MEMBRANE PROPERTIES OF THERMOPLASMA ACIDOPHILA

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
MARY J. RUWART
1974

This is to certify that the

thesis entitled

MEMBRANE PROPERTIES OF THERMOPLASMA ACIDOPHILA

presented by

Mary J. Ruwart

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biophysics

A. Haug

Dr. A. Haug
Major professor

Date Dec. 21, 1973

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ABSTRACT

MEMBRANE PROPERTIES OF THERMOPLASMA ACIDOPHILA

By

Mary J. Ruwart

Plasma membranes were isolated from Thermoplasma acidophila, a mycoplasma-like organism which grows optimally at pH 2 and 59° C. Cells in concentrated suspensions were lysed by titrating to pH 9.3. The membranes were purified by washing at pH 10.0 and centrifuging on a discontinuous sucrose gradient. Membrane purity was assessed by electron microscopy, deoxyribonucleic acid content, and polyacrylamide gel electrophoretic behavior. Small quantities of membrane could be prepared by a combination of osmotic shock and sonication. These membranes were indistinguishable from those prepared via high pH lysis as judged by the above criteria. Gel electrophoretic patterns and amino acid composition of cells and membranes were found to differ significantly. The lipids contained small amounts of fatty acid esters and larger amounts of branched long-chain alkyl ethers.

The fluidity and structure of Thermoplasma acidophila membranes were investigated with electron paramagnetic resonance techniques.

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Due to the relative homogeneity of the lipid matrix, sharp temperature-dependent lipid transitions were detectable with stearate spin labels. When labelling was performed at pH 2, transitions were observed at 15° and at 60° C., although Thermoplasma acidophila was grown at 56° C. Within the growth range of this organism (45° to 60° C.), the fluidity undergoes relatively little change. Order and rotational parameters indicate that Thermoplasma acidophila has one of the most rigid biological membranes known. Partial interaction between radicals located on the sixteen position of the stearic acid were observed at low molar ratios of label to lipid. Upon denaturation of the membranes at temperatures greater than 65° C., this type of spectrum is gradually converted to one characteristic of strong interaction. Extensive interactions were also noted when nitroxy cholestane was incorporated into the native membrane. Our results appear to be consistent with a model wherein at least some of the lipids are arranged in a cluster rather than the conventional bilayer.

MEMBRANE PROPERTIES OF *THERMOPLASMA ACIDOPHILA*

By

Mary J. Ruwart

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biophysics

1974

This di

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DEDICATION

This dissertation is dedicated to my loving husband and especially to my parents whose lifelong encouragement has sustained me these many years.

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ACKNOWLEDGEMENTS

The author wishes to acknowledge the support and encouragement of her husband, relatives, friends, and co-workers. The helpful comments and advice of the committee members were very much appreciated. Special thanks are extended to Dr. D. T. A. Lamport, Dr. G. G. Hooper, and Dr. C. C. Sweeley, as well as their colleagues, for assistance in amino acid analysis, electron microscopy, and mass spectrometry respectively. The technical assistance of Mr. G. Smith, Mr. D. Jaquet, and Ms. N. Brittain is gratefully acknowledged. Very special commendation is made to Dr. A. Haug, whose guidance and encouragement made this project possible. The author also wishes to thank Dr. A. Lang for enabling her to carry out these investigations in the MSU/AEC Plant Research Laboratory.

This research was supported by the U.S. Atomic Energy Commission Contract No. AT-(11-1)-1338. The author was also supported by U. S. Public Health Service Training Grant No. GM-01422 from the U.S. National Institutes of Health and by the College of Osteopathic Medicine, with the cooperation of the Biophysics Department, Michigan State University, East Lansing, Michigan.

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CHAPTER I

GENERAL INTRODUCTION

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INTRODUCTION

Biological membranes have been the subject of much study in recent years because they are intimately involved in the life processes of the cell. Once considered a mere envelope for containing the cytoplasmic matrix, the membrane is now known to actively control transport and to be the site of macromolecular synthesis and function (70). Surface structures generated by these membranes are immunogenic recognition sites (71). Furthermore, it appears that the plasma membrane structure may be one of the limiting factors determining the extremes of environment under which an organism may live (72).

Many models have been proposed for membrane structure, which basically can be classified into two major groups based on their lipid arrangements. The oldest and probably the most substantiated model describes the lipid as a bilayer structure with protein coating or penetrating the lipid matrix. The bilayer can be continuous or broken into segments by the protein (73-76). The second model proposes that the lipids are in clusters, with protein interspersed between or into the globules (77,78). Many excellent reviews are available on this subject and therefore will not further be discussed herein (79).

Probably the most widely studied bacterial membrane is that of Acholeplasma laidlawii. Because it is a mycoplasma, it has no cell wall, making membrane isolation and purification relatively easy (80).

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Lipid composition can be manipulated by supplementing the growth media with fatty acids and cholesterol. Physical studies on this membrane have indicated that its lipids are in a bilayer form. A. laidlawii has been well studied in many respects, including electron paramagnetic resonance analysis of its fluidity and thermal transitions (66).

In 1967, a mycoplasma-like organism was isolated from a coal refuse heap and named Thermoplasma acidophila since its optimal growth conditions were pH 2 and 59° C. (1). It differed from A. laidlawii and other mycoplasma in that it did not require lipid supplementation of its growth media. Because of its lack of a cell wall, however, and its probable relation to a well-studied organism, information could be obtained as to how the hot, acidic environment influenced membrane structure and function. T. acidophila cannot grow at temperatures higher than 62° C. and this lives very near the high temperature limit found empirically for acidophiles (36). Thermophilic bacteria have been discovered which can grow in near boiling water at neutral pH. No organism which requires a pH less than 3 has been found that can also grow at 70° C. Thus, T. acidophila represents the edge of the acidophilic, thermophilic frontier at which life may exist. Since the membrane comes into direct contact with this harsh environment, its structural and functional characteristics may determine the environmental limits at which T. acidophila may grow.

Initial studies revealed that T. acidophila contained branched, long-chain alkyl ethers (2). In this respect, this organism was compared to Halobacterium cutirubrum which had been found to contain

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phytanyl ethers. H. cutirubrum grows at high NaCl concentrations and is solubilized in low saline (81); T. acidophila requires high proton concentrations for growth, and is solubilized at low hydrogen ion contrations. These findings lend support to the hypothesis that certain types of environmental stress, regardless of their specific nature, can evoke similar responses in different organisms.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF THE PLASMA MEMBRANE

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INTRODUCTION

Thermoplasma acidophila, a mycoplasma-like organism with no cell wall, grows optimally at pH2 and 59°C. (1). Investigations of the lipids of this organism revealed the presence of large quantities of long-chain alkyl glyceryl ethers but no fatty acid esters (2). Membrane preparations have been attempted (3), but commonly used lytic procedures have not been successful (4). Herein, we describe properties of purified *T. acidophila* membranes obtained in high yield by rupturing the cells at alkaline pH.

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MATERIALS AND METHODS

Growth of Cells. Thermoplasma acidophila was kindly provided by the American Type Culture Collection. The organism was grown at pH 2, 56°C (1). Yeast extract used in this medium was obtained from a single lot (Difco Control #583197). Several experiments were performed with yeast extract which had been lipid extracted (5). Cultures were inoculated with a 10% (v/v) aliquot taken from a 22 hour culture. Each culture was continuously aerated with sterile, filtered air. Late log phase cells were harvested after 22 hours of growth and treated as described below.

Collection of Cells. Intact cells were obtained by centrifugation at 4200 X g for 5 min. at 25°C. Medium components were removed by gently resusperding cells in T-buffer (0.02% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 0.025% $CaCl_2.2H_2O$, and 0.3% KH_2PO_4, adjusted to pH 2) by shaking on an Eberbach shaker at low speed.

Preparation of Membranes.

1). Cell Rupture.

Osmotic shock: Thermoplasma acidophila cells were exposed to osmotic shock both at pH 2 and pH 7.4. Lysis was attempted with the following four methods: a). Collected, washed cells were diluted 1000-fold with deionized, distilled water and stirred for 2 hours. b). Cells were resuspended in glycerol (20% or 50%), sucrose (25%, 50%, or 70%), 50% glucose, or NaCl (1M or 3M) and stirred for 1 hour prior to dilution as in (a). Methods (a) and (b) were performed at 4°, 23°, or 55°C in order to determine if temperature affected cell rupture. c). Cells were stirred for 1 hour in T-buffer, centrifuged at 34,000 X g for 30 min., and then resuspended in 75% T-buffer. These cells were stirred for 1 hour and the entire procedure

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was repeated with 50% and 25% T-buffer, respectively. d). Gradual osmotic lysis as in (c) was also attempted with beta-buffer (6). Procedures (c) and (d) were performed in the presence or absence of 0.0125 M Na-EDTA.

Denaturants and Solubilizing Agents: Cells were resuspended in 1 M or 5 M urea; 5% or 50% dimethylsulfoxide; 0.1% or 1.0% Triton-X100; or 0.1% sodium lauryl sulfate.

Freezing: Cells suspended in distilled water were frozen in liquid nitrogen and thawed. This procedure was repeated five times.

Mechanical Lysis: Cells were suspended in distilled water or 0.1 M phosphate buffer, pH 7.4, and were treated for 1 hour with the following: a). Branson automatic cleaner sonicator Model D-50, or b). Raytheon Sonic Oscillator, Model DF-101, 0.92 amperes; or 30 minutes with: c). Omnimixer, Ivan Sorvall Omnimixer Inc.,; or d). Sorvall Ribi Cell Fractionator Model RF-1. or e). Biosonik sonicator, 80% full power. Procedures (b) and (c) were also performed in the presence of 110 micron glass beads (Super Bright Size 110).

Lysis by High pH: This method was most successful for rupturing cells. Cells were collected and washed with T-buffer (1 X) and/or water (1-5 X, depending on the experiment). The washed cells were then resuspended in distilled water at a protein concentration greater than 10 mg/ml. The pH was adjusted to 9.3 by adding 2.0 N NaOH dropwise with stirring. The pH was determined with a Beckman Zeromatic II pH Meter.

2). Membrane Purification.

Membranes from High pH Lysis: Membranes were collected at 34,800 X g for 4 hours. The membrane pellet was resuspended in half as much water as was used during lysis and the pH of the suspension was adjusted to pH

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10 with 2N NaOH. The membranes were then collected as before, layered onto a discontinuous sucrose gradient (25%/55%), pH 7.4 and centrifuged on a Beckman L2-65B Ultracentrifuge for 2 hours at 40K with an SW41 rotor. The membranes appeared as a single band at the interface of the two sucrose layers and were collected and freed from sucrose by washing with water.

Lipid Extraction.

Lipids were extracted from cell or membrane pellets after multiple water washings by homogenizing with chloroform-methanol (2:1, v/v). Debris was removed by filtration through a fat-free filter or by low speed centrifugation. After evaporation to dryness, non-lipid contaminants were removed by passing the extract over Sephadex (7) and eluting lipids with chloroform-methanol (19:1) and chloroform-methanol-acetic acid 19:1:4. Silicic acid chromatography (Anisil, 100-200 mesh; 1.5 x 12 cm column) was also employed to separate the lipids into three fractions: 1). neutral (50 ml chloroform), 2). glycosyl diglycerides (50 ml acetone), and 3). phospholipids (50 ml chloroform:methanol, 2:1 v/v; 50 ml methanol). All glassware used in lipid analyses was acid-cleaned prior to use. All solvents were redistilled.

Fatty Acid Ester and Alkyl Ether Derivatives.

Fatty acid methyl esters were prepared by incubation of lipid as follows: 1). in 2.5% methanolic H_2SO_4 for 24 hours at 40°C; or 2). in 10% methanolic H_2SO_4 for 24 hours at 75°C (8); or 3). in 10% methanolic BF_3 and benzene (4:1) for 48 hours at 75°C (9). After methanolysis, water was added and the methyl esters were extracted with hexane and washed with water until neutral. Alkyl ethers were converted to their alkane or acetate derivatives (10). For quantitation by gas chromatography, C_{21} methyl ester or C_{24} alcohol standards were added prior to derivatization.

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Gas-Chromatography.

A Hewlett Packard Model 402 gas chromatograph with a flame ionization detector and with six foot glass columns was used throughout. Fatty acid methyl esters were determined at 170°C on 15% DEGS(80-100 mesh, Hewlett Packard). Alkyl ether derivatives were separated on 1% SE-30 (80-100 mesh, Hewlett Packard). The ethers were eluted isothermally at 320°C or programmed at 8°/min. from 200°C. Molar response factors were determined empirically from standards obtained from Applied Science. Areas of peaks were determined with a planimeter or by height and width-at-half-height measurements.

Thin Layer Chromatography (TLC).

Qualitative TLC of the three lipid fractions was performed on Ana-sil-G (250 micron) plates (Analabs, Conn.) as described by Langworthy et al (2).

Electron Microscopy.

Negative staining was performed with 1.5% or 2.0% phosphotungstic acid at pH 6 and 8, respectively. Samples were fixed in 2% glutaraldehyde, 0.033M cacodylate buffer, pH 6.1 for 2 hours at 4°C. The sample was collected via centrifugation and washed once in 0.1 M cacodylate buffer containing 0.25 M sucrose. The sample was again centrifuged and 2% osmium tetroxide in R-K buffer (11) was carefully added to the pellet without disturbing the surface. After 18 hours at 4°C, the preparation was drained and dehydrated through a graded ethanol series. The sample was embedded in Spurr's epoxy resin (12) and stained with lead citrate and uranyl acetate (13).

Intact cells were dehydrated through graded ethanol and isoamyl acetate, and dried in a Bowmar Critical Point Apparatus. Gold palladium (150-200 Å) was shadow-casted onto the specimen with a Ladd Vacuum Evap-

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orator. A Philips 300 Transmission Electron Microscope and a AMR 900 Scanning Electron Microscope were used to view the specimens.

Mass Spectrometry.

All mass spectra were taken with a LKB 9000 Gas Chromatograph-Mass Spectrometer equipped with computerized data acquisition (Dr. C. C. Sweeley, Dept. of Biochemistry, Michigan State University, East Lansing, Mich.). Fatty acid methyl esters were run on a 6 ft. 3% SE-30 column; alkyl ether derivatives were eluted on a 1% SE-30 (2ft. or 6 ft.)

Analytical Methods.

