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PHYSICAL CHEMISTRY OF MEMBRANES FROM *THERMOPLASMA ACIDOPHILUM*: AN ELECTRON SPIN RESONANCE STUDY

By

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ABSTRACT

PHYSICAL CHEMISTRY OF MEMBRANES FROM THERMOPLASMA ACIDOPHILUM: AN ELECTRON SPIN RESONANCE STUDY

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Thermoplasma acidophilum, which grows optimally at pH 2 and 59°C, has an extremely rigid membrane containing unusual ether-linked, long chain lipids and a mannose-rich glycoprotein. Electron spin resonance (ESR) studies of whole cells, membrane vesicles and vesicles of isolated phospholipid, glycolipid and total lipid fractions labeled with the spin probe 5-nitroxy-stearate reveal the presence of discrete slope discontinuities in plots of the hyperfine splitting vs temperature. A complex gel to liquid-crystalline lipid phase transition occurs in lipid vesicles between about 22°C and 60°C. In the presence of protein a third discontinuity around 40°C is indicative of a membrane event that may determine the lower limit of growth for T. acidophilum. Efforts to produce incompletely glycosylated glycoproteins by inhibition with the antibiotic tunicamycin were inconclusive; membrane fluidity as measured by ESR was not noticeably altered in cells grown with 5 μg/ml tunicamycin, although that concentration inhibited growth.

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TABLE OF CONTENTS

	Page
LIST OF TABLES	v
LIST OF FIGURES	vi
INTRODUCTION	1
Membranes of *T. acidophilum*	2
Nature of the Charged Membrane Surface of *T. acidophilum*	3
Effect of Cations at the Membrane Surface of *T. acidophilum*	4
General Effects of Cations on Membranes	5
Effects of pH on Membrane Fluidity	7
T. acidophilum's Niche as an Archaebacterium	8
T. acidophilum's Susceptibility to Tunicamycin	8
Action of Tunicamycin	9
MATERIALS AND METHODS	11
Growth of *T. acidophilum*	11
Membrane Isolation and Purification	11
Lipid Extraction	12
ESR Sample Preparation	12
ESR Procedure	13
Tunicamycin	16
RESULTS	17
Membrane Vesicles	17
Whole Cells	18
Total Lipid Vesicles	20
Phospholipid Vesicles	25
Glycolipid Vesicles	29
pH Dependence of 5NS	29
Tunicamycin	32

Page

DISCUSSION AND CONCLUSIONS 35

ESR Studies 35
Cation Effect. 39
Correlation of Growth Temperature and Membrane Fluidity 40
Role of Membrane Lipids 42
Tunicamycin 42

BIBLIOGRAPHY 44

LIST OF TABLES

Table		Page
1.	Break Point Determinations From ESR Plots of $2T_{\|\|}$ vs Temperature for Isolated and Intact Whole Cell Membranes	19
2.	Break Point Determinations From ESR Plots of $2T_{\|\|}$ vs Temperature for Lipid Vesicles	24
3.	pH dependence of 5NS.	31

LIST OF FIGURES

Figure		Page
1.	Structures of tunicamycin	10
2.	Typical ESR spectra at 3 temperatures for membranes labeled with 5NS	14
3.	Hyperfine splitting vs temperature for whole cells at pH 6.	21
4.	Hyperfine splitting vs temperature for whole cells.	22
5.	Hyperfine splitting vs temperature for total lipids at pH 6	23
6.	Hyperfine splitting vs temperature for phospholipid vesicles.	26
7.	Hyperfine splitting vs temperature for phospholipid vesicles at pH 6 . . .	27
8.	Hyperfine splitting vs temperature for phospholipid vesicles at pH 2 . . .	28
9.	Hyperfine splitting vs temperature for glycolipid vesicles	30
10.	Growth as a function of time for cells grown with tunicamycin	33
11.	Hyperfine splitting vs temperature for membrane vesicles and membrane vesicles from cells grown with 5 ug/ml tunicamycin; pH 6.	34

INTRODUCTION

The results of previous electron spin resonance (ESR) studies of membranes from Thermoplasma acidophilum indicate extremely rigid acyl chains, a pH-dependent discontinuity in plots of the hyperfine splitting ($2T_{||}$) vs temperature for samples labeled with 5-nitroxy-stearate (5NS), and a calcium effect which rigidified the membrane (1,2).

The present set of experiments is intended to explain these phenomena by studying the individual components of the membrane as well as intact whole cells. By examining vesicles of extracted lipids in the absence of protein, it can be determined whether the observed break points are representative of lipid events and which, if any, are mediated by protein. It is also possible to inspect the specificity of the membrane-calcium interaction and the pH effect on membrane fluidity. In addition, the importance of the major membrane glycoprotein to survival in a harsh environment will be studied by adding a specific inhibitor of glycoprotein synthesis, tunicamycin, to a growing culture of T. acidophilum.

