcium ion is added back to the medium (15). Polyguluronic acid has 20-fold higher affinity for calcium ion than polymannuronic acid. Guluronic acid residues in the M-G sequences also bind calcium selectively (16).

Electronmicroscopically, exine appears to be a multilayered sheet like structure (12, 8). EDTA causes the rupture of cyst exine which in turn causes the release of the intine and central body into the growth medium (17, 12). It is believed that during encystment, calcium ion in the medium activates the polymannuronic-5-epimerase and changes the polymannuronic acids in the cell capsule into polyguluronic acid or polymannuronic-guluronic blocks. Guluronic acid then binds with calcium and together with lipids and protein components they form lipoprotein-lipopolysaccharide leaflets of the exine. Calcium ion presumably forms interchain linkages (15). But as the

exine becomes thicker and thicker, it becomes a physical barrier which may prevent the calcium ion and 5-epimerase from reacting with newly synthesized material. Page and Sadoff (unpublished) have isolated a mutant of A. vinelandii which is unable to encyst having no 5-epimerase.

We wish to see the change of polymannuronic-5-epimerase activity during the course of encystment as well as the calcium uptake during the encystment and coordinate these with other events already known to occur during the encystment.

Experimental

Substrate Preparation.--In a previous study, Haug and Larsen (7) used an alginate prepared from Ascophyllum nodosum to test for the epimerase. Their substrate contained 93% of D-mannuronic and 7% of L-guluronic acid. Page and Sadoff (15) used a commercial alginate from Macrocystis pyrifera but it contained only 60% mannuronic acid (5). An attempt was made to purify some mannuronic acid from the commercial alginate substrate (alginate from Macrocystis pyrifera, Sigma Chemical Co., St. Louis, Mo.). This was subjected to acid hydrolysis and then separated by column chromatographic method described by Haug and Larsen (5). The procedure was very time consuming and only small amount of mannuronic acid was purified each time. Beside that, strong acid hydrosis cleaved the polyuronic acid

into monomers which are not natural substrates of the 5-epimerase. An alternative approach was taken and A. vinelandii was grown in B medium (10) with a low concentration of calcium ion (0.03 mM of $CaCl_2$ in growth medium) for 5 days. The polysaccharide or alginate released in the growth medium was precipitated by the addition of 4 volumes of cold ethanol after cells had been removed, the precipitate was collected by centrifugation, redissolved in distilled water and then boiled for 10 minutes to "kill" the 5-epimerase and denature all the protein in the substrate. The denatured protein was removed by centrifugation. The ethanol precipitation was repeated and the precipitate was redissolved in one fourth the original culture volume of distilled water. The polysaccharide was dialyzed extensively against deionized water at 4 C for 40 hours to remove all the small molecules. If it was stored, a few drops of toluene were added to prevent the growth of mold.

Characterization of the Substrate.--The percentage of uronic acid in the alginate was assayed by the modified carbazole method of Bitter and Muir (1). The protein contamination was estimated by the method of Lowry et al. (13) with bovine serum albumin as standard. Total carbohydrate was estimated by the phenol-sulfuric acid method (3).

Enzyme Preparation.--Cells of A. vinelandii ATCC 12837 were grown in 3 liters of Burk's nitrogen free medium with 1% glucose as carbon source. They were harvested, washed, and resuspended in same amount of Burk's N-free medium with 0.2% BHB to induce encystment (11). A 100 ml sample was taken initially and then every two hours for 40 hours of incubation at 30 C. Samples were spun down immediately after collection and the epimerase activity was tested in both supernant fluid and cells. The enzyme was partially purified and concentrated from the supernant by the method described by Haug and Larsen (7). Enough ammonium sulfate was added to achieve 30% saturation at 0 C and the resulting precipitate was discarded. The ammonium sulfate concentration was raised to 50% and the resulting precipitate was collected by centrifugation. This was washed once with 50% saturated ammonium sulfate solution and then dissolved in 2 ml of water. The epimerase in the cells was released by resuspending the cells in 3 ml of water and rupturing them with sonic treatment. The cell debris was spun down and the supernatant fluid was kept. All enzyme preparations were dialyzed against Burk's N-free medium with calcium ion omitted. The volume was adjusted to 5 ml after dialysis and the preparation stored at -20 C.