Protein was quantitated by the method of Lowry et al. (14). Phosphate was assayed using the Bartlett method (15). Ester and ether derivatives were hydrogenated as reported (16). Acyl esters were measured by the ferric hydroxymate method (17) and by infrared absorption at 5.85 micron (18) on a Perkin-Elmer Model # 621 grating IR Spectrophotometer. Deoxyribonucleic acid content was determined via the Burton modification (19). Assays for the presence of phosphonate (20) and plasmalogens (21-23) were performed. After removal of non-lipid contaminants, lipids were quantitated by a modification of a previously reported colorimetric method (24). Carbohydrate (hexose) was analyzed colorimetrically (25). Glycerol was liberated by 24 hour incubation of lipid at 25°C in BCl_3-chloroform (1:1, v/v) and assayed after periodate oxidation. (26).

Gel Electrophoresis.

SDS polyacrylamide gel electrophoresis was performed as follows: membranes, cells or cytoplasm (precipitated with hot trichloracetic acid) were suspended in 0.1% mercaptoethanol, 0.01 M phosphate buffer (pH 7.1), and 1.0% sodium dodecyl sulfate (SDS) and incubated at 100°C for 2 min. Sucrose and bromophenol blue were then added. Samples were layered onto 5%

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or 10% (8 or 10 cm) polyacrylamide gels containing 0.1 M phosphate buffer (pH 7.1), 0.1% SDS, 0.026 ml of N,N,N-tetramethylethylenediamine per 100 ml of gel, 0.2% methylene bisacrylamide, and 0.065% ammonium persulfate. The upper and lower buffer reservoirs contained 0.1% SDS and 0.1 M phosphate buffer (pH 7.0). Gels were electrophoresed for 15 mins. at 10 mA per gel for 5 hours. Gels were stained overnight in 0.15% Coomassie Blue R, 7.5% acetic acid, 5.0% methanol. Gels were shaken in destaining solution (5.0% methanol, 7.5% acetic acid) for 6 hours and electrophoretically destained for approximately 2 hours. Gels were stored in distilled water at 4°C.

Native polyacrylamide gel electrophoresis was carried out with 2.2%, 4.5%, and 9.0% acrylamide as described by Davis (27) with the following modifications: sample and spacer gels were omitted, pH was adjusted to 8.6, and 10-20 microliter of sample containing 20-200 microgram protein, 10% glycerol, 5% bromophenol blue, and 0.1 M buffer (pH 8.6) were layered directly on each gel. Samples were electrophoresed from cathode to anode at constant current of 1 mA/gel for 10 min followed by 2.5 mA/gel/hr. Gels were stained for protein with Xylene Brilliant Cyanin G prepared by a modification of the Malik-Berrie procedure (28) as described by Blakesly (29). Gels were stained for 5-8 hours. Stained gels were rinsed two times with distilled water and stored in distilled water at 4°C in the dark. The absorbance of the stained gels was monitored at 550 nm with a Gilford Model 2400 Spectrophotometer equipped with a Gilford Linear Transport.

Amino Acid Analysis.

An aliquot of cells or membranes was dried under nitrogen. 6 N HCl was added and 2.5×10^{-3} micromoles of phenol were added per ml of suspension.

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Hydrolysis was complete after 18 hours at 105°C. The suspension was taken to dryness under nitrogen and resuspended in water. Amino acid analysis was performed on a single column accelerated flow Technicon System modified by Dr. Derek Lamport, Plant Research Laboratory, Michigan State University, East Lansing, Mich. Results were computed by a modified Autolab System IV.

Preparation of Flagella-like Filaments.

Filaments were found in the supernatants from T-buffer and water washes of cell preparations (see section "Lysis by high pH"). Filaments were collected by ultracentrifugation of the above supernatants at 40 K for 2.5 hours in a SW-41 rotor. Small pellets containing filaments and cell debris were obtained. Collection of filaments could be faciliatated by washing cells only once in distilled water and stirring the suspension for one hour. Cells, menbranes, and debris were then removed by centrifugation at 34,800 X g for 30 min. and the filaments were harvested from the clear supernatant by ultracentrifugation.

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RESULTS

Growth Characterization. The growth curve of Thermoplasma acidophila at pH 2 and 56°C was determined in order to insure consistent results and to find optimal conditions for harvesting the cells. (Fig. 1). T. acidophila grew similarly on lipid-extracted medium, a finding that is very uncharacteristic of other mycoplasma. As shown in Figure 1, log phase terminated after 24 hours of growth at 56°C. Colonies could not be counted because solid media could not be obtained under growth conditions. Therefore, all cells were harvested or used as inoculum after a 22 hour incubation period. The rapid propagation of this organism is facilitated by vigorous aeration, since standing cultures grown in our laboratory have characteristics similar to non-aerated cultures as reported by Darland et al. (1). Shaken cultures (2) appear to grow at rates intermediate between those reported for non-aerated cells (1) and those reported herein for rapidly aerated cells. T. acidophila appears to be coccoidal in shape and cell diameters vary between 0.5 and 1.0 micron. Some cells have one or two filamentous projections (Fig. 2).

Membrane Preparation. T. acidophila has a more electron dense cytoplasm than Acholeplasma laidlawii (Fig. 3) although the membranes have similar thickness (100 - 80 Å) (30). Isolation of T. acidophila membranes by methods usually employed for mycoplasma were unsuccessful since they gave extremely low yields. T. acidophila is very resistant to osmotic lysis. Nonaerated cultures release no protein when exposed to the osmotic shock procedures described in the Methods section. Cells from aerated cultures appear to be more osmotically fragile; approximately 10% of the total cell protein and a yellow pigment were released by osmotic shock or mechanical treatment such as centrifugation. Additional osmotic shock or mechanical treatments did not

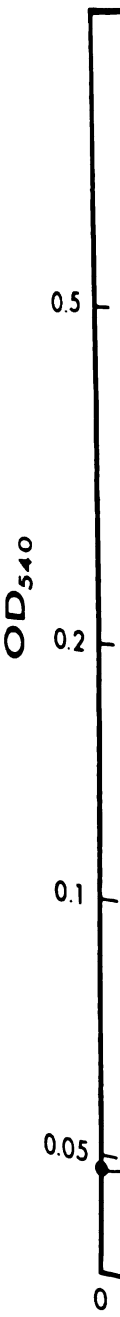

Figure 1. Growth curve of Thermoplasma acidophila at pH 2 and 56° C. Cultures were inoculated with a 10% (v/v) aliquot taken from a 22 hr. culture. Aeration was continuous throughout the growth period.

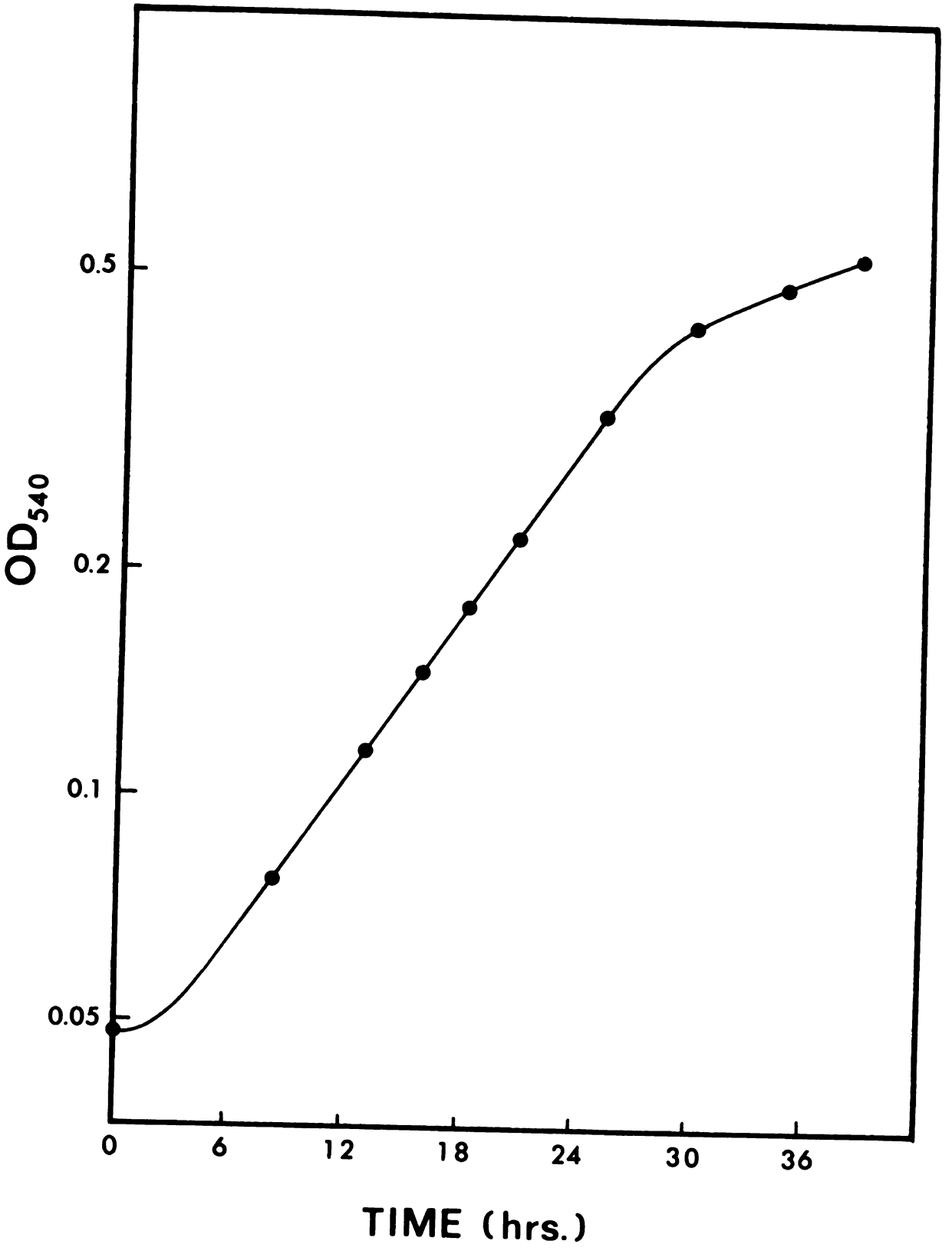

Figure 1

Figure 2. Scanning electron micrograph of critically-point dried late log phase T. acidophila cells shadowed with gold-palladium.

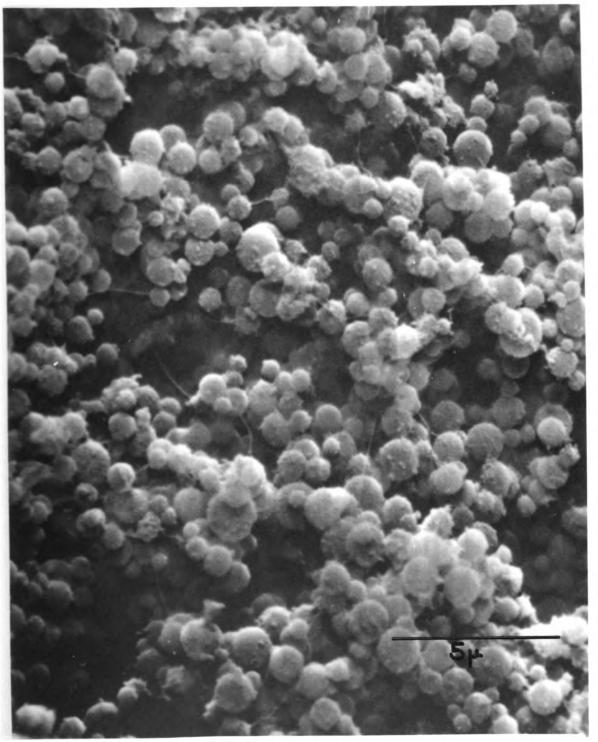

Figure 2

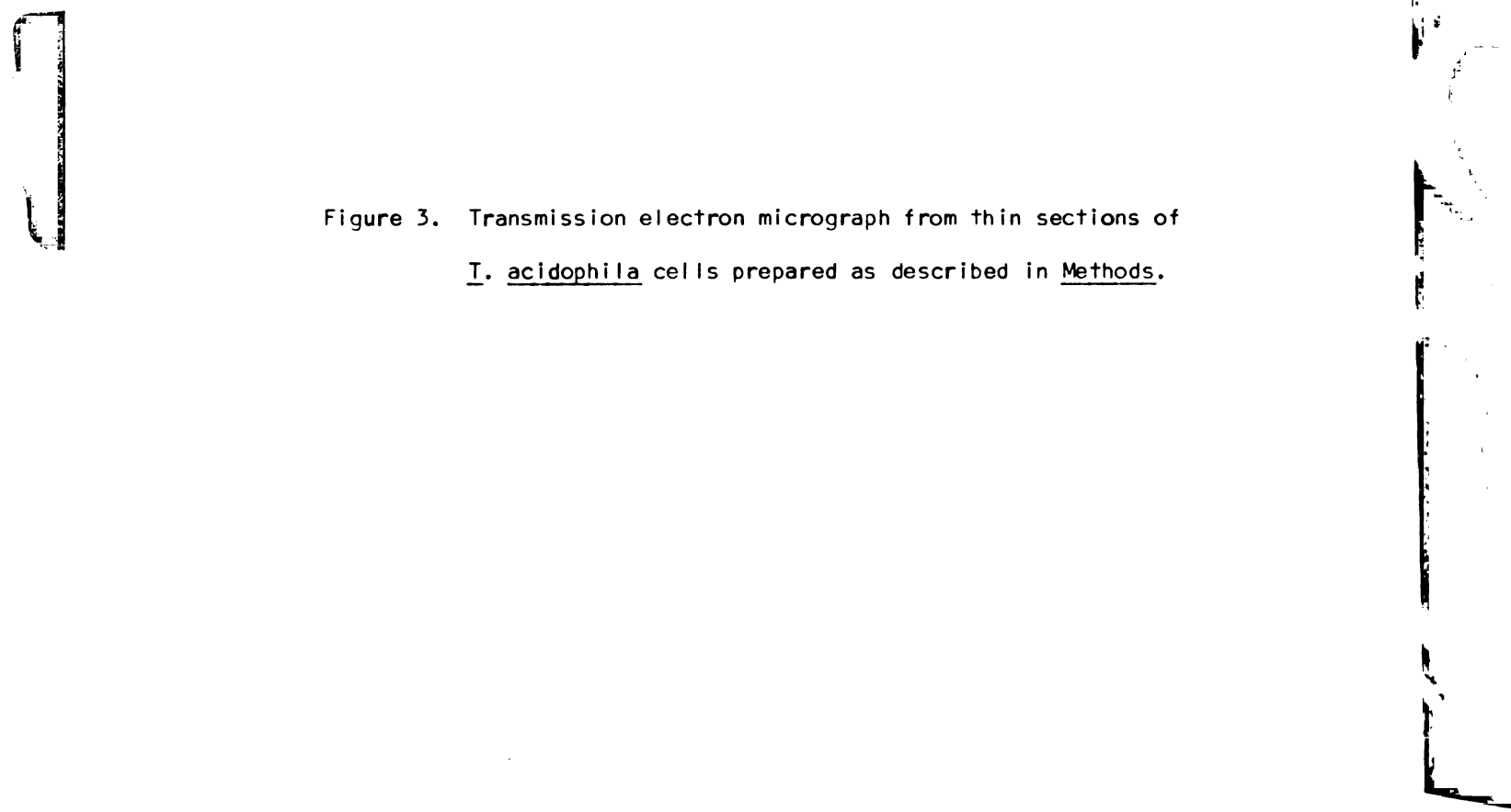

Figure 3. Transmission electron micrograph from thin sections of *T. acidophila* cells prepared as described in *Methods*.