Membranes of T. acidophilum

A revealing but incomplete picture of T. acidophilum's membrane has evolved in recent years. In particular, a thorough characterization of the unusual lipids and the major membrane glycoprotein has cleared the way for a better understanding of the physical chemistry of the membrane.

The presence of unusual diglycerol tetra-ether-linked lipids in T. acidophilum's membrane has been established (3,4). The 40-carbon isoprenoid-derived acyl chains found in these lipids are highly methyl-branched and contain cyclopentane rings. These long chains possibly span the membrane in a monolayer, linking glycerol molecules on opposite sides of the membrane.

Similar long-chain ether-linked lipids have been found in other organisms forced to survive in extreme environments (5). In T. acidophilum, the ether linkages serve to provide stability at low pH; the long chains may confer stability at the high growth temperature of 56°C (6). The rigidity offered by these lipids may be mandatory for survival under circumstances which require the exclusion of protons from the cell without the assistance of a cell wall (7,9).

Another dominating feature of the membrane is a 152,000 MW glycoprotein constituting 32% of the total membrane proteins (8). The highly branched mannose residues of its carbohydrate portion may form a protective mesh

over the cell's surface. A higher glycolipid content (20%) than is found normally in bacteria may also be related to the need for acid and heat stability (9).

Nature of the Charged Membrane Surface of T. acidophilum

At pH 2, T. acidophilum has an abundance of protons and should have few negatively charged phospholipids on the outer surface (5), since all pK values for the phospholipid ionizations lie well above pH 2. As far as is known, despite the unusual nature of the lipid acyl chains in the membrane, T. acidophilum and other archaebacteria have analogies of the major lipid headgroups found in conventional bacteria (10). Phospholipids, glycolipids and neutral lipids are isolated in a ratio of 3:2:2 from T. acidophilum's membranes (11).

Conceivably, ion effects may still be seen at pH 2 without a highly charged surface. Electrophoretic measurements of cell mobility indicate the presence of a net negative surface charge at pH 2 (12). At pH 6, a larger negative surface charge results from the increase in headgroup ionization. Calcium has been observed to decrease the cell mobility; at pH 2 a larger concentration (0.01 M $CaCl_2$) is required than at pH 6 ($2.5X10^{-4}$) to reduce the mobility by 50%. Obviously, protons and calcium are in competition for sites suggesting a specificity of calcium binding. Whether binding occurs by proton displacement is not known.

The intracellular pH has been estimated at 6.4-6.9 as determined by the distribution of a radioactive weak

organic acid, 5,5-dimethyl-2,4-oxazolidine-dione (DMO)(13). Thus the inner surface of the bilayer faces a quite different environment than the outer surface. The membrane potential of 109-125 mV, positive inside, is not affected by the addition of metabolic inhibitors such as 2,4-dinitrophenol or NaN_3 (14), indicating that the large pH gradient of 4.4 pH units is maintained passively. The protons may be excluded from the cell by the rigid nature of the membrane components (5).

Effect of Cations at the Membrane Surface of T. acidophilum

Convincing evidence for ion-membrane interactions comes from ESR studies on the effect of aluminum and calcium on membrane fluidity as measured by the spin probe 5NS. The addition of 1 mM $AlCl_3$ to membrane vesicles at pH 4 causes a simultaneous shift of a 15°C and 35°C break point to higher temperatures. 10 mM $AlCl_3$ causes a dramatic increase of the low temperature break point by 39°C, possibly the largest ion-induced shift yet reported. A similar, less pronounced effect was seen on intact whole cells. No response of membrane vesicles was observed to the presence of aluminum at pH 2 although slightly reduced $2T_{||}$ values were seen in the presence of the trivalent ion (15).

The addition of $CaCl_2$ to membrane vesicles has been reported to induce an upward shift in a low-temperature break point. Concentrations tested ranged from 0.1 mM to 100 mM (2).

General Effects of Cations on Membranes

Ion-phospholipid interactions depend on the ionization state of the membrane, and consequently, on the pH. In the case of calcium both specific interactions with the charged phospholipid headgroups and nonspecific screening effects occur.

Screening occurs when calcium ions exert their electrostatic effect at a distance of a few angstroms from the membrane (16). They are located in a diffuse, mobile ion layer attracted to the membrane by the negatively charged phospholipids. The resulting charge neutralization stabilizes the membrane; it allows a reduction in the distance between headgroups, and a closer packing of the lipid acyl chains (17). This type of nonspecific binding, which is adequately described by the Stern treatment of the Gouy-Chapman diffuse double layer theory, has been shown to describe the association of calcium with phosphatidylcholine vesicles (18).