Enzyme Assay.--Enzyme activity in the sample was assayed by the method of Haug and Larsen (7). The

reaction mixture was composed of 0.25% alginate, 0.5 ml; 0.05 M collidine buffer, pH 6.8, 1.65 ml; 34 mM calcium chloride, 0.35 ml; and enzyme preparation, 0.5 ml. In controls, 0.35 ml of 0.1 M EDTA was used instead of the calcium chloride solution. The solution was mixed and incubated at 30 C for 20 hours. The reaction was stopped by the addition of 0.5 ml 0.1 M EDTA solution. To the control, both 0.15 ml of 0.1 M EDTA and 0.35 ml 34 mM $CaCl_2$ was added so that the final concentration of the chelator and calcium was identical in control and reaction mixture. The enzyme activity was determined by assaying the amount of mannuronic acid changed into guluronic acid with carbazole at 55 C without borate (9). When reacted with carbazole, 50 µg of mannuronic acid produces an absorbance of 0.06 at 530 nm, whereas the same amount of guluronic acid has an absorbance of 0.23. Hence, for every 50 µg of mannuronic acid converted, there is a 0.17 increase in absorbance. By measuring the difference in absorbance between the reaction mixture and the control, we can assay enzyme activity.

Calcium Uptake.--Radio active calcium (Ca^{45} New England Nuclear Co., Boston, Mass.) was used in this experiment. Cells of A. vinelandii were grown in 100 ml of Burk's N-free medium with 1% glucose as carbon source until late exponential growth. The culture was divided into two parts and each was spun down and washed once with

distilled water. One part was resuspended in Burk's N-free medium with 0.2% BHB (β-hydroxybutyrate) plus 50 μCi of radioactive calcium. The other part was resuspended in non radioactive Burk's N-free medium with 0.2% BHB. Both cultures were incubated in a Gyrotory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 30 C shaked at 240 rpm. Samples were taken at 0 time and then every two hours. These were cooled in ice for 2 minutes, collected on membrane filters (pore size 0.8 μ Gelman Instrument Co., Ann Arbor, Mich.), washed once with 1 ml Burk's N-free buffer and dried. Filters were put in glass scintillation vials to which 10 ml of liquid toluene based scintillation fluid added and counted in a Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co., Downer Grove, Ill.) for 1 minute. The non radioactive control was treated by the same procedure except that after cooling in ice, 1 μCi of radioactive calcium was added and mixed before filtration.

Results and Discussion

Substrate.--Although commercial alginate from Macrocystis pyrifera is said to contain more than 70% of mannuronic acid, assays indicate it contains about 60% mannuronic acid and 40% guluronic acid, data which correspond to the result of Haug and Larsen (5). An attempt to purify mannuronic acid from commercial alginate was

discontinued because it was laborious and the yield was small. Growth of A. vinelandii in medium B with low calcium yielded large amounts of extracellular polysaccharide which had a high percentage of mannuronic acid. After 5 days of incubation, 2 liters of B medium with 0.035 mM calcium yielded after purification 1.7 g of polysaccharide contaminated with 60 mg of protein. This material contained 81.3% mannuronic acid. The extracellular polysaccharide of A. vinelandii also contained small amount of glucose and rhamnose (2) which formed a chromogen in the carbazole assay (9) and caused some error in determining the amount of uronic acid in the substrate. The substrate was free of epimerase.

Enzyme Activity.--It is very likely that C-5-epimerase is synthesized in the cell and then excreted through the membrane to the outer cell layers. Haug and Larsen (7) isolated the enzyme from the supernatant of the growth medium. Page and Sadoff (15) found that in the mature cyst 91.4% of the C-5-epimerase is in central body and 8.6% in supernatant. So, the C-5-epimerase activity was assayed in both cells and in the supernatant of all the samples. In order to control the calcium concentration in the reaction mixture, all enzyme preparations were dialyzed against Burk's N-free medium without calcium for 40 hours at 0 C to eliminate the calcium ion and ammonium sulfate. The final volume of each enzyme

preparation was readjusted to the same amount with H_2O before it was assayed. The enzyme activity in all cases was too low to detect any significant difference between the reaction sample and control. Because calcium ions are important to the activity and stability of this enzyme, its activity could have been destroyed during the dialysis. In later trials the enzyme preparations were all dialyzed against Burk's N-free medium with 0.34 mM calcium ion, but the enzyme activities observed were still very low. Lowry protein assay revealed that the supernatant of encysting cells contained only 1.5-5 mcg of protein per ml. This explains why very little protein was precipitated from the supernatant by ammonium sulfate. Conceivably, there was no epimerase in the supernatant culture fluid.

The method of enzyme assay is particularly poor, especially when the epimerase activity is very low. Any small variation in sample size could cause great error, sometimes even resulting in negative activity. Another built in error results from the fact that the colors developed from uronic acids are not stable and the reaction conditions are not easy to control.