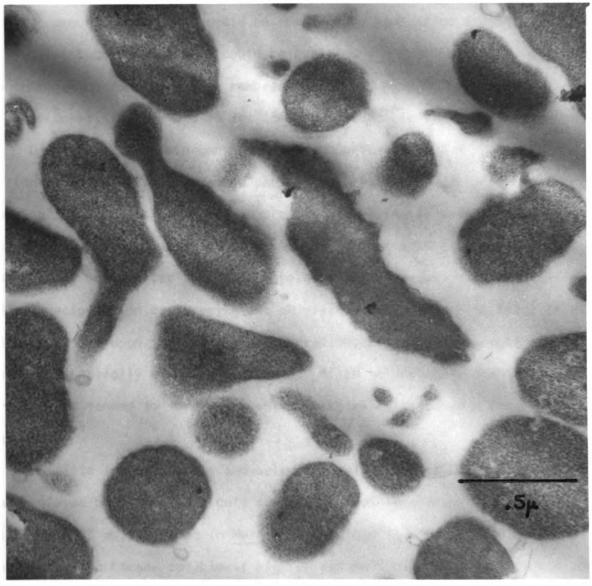

Figure 3

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release more protein. Repeated freezing and thawing of the aerated cells caused loss of viability, but only minimal loss of protein. No additional protein was released when cells, treated as described above, were washed in one of sixteen different buffers of varying molarity (1-5M) or pH (2 -7.8). A small amount of membrane could be obtained by sonication and osmotic shock (Fig. 4). Such preparations could be purified via sucrose density gradient centrifugation to give a pure membrane fraction, but the method was considered unsatisfactory due to the low, irreproducible yield (0-5%).

Denaturants and detergents at high concentrations (5 M urea, 1% Triton-X100, or 0.1% SDS) caused partial solubilization of cellular material, leaving intact cells or amorphous protein aggregates as determined by electron microscopy. Attempted lysis by raising the pH of dilute cell suspensions solubilized the cells completely in agreement with previous reports (3). Precipitation by lowering the pH yielded an amorphous material (Fig. 5) which depended upon the protein concentration, but generally began at pH 6.0 and was essentially complete at pH 4.5. At pH values lower than 4.5, the precipitate tended to form large white vesicles rather than a flocculent material.

Complete lysis of cells and selective solubilization of cytoplasm was achieved by titrating a cell suspension (not less than 10 mg protein/ml) to pH 9.3 as described in Methods. Care had to be exercised since membranes also became soluble if high pH was maintained and the protein concentration was reduced. After multiple washings with high pH buffers, membranes from such solubilized preparations was not possible. Titrating cell suspensions to pH values lower than 9.0 resulted in incomplete lysis as evidenced by the presence of intact cells collected after centrifugation for 20 min. at 34,800 X g.

Figure 4. Transmission electron micrograph of thin sections of T. acidophila stained as described in Methods. Cells were suspended in water and sonicated for one hour as described in Methods. Sedimentable material was washed ten times with 0.1 M phosphate fuffer, pH 7.4.

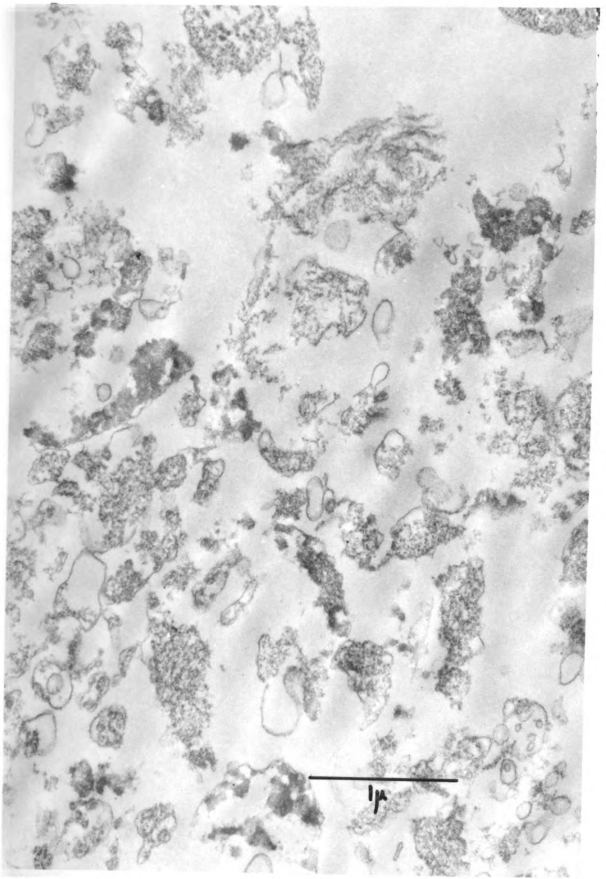

Figure 4

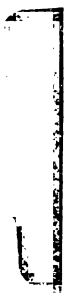

Figure 5. Transmission electron micrograph of negatively stained "amorphous" material collected after cytoplasm, solubilized by high pH, was reprecipitated by titrating to acidic conditions (pH less than 5.5).

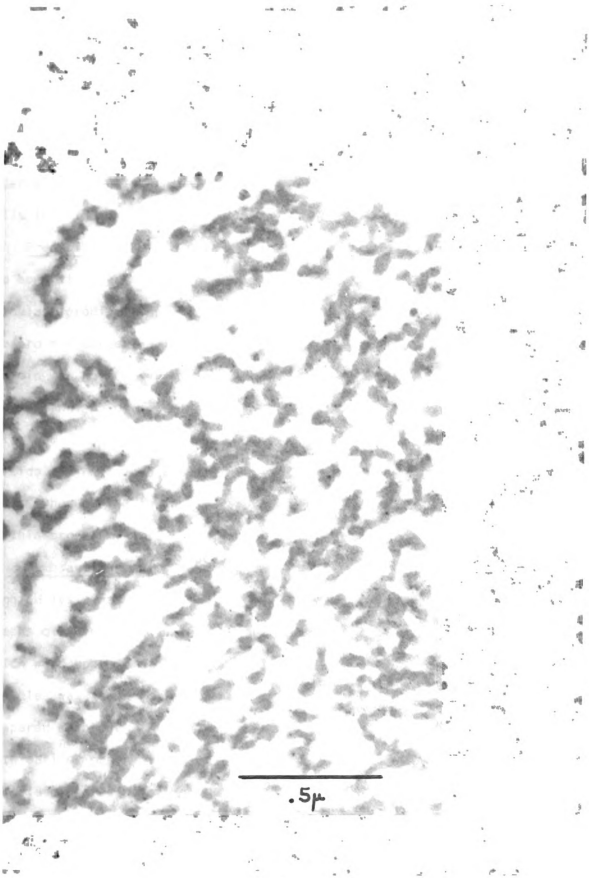

Figure 5

In more dilute cell suspensions (less than 10 mg protein/ml), lysis was completed between pH 6.5 and pH 7.5 (Fig. 6), indicating that cell lysis was concentration dependent. Membrane solubilization as indicated by protein release at high pH (greater than pH 9.5), continued until no sedimentable matter was collectable (pH 12). Similar curves were obtained (Fig. 6) when the volume of base added was plotted versus pH in order to observe the release of buffering components (i.e. protein) from cells into the medium.

Precipitation of Soluble Cytoplasm. When supernatants of cells which had been titrated to pH 9.3 were adjusted to acidic pH, the solubilized cytoplasm precipitated. The onset of precipitation was dependent upon the protein concentration in the supernatant fluid. It appears that this protein was more soluble at high pH. From these data, one can infer that the internal pH of T. acidophila is probably in the neutral range unless its cytoplasm is particulate. These data further suggest that the inside and outside of the membrane differ greatly, since neutral external pH causes lysis, whereas the intracellular neutral pH is physiologically acceptable to this organism.

Membrane Purity. One criterion for purity of membranes prepared by high pH lysis was the absence of cytoplasmic components. Electron micrographs of thin sections (Fig. 7) revealed the presence membrane vesicles which were approximately the size of intact cells. The interior of the vesicles was free from electron dense cytoplasm. In contrast, sonically prepared membranes (Fig. 4) usually contained smaller vesicles in agreement with earlier observations (3). The major drawback to this procedure is the cytoplasmic precipitation which occurs at low pH and probably accounts for the amorphous material previously reported. Failure to remove this cyto-

Figure 6. Effect of pH on cell lysis.

●—● pH change in an aqueous cell suspension (6 mg/ml) upon dropwise addition of 0.04 N NaOH.

X—X Release of protein observed when an aqueous suspension of cells (6 mg/ml) is diluted ten-fold by buffers of varying pH. All buffers used were I M. Buffers at pH 9, 10, and 11 were glycine-sodium chloride; at pH 8 and pH 4, citrate-NaOH; at pH 7, 6, and 5, phosphate-NaOH.

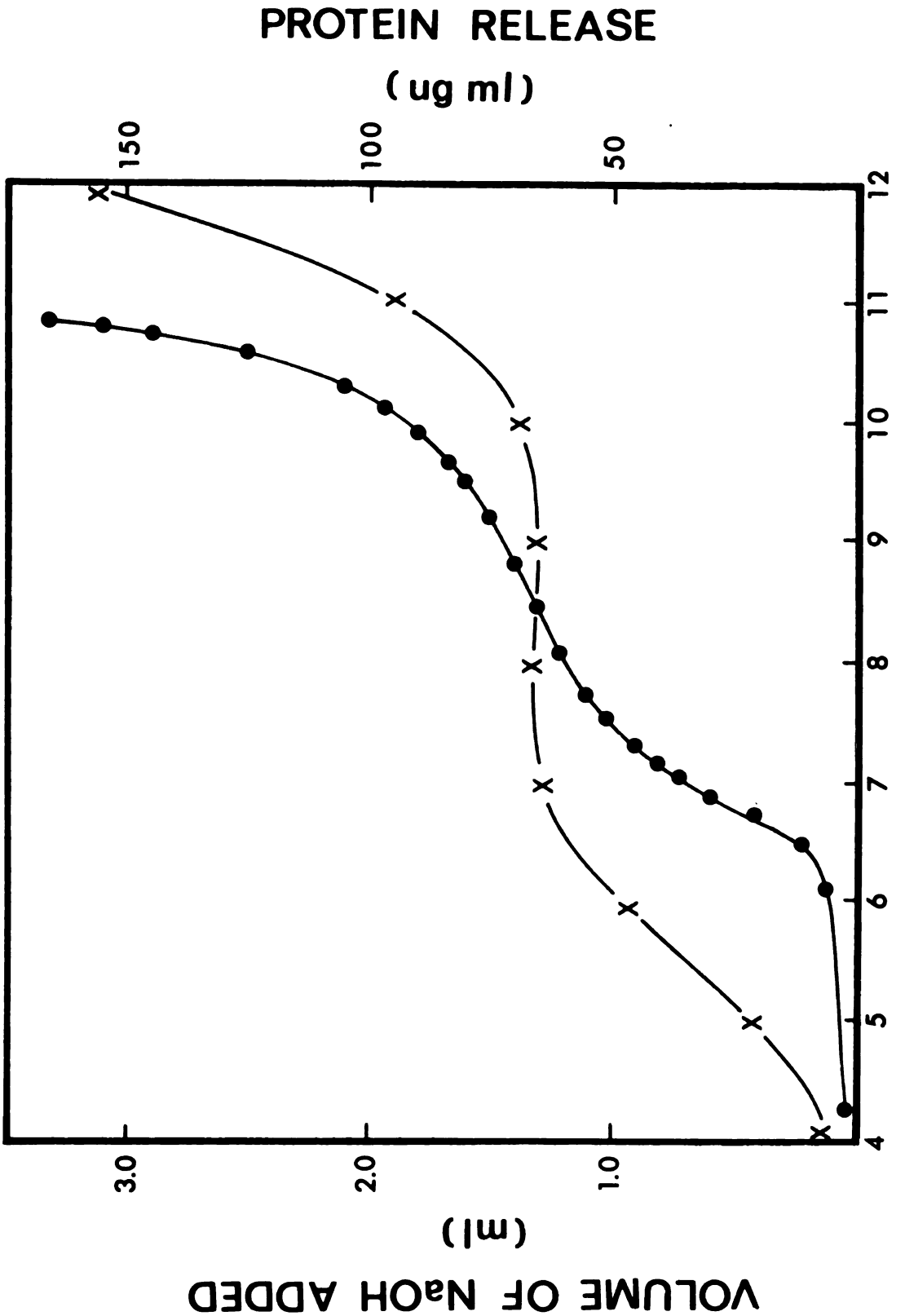
PROTEIN RELEASE
(ug ml)
150
100
50
12
11
10
9
8
7
6
5
4
pH
3.0
2.0
1.0
(ml)
VOLUME OF NaOH ADDED

Figure 6

Figure 7. Transmission electron micrographs of thin sections of T. acidophila membranes prepared by high pH lysis, washed once at pH 10, purified by density gradient centrifugation, and washed two times with distilled water. The membranes were stained as described in Methods.