The specific effects of calcium on membrane fluidity have been studied in detail. Träuble and Eibl observed the onset of phase transitions in phosphatidic acid vesicles reflected by changes in the fluoresence intensity of the partition probe N-phenyl-naphthylamine (19). In phosphatidic acid vesicles, specifically bound calcium increases the transition temperature by reducing the surface charge. The result is a more rigid membrane, providing further support for calcium's postulated role as a membrane stabilizer.

Manganese interactions with membranes provide insight into analogous calcium binding. In phosphatidylserine vesicles, ESR determinations of free and bound manganese revealed two modes of manganese binding. A nonspecific adsorption occurred below pH 7.3, and a higher affinity binding involving proton displacement dominated above pH 7.3, causing acidification of the medium (20).

In liposomes of mixtures of acidic phospholipids, calcium produced aggregation and some degree of phase separation (21). A headgroup crosslinking effect was proposed. A differential scanning calorimetry study showed that in situations where similarly charged phospholipid vesicles vary only by headgroup type, calcium still exerted differential effects, indicating that the character of the headgroup was influential in calcium binding (21).

More evidence for specific binding of calcium comes from studies of the addition of calcium to phosphatidylserine vesicles, which are singly-ionized above pH 4.4. A phosphatidylcholine headgroup-labeled spin probe is excluded from phosphatidylserine domains created by calcium bridging between the phosphatidylserine headgroups (22).

Although most studies have been done with phospholipid monolayers and vesicles, it is possible that divalent cations also interact with glycolipids, altering headgroup configurations (23).

Effects of pH on Membrane Fluidity

Several studies reveal the importance of the pH in regulating membrane fluidity. Träuble and Eibl also studied pH-induced changes in phosphatidic acid vesicles. For phosphatidic acid a primary ionization occurs at pH 4; a secondary proton is lost from 8.1 to 8.5. This progressive ionization is reflected in a stepwise decrease in the temperature of the liquid crystalline to gel phase transition. Near either of these pK values under the proper conditions, an incremental change in pH can actually trigger a phase transition (19).

A study of phosphatidylserine vesicles demonstrated a proton-induced phase separation. As the pH was lowered, the protonation of the serine carboxyl group at 4.4 induced a transition to the gel state. This was reflected by the exclusion of a phosphatidylcholine headgroup-labeled spin probe from phosphatidylserine domains (25).

Studies of the pH-dependent fusion between the Semliki Forest virus membrane and phospholipid liposomes provide evidence for a different effect of pH on membranes. A strict dependence of fusion on pH is observed which most likely involves a change in a crucial viral membrane glycoprotein- the "fusion protein". Studies with the isolated glycoprotein indicate a low-pH induced change in the molecule itself which may be required for the interaction with host membrane lipids. This suggests a pH induced change in the ionization or configuration of a protein rather than the membrane lipids (26).

T. acidophilum's Niche as an Archaebacterium

T. acidophilum has recently been classified as a member of the archaebacteria group of the prokaryote kingdom, a group which includes such diverse species as halophiles, thermophiles and methanogens. The absence of a cell wall was not sufficient to classify T. acidophilum with the mycoplasmas; it is evolutionarily distinct from the eubacteria, including mycoplasmas (27).

The classification as an archaebacterium is based on the presence of characteristic transfer RNAs and ribosomal RNAs, the absence of a peptidoglycan cell wall, the presence of ether-linked lipids, and growth in an extreme habitat. T. acidophilum is distinct from other archaebacteria in its total absence of a cell wall (28).

T. acidophilum's Susceptibility to Tunicamycin

The position of T. acidophilum and other archaebacteria in this classification scheme suggests that in some respects their intermediary metabolism may operate at a more primitive level of organization than is found in ordinary bacteria. A better understanding of prebiotic chemistry may result from studies of the components and metabolism of the archaebacteria. However, the archaebacteria resemble eukaryotes in many respects, including sensitivity to specific inhibitors of translation (28). For this reason, it is not unreasonable to expect tunicamycin to act as specifically against protein glycosylation in T. acidophilum as it does in other organisms studied so far. The presence of the

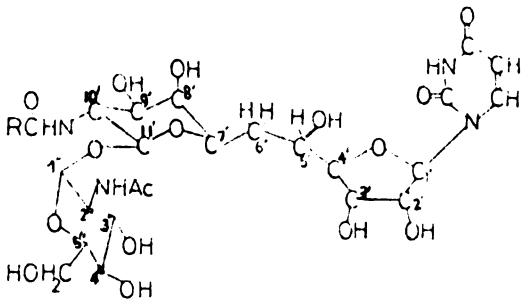
glycoproteins in membranes is thought to have occurred evolutionarily before the division of archaebacteria from the eubacteria (28).