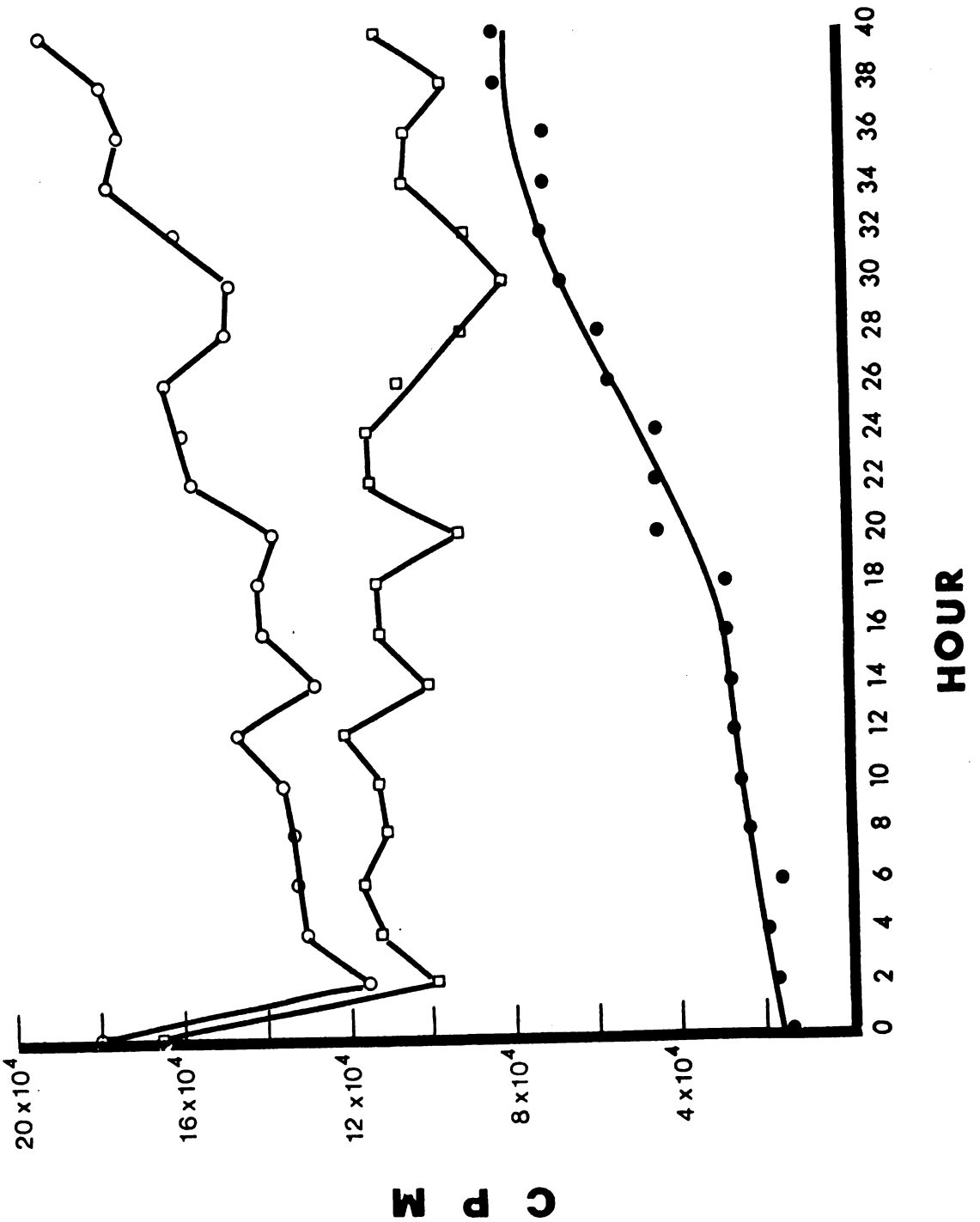
The conclusion was reached that the principal cause of failure in this investigation was the inadequate enzyme assay.

Calcium Uptake.--The results of calcium uptake studies during the encystment of A. vinelandii is shown in

Fig. 3. The uptake of calcium occurs at a near constant rate for about 20 hours, increases to a new value during the next 12 hours, and ceases by 36 hours. This possibly reflects the amount being bound to the uronic acids of the cyst coat. This probably results from the increase of uronic acids outside the developing cysts and the change of mannuronic into guluronic acid which produces a greater affinity of the polymer for calcium (15, 16).

The high background count in the control is due to the Burk's buffer. This medium contains phosphate which, on mixing with Ca^{45} forms radioactive precipitate which is retained on filters.

Fig. 3.--Calcium uptake during encystment of A. vinelandii. (o) encysting cells incubated in medium containing radioactive calcium. (□) control. (●) net increase of radioactivity in encysting cells.


CPM
20 x 10^4
16 x 10^4
12 x 10^4
8 x 10^4
4 x 10^4
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
HOUR

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