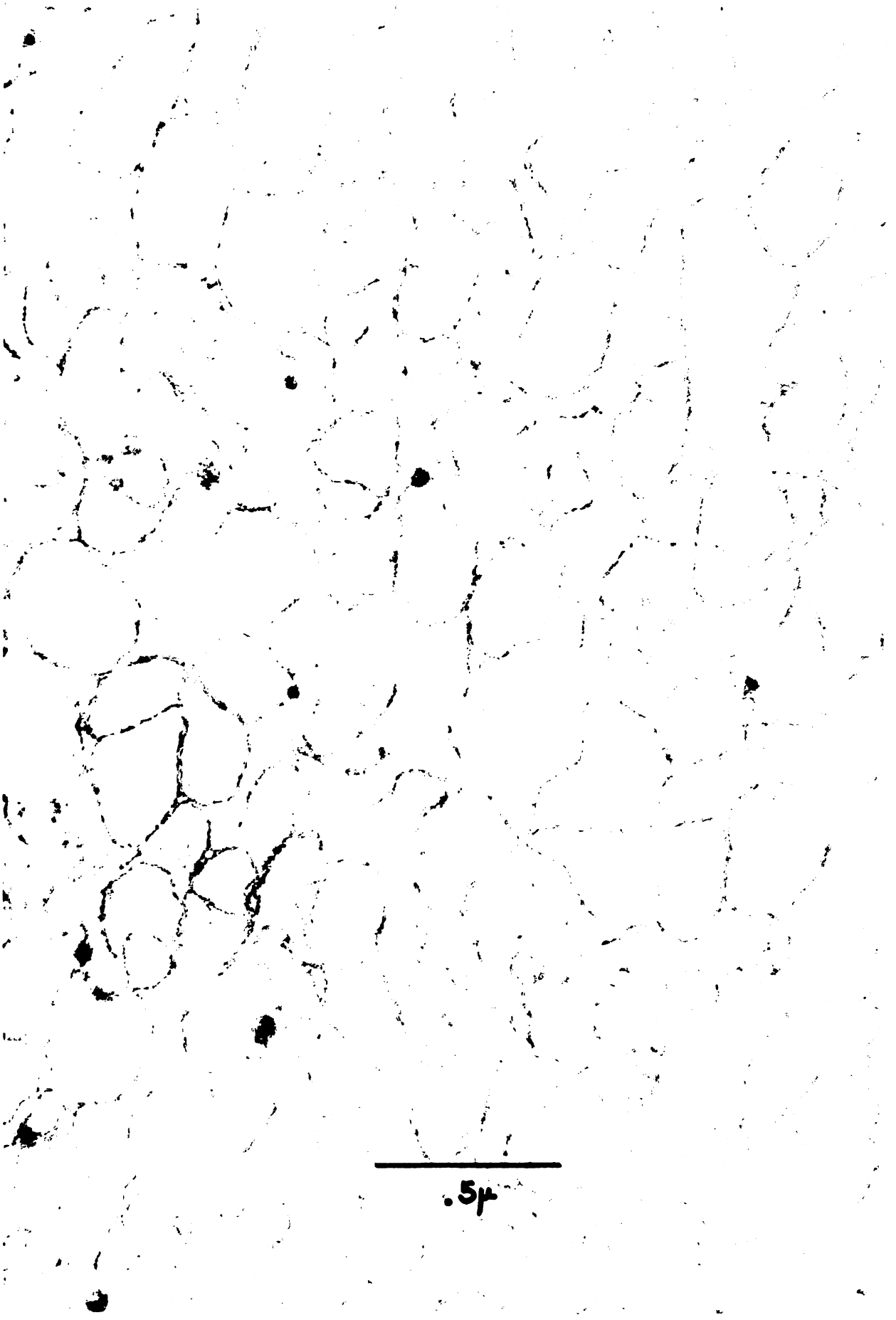

Figure 7

plasmic material was probably due to the acidic conditions chosen (pH 5).

A second criterion of membrane purity was the failure of contaminating cytoplasmic protein to penetrate low percentage polyacrylamide gels (native), even when gels were loaded with 200 micrograms of protein. In contrast, others (3) found that their preparations, which contained "amorphous" material, easily penetrated native polyacrylamide gels. Similar gel patterns were obtained in our laboratory on 10% SDS polyacrylamide gels of cytoplasmic components present in the supernatant of cells sonicated at pH 6.5 (Fig. 8).

Thirdly, the membrane preparation used herein was essentially devoid of deoxyribonucleic acid. The membranes contained less than 0.1% (w/w) DNA. When comparing our results with those of other laboratories, caution must be exercised, since different strains, of T. acidophila may have been selected for as previously suggested (31).

Membrane Characterization. The purified membrane was composed of 19% lipid, 5% carbohydrate, and 76% protein. Chloroform-methanol (2:1) extractions with or without acid (0.1 NHCl) contained large amounts of hydrophobic protein which must be removed by column chromatography for accurate quantitation of lipids (30). The exact quantity of protein present in each extract varied with the length and type of homogenization during the extraction and with the total volume of organic solvent used. These extracts always contained a 2 to 15 times as much protein as lipid (w/w). These considerations may account for the high lipid-to-protein ratio reported by workers (3) who report the presence of amorphous material in their preparations.

Protein Composition

Membranes solubilized and electrophoresed on SDS polyacrylamide gels had a pattern strikingly different from solubilized Thermoplasma acidophila

Figure 8. Sodium dodecyl sulfate polyacrylamide gel of proteins precipitated from sonication fluid with 5% trichloroacetic acid in boiling water (10 mins.). The gel was subjected to electrophoresis and stained with Coomassie blue as described in Methods.

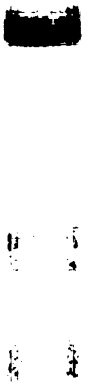

Figure 8

TABLE I

Amino A	
Lys	
His	
Arg	
Asp	
Thr	
Ser	
Glu	
Pro	
Gly	
Ala	
Cys	
Val	
Met	
Isoleu	
Leu	
Tyr	
Phe	

a ave

TABLE I: AMINO ACID COMPOSITION OF CELLS AND MEMBRANES FROM *T. ACIDOPHILA*

Amino Acid	Cells[a] (mole percent)	Membranes[a]
Lys	7.6 ± .00	4.05 ± .35
His	1.7 ± .10	1.15 ± .25
Arg	5.2 ± .10	3.25 ± .05
Asp	11.4 ± .40	8.40 ± .01
Thr	4.8 ± .10	6.40 ± .30
Ser	4.9 ± .00	6.35 ± .35
Glu	9.6 ± .00	6.0 ± .00
Pro	3.8 ± .10	5.25 ± .05
Gly	8.9 ± .40	7.75 ± .55
Ala	7.1 ± .10	8.40 ± .30
Cys	0.8 ± .00	5.75 ± .05
Val	8.2 ± 1.10	6.90 ± .30
Met	2.7 ± .4	2.95 ± .05
Isoleu	7.4 ± .20	7.20 ± .20
Leu	7.6 ± .20	9.60 ± .00
Tyr	3.7 ± .10	4.35 ± .00
Phe	4.5 ± .40	6.25 ± .05

[a] average of three determinations

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TABLE 2: LIPID DISTRIBUTION IN THERMOPLASMA ACIDOPHILA[a]

Moiety	Neutrals	Glycolipids	Phospholipids
		(μmoles/mg lipid)	
Ester	0.14	0.07	0.07
Carbohydrate	0.31	1.15	0.58
Glycerol	0.27	0.75	0.84
Ether	0.09	1.08	1.00
Phosphate	----	----	0.76
Approximate Percent of weight accounted for	67	95	83
Pigmentation	yellow	yellow-brown	red-brown

[a] Error is estimated at 5 to 7% after five determinations.

cells (Fig. 9). Cells had approximately 32 protein bands on SDS gels; molecular weights varied from 10,000 to 125,000. Membranes contained 15 protein bands and four components accounted for approximately 70% of the membrane protein. Ninety percent of the membrane proteins had molecular weights less than 80,000. These results were virtually identical to those obtained with membranes prepared osmotically.

The amino acid composition of membranes differed from that of whole cells (Table 1). The cells contained more basic and acidic amino acids, while membranes were enriched in tyrosine and cysteine. The amount of hydrophobic amino acids was approximately the same in both membranes and cells. The previously reported amino acid composition (3) was similar to that of our cells.

Lipid Composition. General Distribution. Lipids from T. acidophila were fractionated into neutrals, glycosyl diglycerides, and phospholipids (17%, 8%, and 75%, respectively). Glycolipid and phospholipid thin layer chromatography (TLC) patterns were similar to those previously described (2). TLC profiles of the neutral fraction differed from that obtained by Langworthy et. al. (2): cholesterol was undetectable and vitamin K_2-7 was present in large amounts (33% of neutral fraction). Neutral lipids were observed which migrated similarly as components Q, R, S, T, V, and W (2). An additional component appeared halfway between V and W with a retention time approximating that of monoether standards. The reasons for these deviations may lie in the growth conditions employed.

The distribution of lipid moieties in Thermoplasma acidophila lipids is listed in Table 2. One component of the phospholipids comprising approximately 10% of the fraction weight contained no phosphate (2); it most probably accounted for the high glycerol to phosphate ratio in the fraction. Carbo-

Figure 9. Schematic profiles of sodium dodecyl sulfate polyacrylamide gels of cell and membrane preparations from Thermoplasma acidophila. Absorbance was measured at 550 nm. Gels were subjected to electrophoresis and stained as described in Methods.

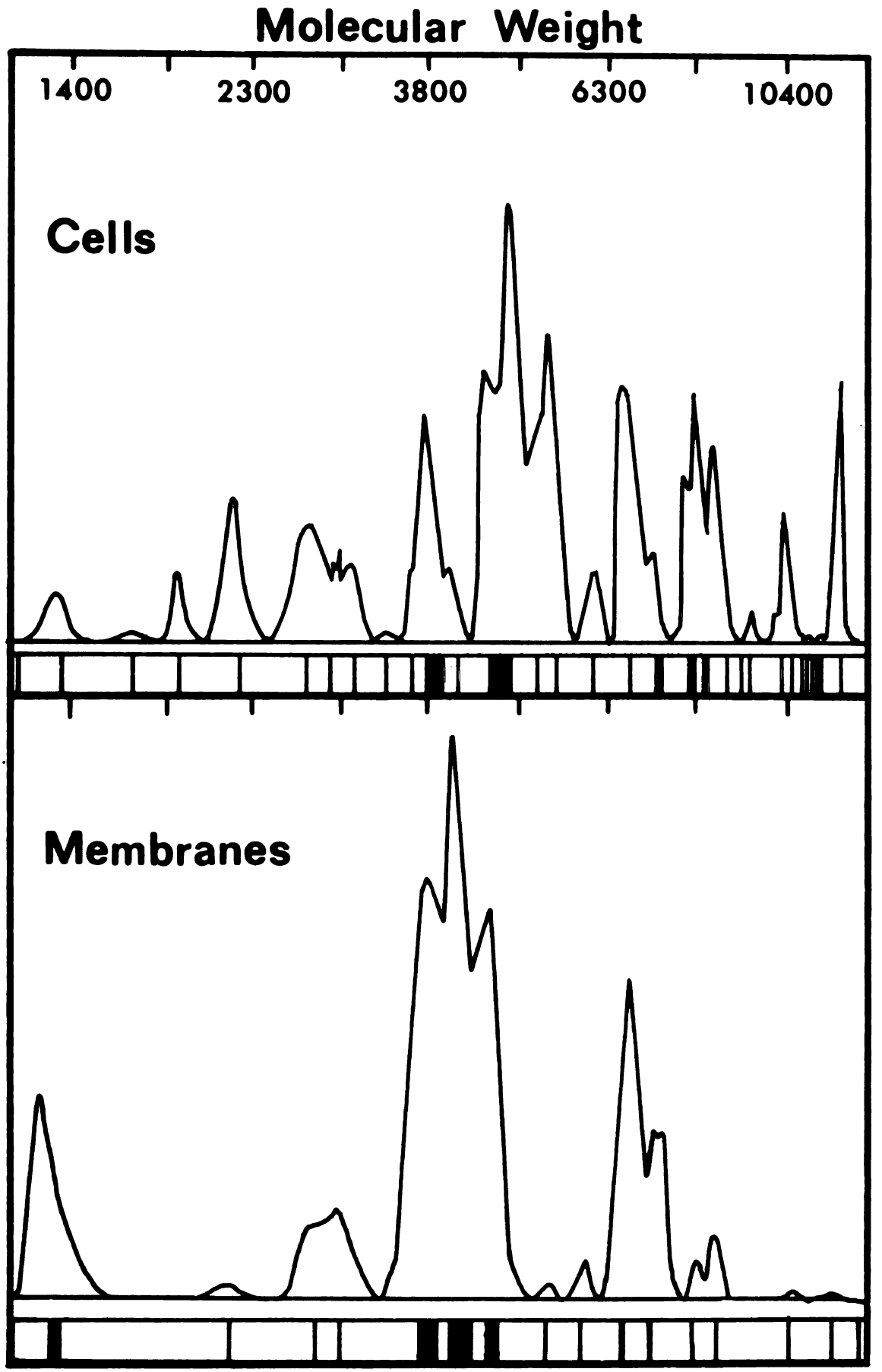
Molecular Weight
1400
2300
3800
6300
10400
Cells
Membranes

Figure 9

hydrate was present in all fractions, as was glycerol. In the glycolipid and phospholipid fractions more ethers were found concomitantly with a decrease in fatty acid esters. Since ether bonds are generally more stable than ester bonds, such findings may suggest that the polar lipid classes are closer to the membrane surface.

Only 67% of the neutral lipid fraction was accounted for by these analyses, but the remainder consisted of large amounts of vitamin K_2-7 (30%) and small quantities of non-glyceride components. Ninety-five percent of the glycolipids and 83% of the phospholipids were accounted for by weight. Langworthy et al. (2) showed that the major component of the phospholipid fraction has two ether moieties, one phosphate group, and one hexose unit per glycerol. Thus, about 17% of the glyceryl hydroxyl groups in the phospholipid fraction were either free (lyso) or were bound to an unidentified moiety. However, it is highly unlikely that lysophospholipids would be present in such large amounts since they are very disruptive to membrane structure. Therefore, it seems more probable that the unaccounted for 17% phospholipid weight (Table 2) contains groups which have not yet been analyzed.

Fatty acid esters. Three methods were used to establish the presence of the fatty acid esters (Table 3), although each gave different values. Semiquantitative infrared spectroscopy consistently yielded high values, while gas chromatography-mass spectrometry gave lower ones. Mass spectrometry was employed to identify all methyl ester derivatives of fatty acids (Table 4). The ester distribution is similar in all lipid fractions except for the increase in $C_{16:1}$, $C_{20:0}$, and $C_{20:1}$ fatty acids and the decrease in $C_{18:2}$ in the phospholipids. Several methylation techniques were attempted in order to ensure complete derivatization of fatty acid esters. Forty-eight hour incubation at 75°C in the presence of methanolic BF_3 and benzene (see Methods) gave the

TABLE 3: FA

Fraction	
Neutral	
Glycolipid	
Phospholipi	

a All valu
of lipi

b Average

TABLE 3: FATTY ACID ESTER DETERMINATION IN THERMOPLASMA ACIDOPHILA[a]

Fraction	Hydroxymate[b]	Infra-red[b]	GC-MS[b]
	(μmoles ester/mg lipid)		
Neutral	3.3 ± 0.4	8.2 ± 2.0	3.7 ± .8
Glycolipid	7.1 ± 1.0	10.4 ± 2.0	1.9 ± 0.7
Phospholipid	2.4 ± 0.2	4.3 ± 0.3	1.7 ± 0.8

[a] All values are expressed in microequivalents ester per milligram of lipid.

[b] Average of five determinations. Procedures are described in Methods.

TABLE 4: Percentage Fatty Acid Composition of *Thermoplasma acidophila* Lipids[a]

Fatty Acid Chain Length	Lipid Fraction (mole percent fatty acid ester)		
	Neutral	Glycosyl diglycerides	Phospholipids
10:0	0.3	0.9	1.1
12:0	1.7	0.8	1.2
14:0	5.3	5.8	5.1
14:1	2.6	2.4	2.6
16:0	20.1	21.1	19.4
16:1	13.7	9.8	17.3
18:0	10.9	11.5	8.1
18:1	21.6	20.4	21.5
18:2	17.4	16.8	2.5
20:0	4.4	7.4	14.0
20:1	2.0	3.1	7.0

[a] Average of three determinations. Variation is between 5% and 10% for each fatty acid listed.

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largest yields and highly reproducible results. For shorter incubation times, hydrolysis was incomplete. The presence of long chain alkyl ethers or other moieties in Thermoplasma acidophila lipids probably stabilize the fatty acid esters. To insure adequate amounts of materials, ester determinations were routinely made with lipid extracted from thirty-six litre cultures. Furthermore, glassware was acid-cleaned, cells and membranes washed with water at least three times to prevent contamination from media components. Other workers did not detect fatty acids in their T. acidophila lipid extracts (2).

Fatty acids were present in T. acidophila grown on lipid-free media; presumably the organism is capable of synthesizing its own fatty acids. In this respect T. acidophila differs from most mycoplasmas which require fatty acid and/or cholesterol supplementation for growth. Oleic acid supplementation (5 mg, 2.5 mg, or 1.25 mg/l) prevented growth of T. acidophila.

Long-Chain Alkyl Ethers. The distribution of the alkyl ethers in T. acidophila was analyzed by gas chromatography-mass spectroscopy (Table 5). The molecular weights were determined with acetate derivatives which indicated multiple branches per molecule. The mass spectra of the acetates of two major ethers were consistent with $C_{40:0}$ and $C_{40:1}$ long-chain compounds in agreement with previous reports (2). Two smaller components appeared to be C_{41} and C_{42} by gas chromatography retention time studies. The distribution of these ether moieties (Table 5) indicated that the neutral lipids contained predominantly $C_{40:0}$ while phospholipids and glycolipids contained an equal amount of $C_{40:1}$. The C_{18} to C_{29} alkanes reported previously (2) were not found to be present in significant amounts.

No phosphonates or plasmalogens were found in the lipids of T. acidophila.

TABLE 5: DISTRIBUTION OF ALKYL ETHERS IN *THERMOPLASMA ACIDOPHILA* LIPIDS[a]

CARBON NUMBER	NEUTRALS	GLYCOLIPIDS	PHOSPHOLIPIDS
40:0	1.00 ± 0.10	1.00 ± 0.04	1.00 ± 0.01
40:1	0.06 ± 0.00	1.08 ± 0.02	0.93 ± 0.01
41:0	0.03 ± 0.00	0.22 ± 0.04	0.31 ± 0.10
42:0	0.00 ± 0.00	0.00 ± 0.00	0.06 ± 0.02

[a] Average of two determinations. All quantities normalized to $C_{40:0}$.

Flagella-like Filaments. Thermoplasma acidophila appears to be coccoidal in shape and cell diameters vary between 0.5 and 1.0 micron. Some cells have one or two fiamentous projections (Fig. 10). When cells were viewed individually (Fig. 10), it became apparent that each cell had one or two filaments which were often lost from the organism during preparation for electron microscopy. Washing T. acidophila especially in distilled water, removed these filaments from the cells. Flagella-like filaments collected from these washes as described in the Methods section are shown in Fig. 11. Each filament is approximately 100 Å in diameter and as much as 30,000 Å in length. The presence of these filaments suggests that Thermoplasma acidophila may be an L-form rather than a true mycoplasma.

Figure 10. Transmission electron micrograph of a T. acidophila cell. This specimen was critically-point dried and shadowed with gold-palladium.

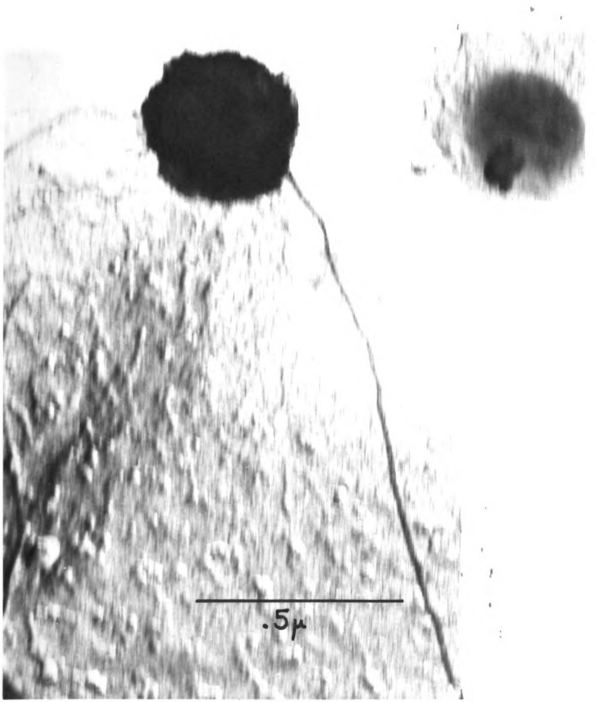


Figure 10

Figure 11. Transmission electron micrograph of flagella-like filaments collected from T. acidophila. Filaments were negatively stained with phosphotungstic acid.

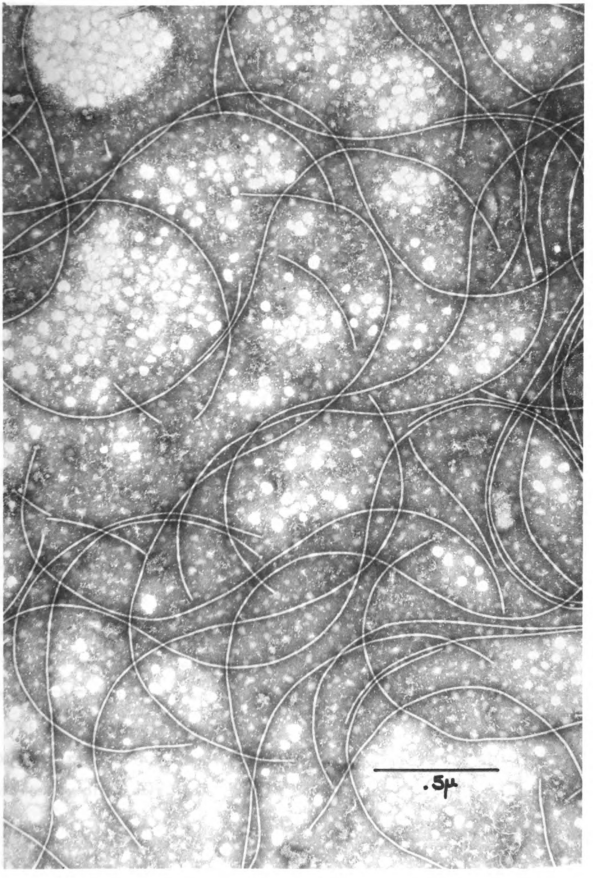


Figure 11

DISCUSSION

These studies have been undertaken in order to determine which characteristics of T. acidophila may account for its thermophily, acidophily, and its ability to withstand osmotic shock despite its lack of a cell wall. The results indicate that these properties may be related. The lack of cell wall indicates that this organism is probably either a mycoplasma or an L-form. Although the classification is still unresolved, the existing evidence appears more consistent with the organism being an L-form. The reasons for the original classification of T. acidophila as a mycoplasma were the following: 1). its size (0.3-2.0 micron), 2). its lack of a cell wall, 3). its sensitivity to novobiocin, and 4). its low Guanidine plus Cytosine content (1). However, the organism has such a large range of size, that classification according to size is meaningless. The lack of cell wall is also characteristic of L-forms. In addition, the presence of flagella-like filaments has never been demonstrated in mycoplasmas, but flagella can be retained in L-forms of flagellated bacteria. Novobiocin inhibits growth of mycoplasma and T. acidophila, but the antibiotic's action may be due to its specificity for cells lacking a cell wall rather than to a specificity for mycoplasmas. Other workers (32) have not found the low G+C content reported by Darland et al.(1), possibly because the initial isolate was not a pure culture. Furthermore, Thermoplasma acidophila does not require fatty acids or cholesterol for growth and is, in fact, inhibited by oleic acid.

In spite of its lack of cell wall, T. acidophila is unusually resistant to osmotic and mechanical stress. This phenomenon is uncharacteristic of mycoplasma. The stability of the T. acidophila membrane may be related to its extreme rigidity as evidenced by electron paramagnetic resonance (EPR) spectra (35). It is also conceivable that the four major polypeptides (Fig. 9) represent "structural" element in the protein-rich (76%) membrane.

Brock and Devaux have noted that acidophily and thermophily are probably incompatible, since no organism has been found that grew at pH 3 or lower and simultaneously at temperatures higher than 70°, while bacteria growing at alkaline pH can live in boiling or near-boiling water. Since T. acidophila grows at the upper temperature limit for acidophiles, it is of interest to investigate which membrane properties are mainly determined by the low pH and which properties are related to the high temperature of its envrionment.

Lipids in thermophiles generally have higher melting points than those of mesophiles; this condition is promoted by increase in length, saturation and branching of the lipids. The lipids of T. acidophila are very long, branched, and 50% saturated and thus probably have the high melting point characteristic of thermophiles. Furthermore, the high ether and the low ester content confer chemical stability to the membrane lipid matrix. Moreover, the high ester content of the neutral fraction would be in accordance with a membrane model wherein the most non-polar lipids are relatively shielded from the harsh environment.

Enzymes from thermophiles are generally more resistant to heat denaturation than their mesophilic counterparts. The reasons for this are not clear, although hydrophobic forces are almost always invoked as one of the major

stabilization mechanisms (37). Hydrophobic interactions have maximum stability at 60° (83), which, coincidentally, is very near to the upper temperature limit at which acidophiles have been found to grow. Although flagella from thermophilic organisms have been found to have few charged groups (40), there is a growing body of evidence that thermostable enzymes require more acidic amino acids than their mesophilic counterpart (39,44).

The solubilization of T. acidophila at high pH is probably related to the charge distribution of the amino acids (3). At high pH (greater than 4) carboxyl groups are ionized, resulting in charge repulsion, destabilization, and solubilization of the membrane. However, in T. acidophila, these effects are minimized, since the organism has only half as many carboxyl and amide groups as mesophilic mycoplasma (3). Moreover, the T. acidophila membrane has fewer -COOH and $-NH_2$ groups than the total cellular material (Table I). Thus, at high pH, the cytoplasm of T. acidophila will be solubilized before the membrane protein, as is indeed found.

With these observations in mind, it is possible to speculate as to why no acidophiles have been found to grow at temperatures above 70°. As thermophiles, these organisms may require a high acidic amino acid content; as acidophiles, they require a minimum of ionizable groups. Apparently the highest temperature at which both requirements are met is, to a first approximation, the same one at which hydrophobic bonds begin to weaken (i.e. 60°).

CHAPTER III

ELECTRON PARAMAGNETIC RESONANCE STUDIES ON *THERMOPLASMA ACIDOPHILA*

INTRODUCTION

Thermoplasma acidophila grows optimally at pH 2 and 59° C (1). This organism has been classified as a mycoplasma, since it lacks a cell wall. Moreover, T. acidophila has several similarities with Halobacterium cutirubrum. Both microbes have a low lipid content (less than 20% of the membrane weight) and a high incidence of branched alkyl ethers (45, 10). H. cutirubrum requires a high salt concentration (greater than 3 M) for growth and lyses readily when the salt concentration is lowered (below 1 M) (82). Similarly, T. acidophila has a high proton requirement (greater than 10^{-4} M; i.e. less than pH 4) and lyses when the hydrogen ion concentration is decreased (less than 10^{-5} M; i.e. greater than pH 5). The proteins of the membrane are hydrophobic and are apparently destabilized at high pH values due to the ioniztion of carboxyl groups (3). Function and structure of the membranes of H. cutirubrum and several strains of mycoplasma have been investigated with electron paramagnetic resonance techniques (epr). Spin label studies with H. cutirubrum revealed the presence of a highly ordered membrane (46).

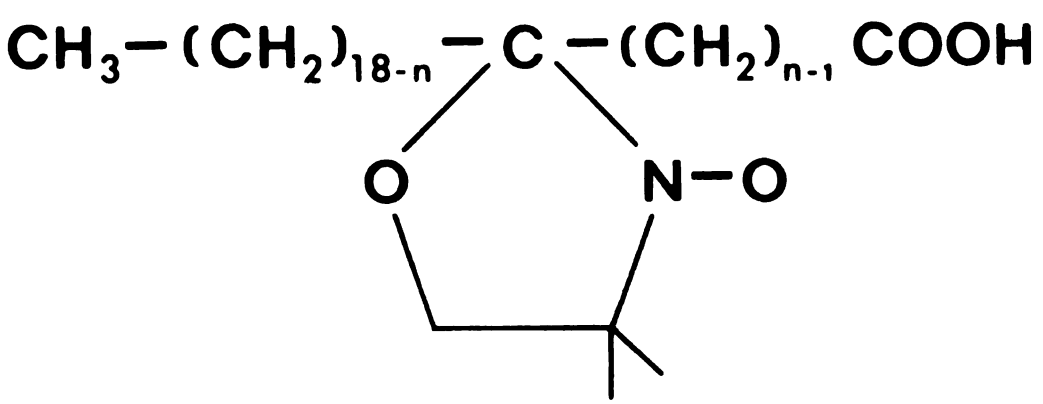
Herein, we report the results of investigations upon the membrane properties of T. acidophila. Comparison of these data with those previously for H. cutirubrum (46) should yield insight into the ability of the organisms to withstand their harsh environments. Since 50% of the T. acidophila lipid by weight is composed of two long-chain alkyl ethers,

this organism should also be an excellent tool with which to study co-operative phase transitions in membranes.

MATERIALS AND METHODS

T. acidophila was grown in aerated cultures at pH 2, 56° C. Membranes were purified after cell lysis at pH 9.3 (45), and washed two times with 40 volumes of distilled water after removal from sucrose density gradients. Protein was determined by the method of Lowry et al. (14). Lipids were extracted with chloroform: methanol (2:1); non-lipid contaminants were removed by Sephadex and silicic acid chromatography (45). Lipids were assayed colorimetrically by a modification of the method of Saito and Sato (24). Organic solvents were redistilled prior to use. All glassware employed in lipid analyses was acid-cleaned.

Fatty acid spin labels were purchased from Synvar (Palo Alto, Calif.) and were of the general formula:



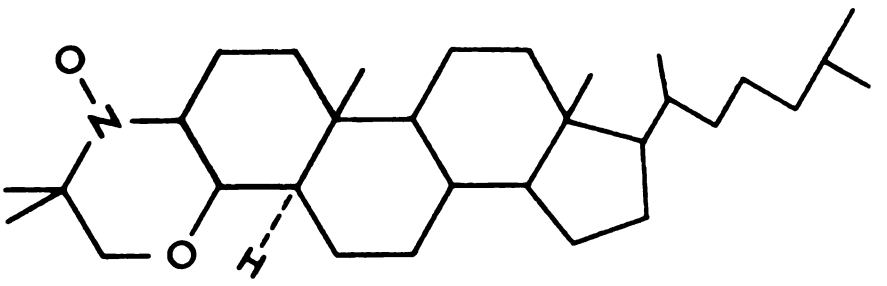
n-nitroxy-stearate (nNS)

16NS = 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl

12NS = 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl

5NS = 2-(3-carboxydecyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl

Spin labelled cholestane (4',4'-dimethyl-spiro-(cholestane-3-2'-(1',3'-oxazolindine-3'-oxyl))) had the following structure:



Aliquots from a stock solution of spin label dissolved in hexane were measured into small glass test tubes; hexane was removed by evaporation. Membrane vesicles were suspended either in water at pH 6 or in T-buffer (45) at pH 2, and added to the spin label. The mixture was then sonicated for 10 min. on a Branson sonicator. The protein concentration of the membrane vesicles was between 10 and 20 mg/ml. Unless otherwise stated the spin label concentration approximated 0.1% of

the lipid weight of the vesicles. Assuming an average molecular weight of 1500 for the lipid (based on the composition of the major diether phospholipid)(2) the lipid to stearate spin label molar ratio was about 250:1.

All electron paramagnetic resonance (epr) spectra were determined on a Varian V-4502-15 spectrophotometer equipped with a Varian variable-temperature accessory, V-4540. An aqueous sample cell was used throughout. The rotational motion of the spin label was calculated from the following equation (47):

$$\tau_n = 6.5 \times 10^{-10} W_o \{(h_o/h_{-1})^{1/2} - 1\} \qquad (1)$$

The order parameter S_n was calculated as previously described (46, 48-50):

$$S_n = 0.568 (T'_{11} - T'_{\perp})/a' \qquad (2)$$

where $a = 1/3 (T'_{11} + 2T'_{\perp})$

Samples were prepared for electron microscopy with 1.5% phosphotungstic acid, pH 7.0. Specimens were viewed on a Phiips 300 Transmission Electron Microscope.

RESULTS

Typical epr spectra of T. acidophila membranes in distilled water (pH 6.0) at 56° C are shown in Fig. 12. Since ascorbic acid does not

Figure 12. Electron paramagnetic resonance spectra of fatty acid spin labels in T. acidophila membranes at pH 2 and 56° C.

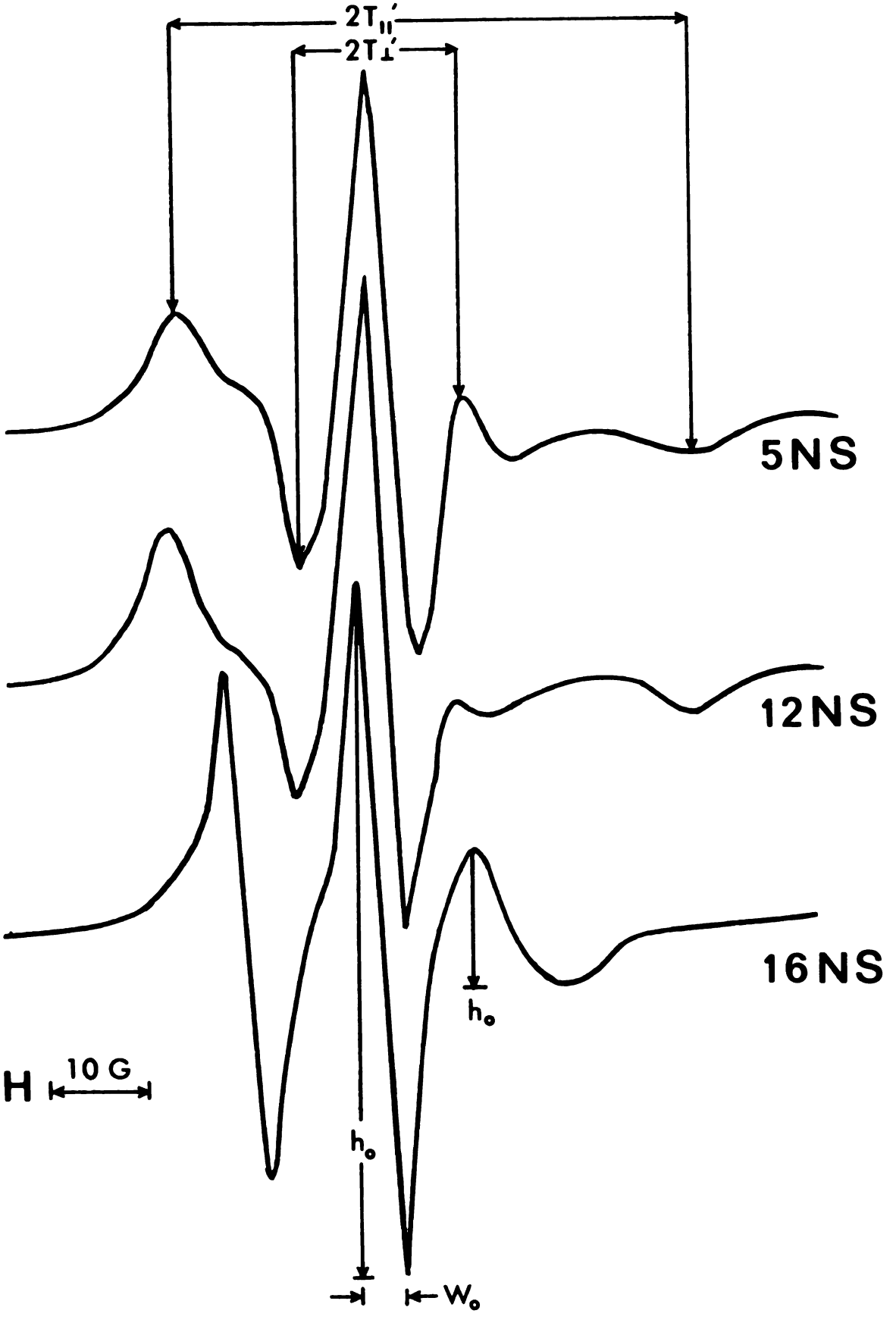


Figure 12

reduce the signal height, it is assumed that the fatty acid spin label has been incorporated into the membrane lipid matrix. Ascorbic acid would destroy unincorporated spin label or that exposed on the surface (51). Immobilization of the spin label is evident at all temperatures tested, but is especially pronounced at low temperatures (Table 6). This immobilizatin is indicative of a rigid membrane structure. Other investigators have found that as the nitroxide group is placed closer to the hydrocarbon end of the stearate molecule, the rotational freedom of the group usually increases (51), since the free radical penetrates deeper into the hydrocarbon region of the membrane bilayer. At temperatures lower than 40° C, 5NS in T. acidophila membranes reflects greater or equivalent membrane rigidity as compared to that seen by the 12NS label (Table 6 and Fig. 13). However, above 40° C, the 12NS label apparently experiences an environment with greater rigidity than that of the 5NS label (Table 6 and Fig. 13).

The temperature dependence of T. acidophila membrane fluidity is illustrated in Fig. 13. When the membranes were labeled at pH 6.0 with 5NS, transitions were observed at 15° and 45° C. No differences were observed when membranes labelled at pH 6.0 were resuspended in pH 2.0 buffer. However, if labelling was performed at pH 2.0, the 45° C transition was abolished and another transition appeared at 60°C. This suggests that the pH at the time of labelling determines the membrane domain to which the 5NS spin label migrates.

The 12NS spin label consistently indicated a transition temperature at 60° C, regardless of the pH at the time of labelling. A transition

TABLE 6. SPECTRAL PARAMETERS OF FATTY ACID SPIN LABELS INCORPORATED INTO THERMOPLASMA ACIDOPHILA MEMBRANES[a]

Label	Temperature	S_n	a'	$2T_{11}$(G)	T_n (nsec
5NS	37°	1.5	15.4	57.2	---
12NS	37°	---	---	57.2	---
16NS	37°	---	---	53.8	8.3
5NS	55°	0.71	14.4	55.5	5.6
12NS	55°	0.71	14.5	---	11.7
16NS	55°	---	---	45.2	2.2
5NS	80°	0.54	16.8	47.9	2.9
12NS	80°	0.54	14.3	---	5.6
16NS	80°	---	---	---	1.4

[a]Membranes were labelled and spectra were taken at pH6. Where no values for a particular parameter are given, the spectral shape was such that measurement and calculation of that parameter was not possible. Error is estimated at 10% for all parameters except $2T_{11}$, where the error is within 3%.

Figure 13. Temperature-dependence of $2T_{||}$ or τ in spin-labeled *T. acidophila* membranes.

a. $2T_{||}$ as a function of temperature for 12NS labelled *T. acidophila* membranes. ●——● pH 6; X——X pH 2.

b. $2T_{||}$ as a function of temperature for 5NS labelled *T. acidophila* membranes. ●——● pH 6; X——X pH 2.

c. τ as a function of reciprocal absolute temperature for 16NS labelled *T. acidophila* membranes. ●——● pH 6.

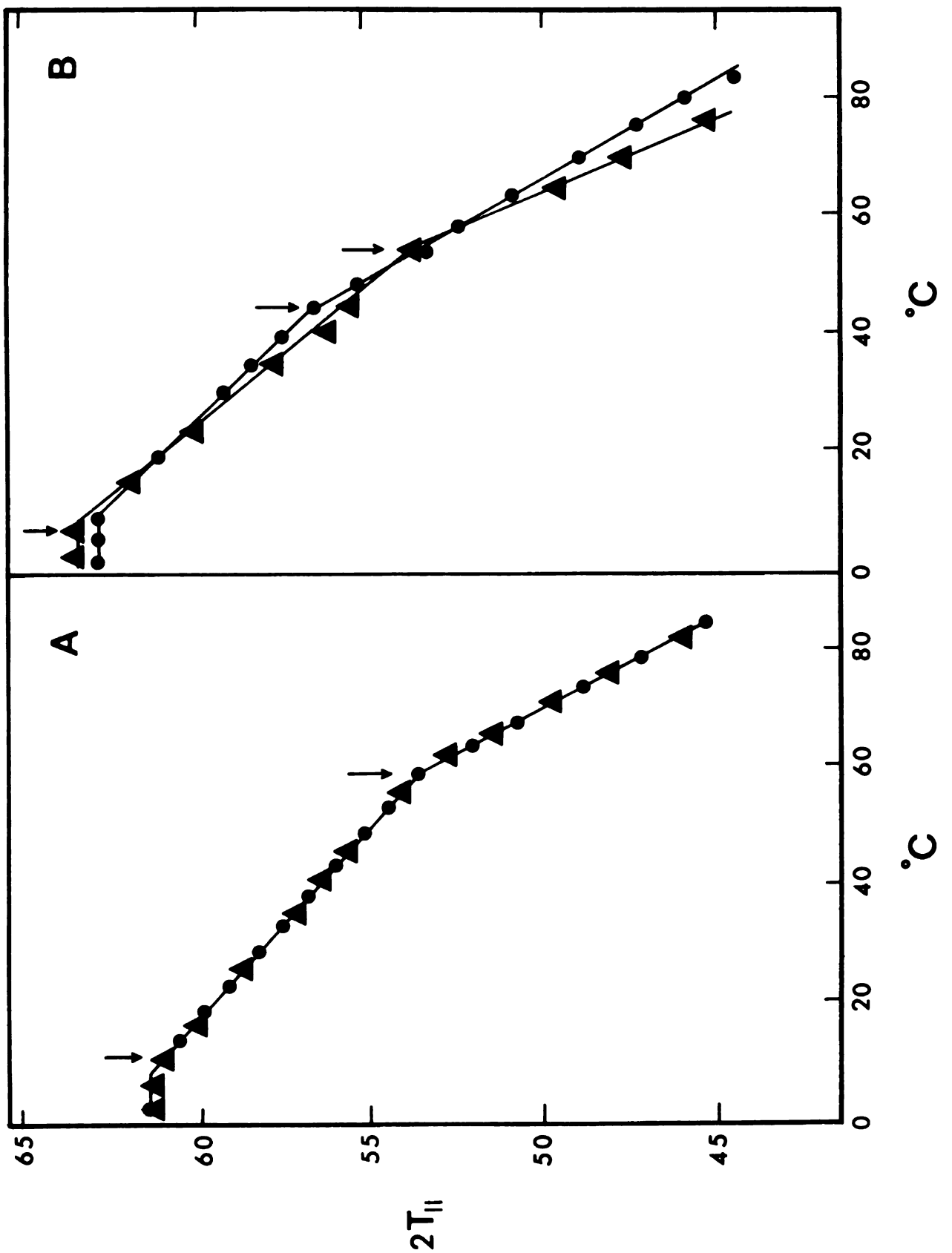


Figure 13

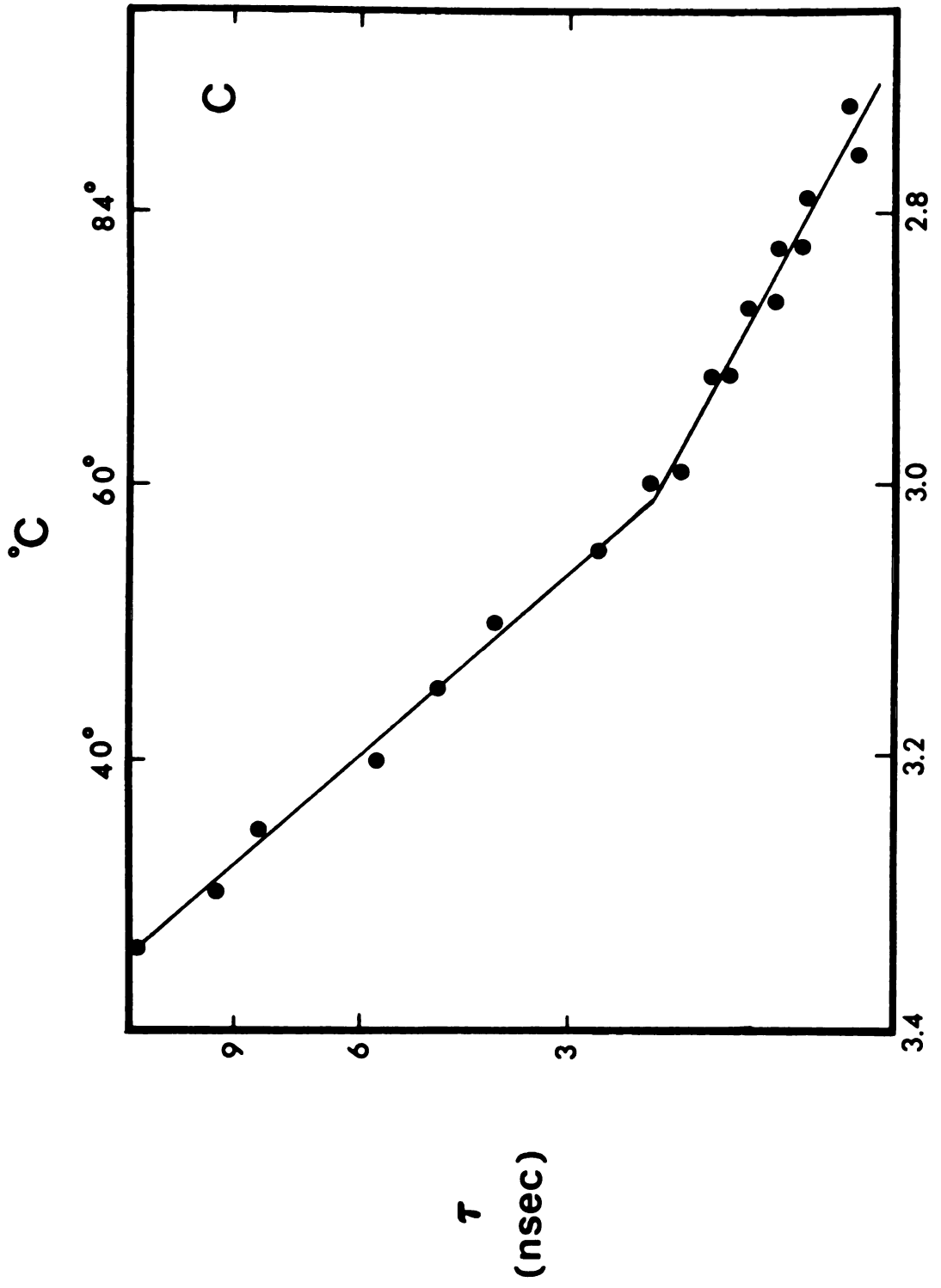


Figure 13 continued

also occurred at 15° C. The transition at 60° C was also observed with 16NS; however, the lower temperature transition could not be detected since spectra at those low temperatures did not allow calculation of τ values.

The spectra of 16NS labelled membranes (Fig. 12) were obtained with membranes which had been stored at 4°C for four days or more. Fresh preparations were found to produce spectra such as shown in Fig. 14c. This type of spectrum appears to be composite of the normal 16NS spectrum (Fig. 14b) and one which can be generated by placing the spin label groups in close proximity to each other (Fig. 14d). Earlier investigators (52-54) observed this type of spectrum only when the membranes were highly doped (greater than 10%) with spin label. The spectrum shown in Fig. 14c was obtained only with fresh membrane preparations and was stable for two days at 5° C. When the temperature of these labelled membranes was raised above 65° C, however, the spectrum converted to a virtually complete spin-spin interaction spectrum (Fig. 14d). This irreversible conversion occurred within 10 to 60 min., depending upon the temperature and the sample used. The exact temperature at which the conversion began and the time taken for complete conversion varied (±5°C, ±30 min.) with the preparation. This conversion caused major alterations in the membranes (Fig. 15). The membrane vesicles decreased in size, retained the phosphotungstic acid stain, and aggregated.

Since the partial spin-spin interaction spectrum indicated that the nitroxide radicals were in close proximity (Fig. 14c), it was of interest to determine whether some of the 16NS spin labels were forming a

Figure 14. EPR spectra of 16NS under various conditions.

A. 16NS in water dispersion at 20° C.

B. 16NS bound to four-day-old T. acidophila membranes at 20° C, pH 6.

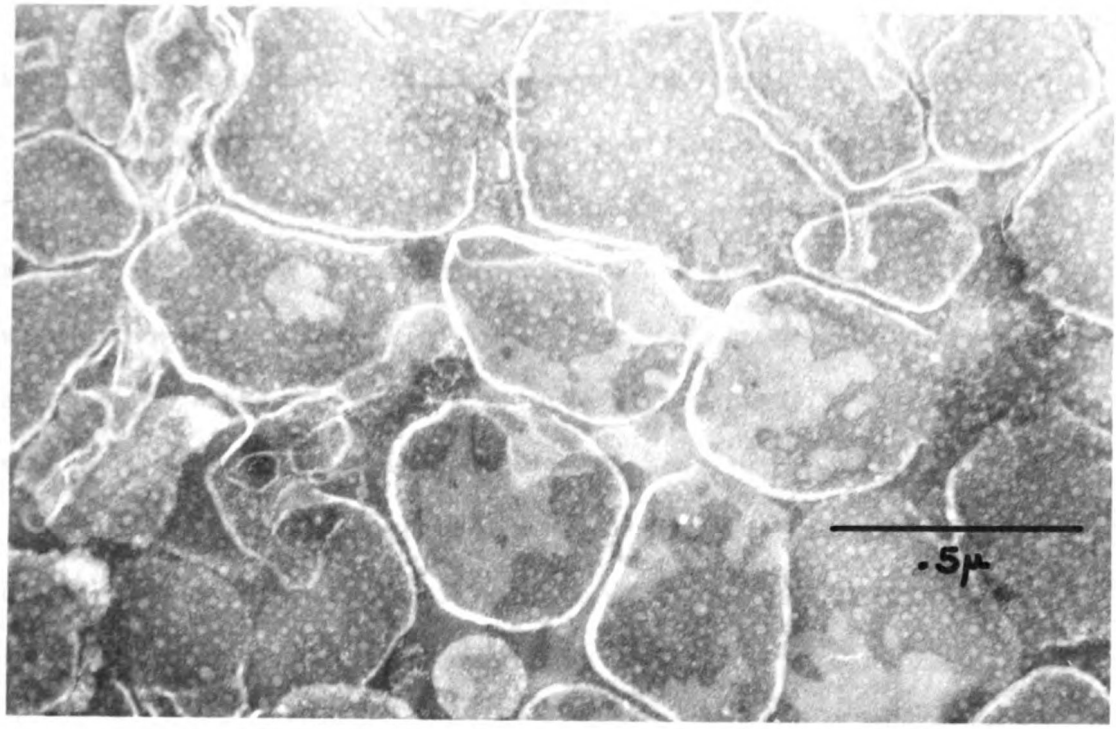
C. 16NS bound to one-day-old T. acidophila membranes at 20° C, pH 6.

D. 16NS bound to one-day-old T. acidophila membranes subjected to 80° C for one hour and cooled to 20° C. The pH is 6.

Figure 15. Electron micrographs of T. acidophila membranes.

A. Negatively-stained native membranes.

B. Negatively-stained membranes after one hour at 80° C.



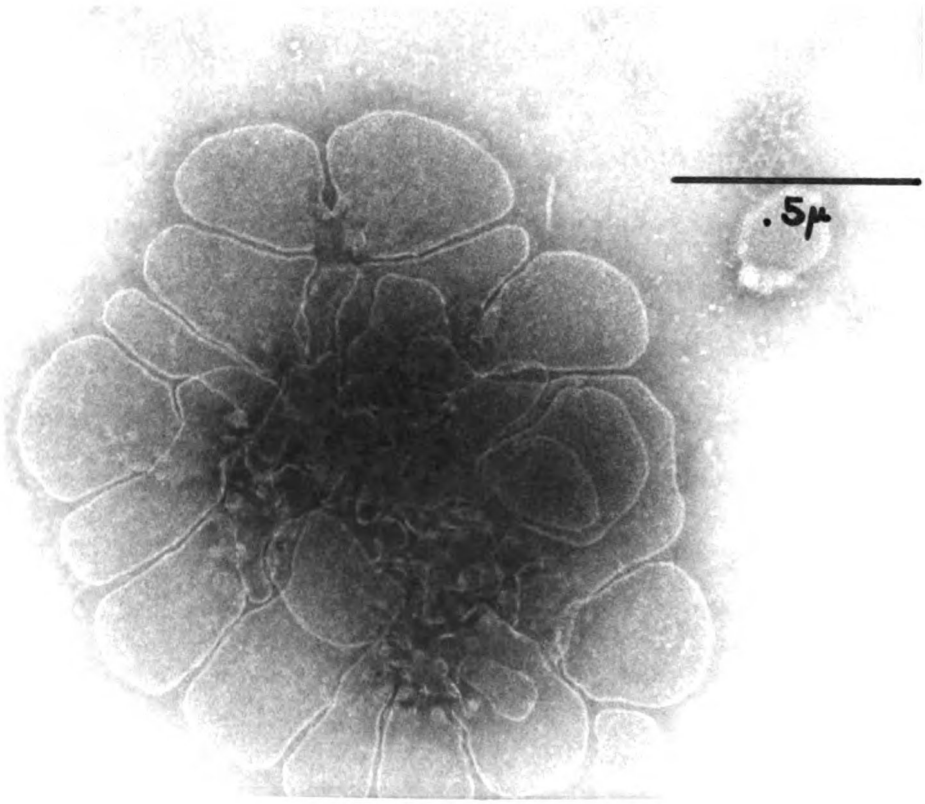


Figure 15

cluster due to low solubility in the membrane lipid. If this were the case, one might expect that further addition of 16NS label would increase the interaction between spin labels. However, this was not observed. Addition of more 16NS resulted in a spectrum with less interaction characteristics (Fig. 16). Thus, it appeared that there were a limited number of sites available wherein 16NS free radicals were able to interact with each other. Apparently, these sites were the first ones filled or modified in the presence of excess 16NS. Addition of unlabelled cholesterol also reduced the interaction between 16NS molecules in the labelled membranes (Fig. 17).

T. acidophila membranes labelled with the spin label cholestane exhibited a spectrum indicative of close interaction between spin labels (Fig. 18). Little change was noted in this spectrum as a function of temperature from 5^{o}-60^{o} C.

DISCUSSION

Previous investigations with physical methods support the theory of a bilayer lipid organization in biological membranes (55-64). From order parameter studies on fatty acids with spin labels at different positions along the alkyl chains, rigidity gradients have been detected in membrane lipids (62, 65-67). In established bilayer membranes such a gradient has been demonstrated and its presence has been used to infer a bilayer structure in less studied systems (68). At temperatures below 40^{o} C, 5NS appears to sense a similar environment as 12NS, which is located in a more rigid lipid region than 16NS in

Figure 16. Concentration dependence of EPR spectra of 16NS labelled *T. acidophila* membranes.

—— One-day-old membranes at 20^{o} C, pH 6. The label: lipid ratio is 4×10^{-3}.

--- The above preparation with the addition of 16NS label giving a final label: lipid ratio of 8×10^{-3}.

●-● The above preparation with the addition of 16NS label giving a final label: lipid ratio of 24×10^{-3}.

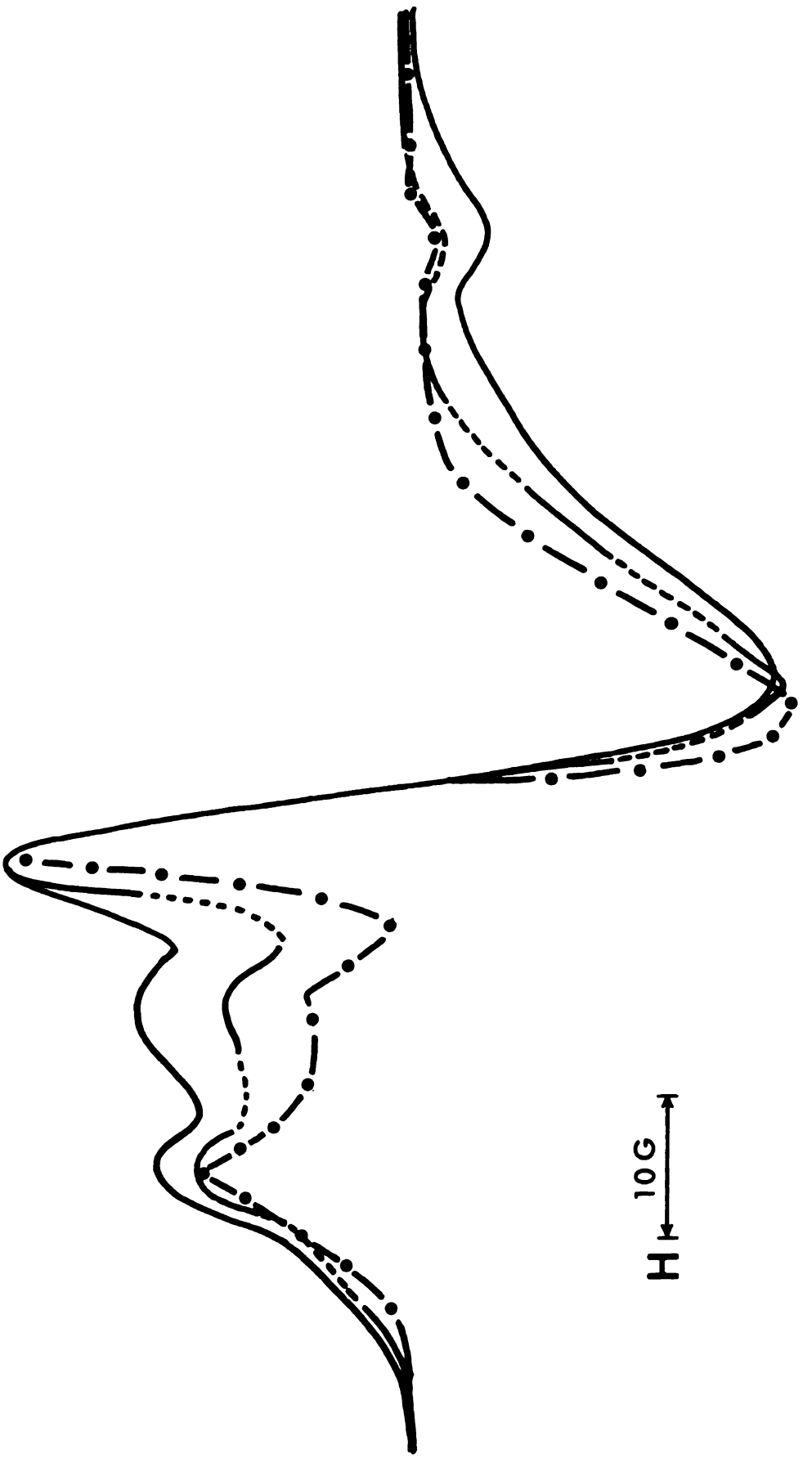


Figure 16

Figure 17. Effect of cholesterol addition on 16NS EPR spectra of labelled T. acidophila membranes.

—— One-day-old membranes labelled with 16NS at pH 6 and 30°C.

--- The above preparation after the addition of non-labelled cholesterol in amounts equal to five times the spin label concentration (w/w).

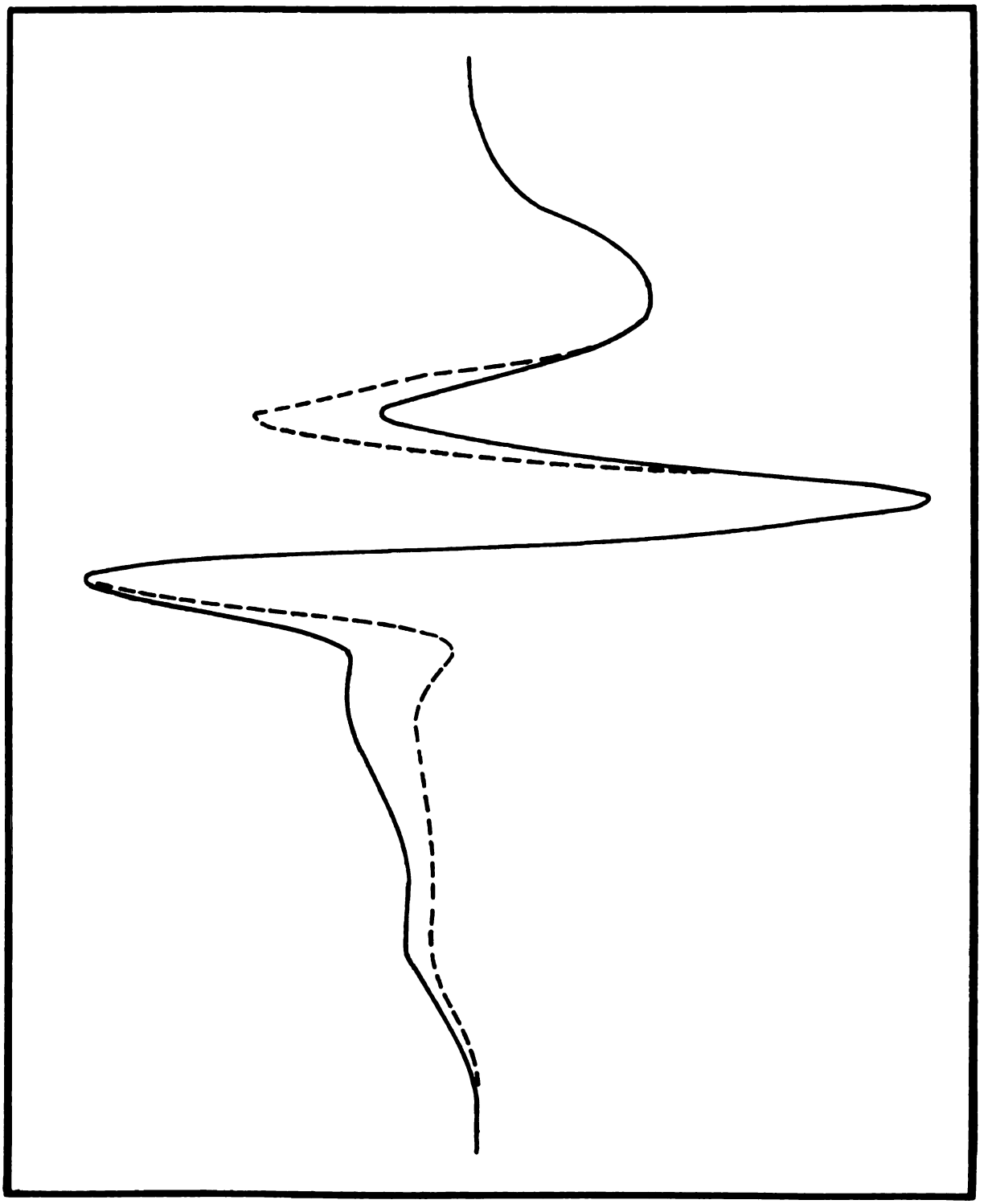


Figure 17

T. acidophila membrane vesicles (Table 6 and Fig. 13). Similar observations at these temperatures have been made with H. cutirubrum (46). Above 40° C, however, 12NS in T. acidophila membranes was apparently in a more rigid environment than 5NS and 16NS labels. This behavior has not been observed in established bilyer systems.

The lipid regions of T. acidophila membranes may best be described as "rigid", since for all labels tested, both the order and rotational parameters indicate less fluidity than has been reported for H. cutirubrum, the most highly ordered membrane known. This rigidity appears consistent with the high resistance to osmotic shock noted in T. acidophila (4). No osmotic procedure has ever been reported to lyse more than 50% of these organisms (3, 4) although it lacks a cell wall. Evidently, in T. acidophila, the membrane is strong enough to protect the cells against osmotic shock possibly by maintaining a greater rigidity than that found in the membranes of other organisms sensitive to osmotic changes. The presence of high amounts of branched long-chain alkyl ethers (molecular weight = 532 to 560) and the large protein: lipid ratio (4:1, w/w) probably contribute to the high rigidity observed in T. acidophila.

Sharp transitions were detected in T. acidophila membranes with all stearate spin labels tested. This observation is consistent with the relative lipid homogeneity (over 50% of the lipid weight is composed of two alkyl ethers (45)) and with the assumption that the fatty acid labels are located in the membrane lipids (51).

Fresh T. acidophila membrane preparations labelled with 16NS resulted in spectra (Fig. 14c) similar to those observed in phosphatidyl

ethanolamine vesicles highly doped (more than $3.5X10^{-2}$ mole steroid/ mole lipid) with spin-label cholestane (52-54). Upon application of spin-label to a limited region of an artificial lipid bilayer, lateral diffusion was accompanied by a gradual change in spectral type; namely, from exchange spectra (Fig. 14d) to the usual three line pattern (Fig. 14b) (69). Although the molar ratio of spin-label to lipid is only $4 X 10^{-3}$ in our experiments, interaction is unquestionably observed (Fig. 14c). The decrease in interaction observed upon further addition of 16NS is inconsistent with the concept of a uniform bilayer or a spin label pool devoid of membrane lipids, since either hypothesis would predict stronger interaction with increased concentration of spin label. These findings could possibly be explained by assuming that at least some of the lipids in T. acidophila are arranged in a cluster, perhaps as illustrated in Fig. 19. Addition of excess label or of unlabelled cholesterol could cause expansion or possibly disruption of the cluster, resulting in less interaction between labels (Figs. 16 and 17). Alternately, only a few cluster sites may be available where 16NS spin labels can interact with each other. Once these cluster sites are filled, spin labels migrate to other membrane regions (e. g. bilayer), thus producing a spectrum with less interaction characteristics. If membranes are labelled only with a spin-label cholestane probe, strong interaction is observed in T. acidophila membranes. This might be expected since non-polar molecules would prefer to migrate into the center of the lipid cluster. Hydrophobic proteins might also penetrate into regions of this cluster, increasing the rigidity of

Figure 18. Electron paramagnetic resonance spectra of cholestane spin label in one-day-old T. acidophila membranes at pH 6, 20°C. The molar label: lipid ratio is 4×10^{-3}.

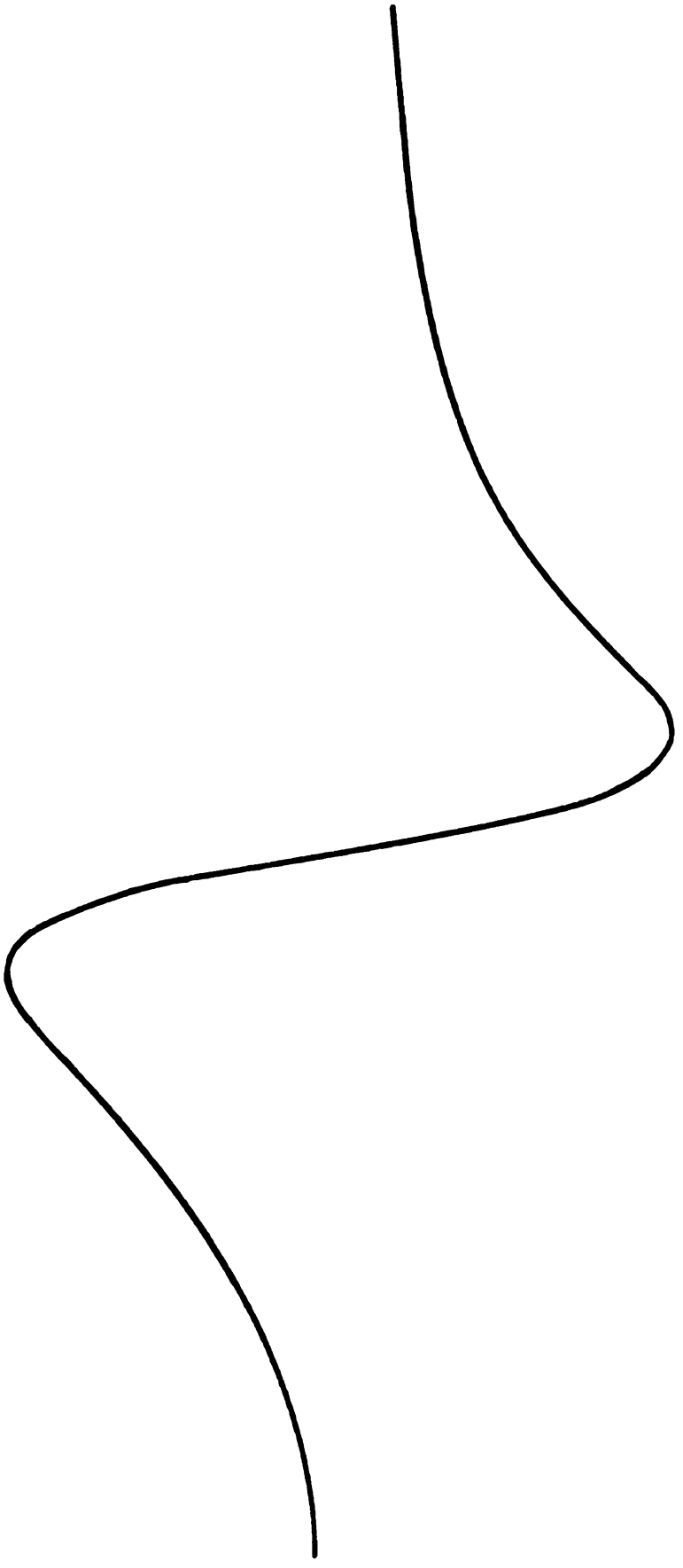


Figure 18

Figure 19. Membrane models.

A. Schematic drawing of a bilayer membrane illustrating the distance between evenly distributed spin labels.

B. Schematic drawing showing a lipid cluster model membrane illustrating the proximity of evenly distributed spin labels.

The number of lipid molecules in each model is the same.

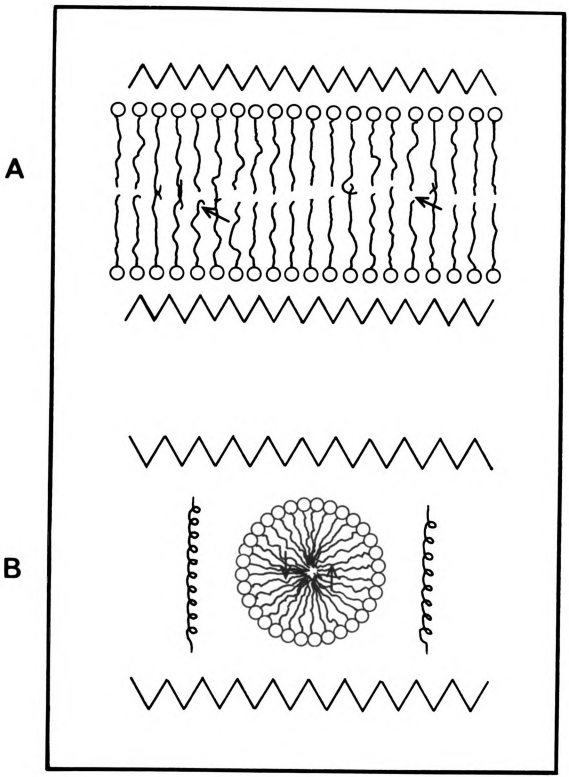


Figure 19

the interior of the cluster, and accounting for the observation that regions near the 12NS label are less fluid than those of 5NS at temperatures above 40° C.

Thus, the results presented herein indicate that T. acidophila membranes are the least fluid biological membranes yet reported. Much of the spin label behavior in the lipid matrix appears to be more consistent with lipid clusters than with a lipid bilayer.

CHAPTER IV

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BIBLIOGRAPHY

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