Action of Tunicamycin

Tunicamycin has been shown to specifically inhibit protein glycosylation in bacterial, plant, lower eukaryotic and mammalian cells (29). It has become increasingly valuable in studies of receptor function (30), human fibroblast interferon (31), glycoprotein function in membrane transport (32), altered glycoproteins in transformed cells (33,34), and antibody secretion (35).

Tunicamycin is a specific inhibitor of a specific step in the biosynthesis of asparagine-linked oligosaccharides (36,37). It prevents the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to a lipid intermediate containing dolichol pyrophosphate to form polyisoprenyl-N-acetylglucosaminyl pyrophosphate.

Tunicamycin is a mixture of at least 10 homologous antibiotics (Figure 1) (38). It is produced by the microorganism Streptomyces lysosuperficus (39).



R =

I	$(CH_3)_2CH(CH_2)_7CH{=}CH-$	VI	$(CH_3)_2CH(CH_2)_{11}$
II (C)	$(CH_3)_2CH(CH_2)_8CH{=}CH-$	VII (B)	$(CH_3)_2CH(CH_2)_{10}CH{=}CH-$
III	$CH_3(CH_2)_{10}CH{=}CH-$	VIII	$CH_3(CH_2)_{12}CH{=}CH-$
IV	$C_{12}H_{25}CH{=}CH-$	IX	$C_{14}H_{29}CH{=}CH-$
V (A)	$(CH_3)_2CH(CH_2)_9CH{=}CH-$	X (D)	$(CH_3)_2CH(CH_2)_{11}CH{=}CH-$

Figure 1. Structures of Tunicamycin. (Source: Ito, T., Takatsuki, A., Kawamura, K., Sato, K., Tamura,G. (1980) Agric. Biol. Chem. 44, 695.)

MATERIALS AND METHODS

Growth of T. acidophilum

T. acidophilum was grown at 56°C in medium containing 0.02% $(NH_4)_2SO_3$, 0.05% $MgSO_4$, 0.025% $CaCl_2 2H_2O$, 0.3% KH_2PO_4, 1.0% glucose and 0.1% yeast extract. The medium was adjusted to pH 2 with concentrated H_2SO_4. Air was continuously bubbled through the cultures, which were continuously subcultured by a 10% (v/v) innoculum into autoclaved medium; cells were harvested in late log phase at an optical density of 0.3 (540 nm). Cells were collected by centrifugation for 10 minutes at 8288 g using a Sorvall RC2-B centrifuge, followed by three water washings at 12,062 g for 15 minutes each.

Membrane Isolation and Purification

Cells were lysed in 1.0 M glycine buffer (pH = 9.3). Membranes appeared as a gel-like pellet after centrifugation at 34,858 g for two hours and were purified on a discontinuous (25%/55%) sucrose gradient. A Beckman L2-65B with SW41 rotor was used at 35,000 rpm for two hours. Membranes, which appeared as a single band at the interface, were drawn out and washed three times with water at 35,000 rpm for two hours each. High purity membranes and high yields could be obtained in this manner (11).

Lipid Extraction

All glassware was acid-washed and solvents were glass-distilled. After washing, lipids were twice extracted from cells by stirring for 1 hour with 2:1 (v/v) chloroform:methanol in a solvent to cell ratio of 20:1, then filtered. The filtrate was further purified by a Folch extraction in a 0.2% NaCl solution of chloroform:methanol: water in a ratio of 8:4:3 overnight. The lower phase, containing the total lipid fraction, was evaporated to dryness and added with 3 ml chloroform to a chloroform-washed silicic acid column in order to separate the lipid classes. Neutral lipids were eluted with 20 column washings of chloroform; glycolipids with 40 column washings of acetone, and phospholipids with 20 column washings of methanol. When necessary, total lipid fractions were further purified by chromatography on a coarse G-25 Sephadex column. Lipids were stored in 2:1 chloroform:methanol at -5°C under nitrogen.

ESR Sample Preparation

Whole cells to be used in ESR experiments were harvested in late log phase at an optical density of 0.3 (540 nm). They were washed once with 10 mM EDTA and three times with water at 12,062 g for 10 minutes each. Cells were suspended at either pH 2 or pH 6 (adjusted with 1 M HCl and 1 M NaOH) and 5NS was added with gentle vortexing. Membrane vesicles were formed at pH 2 or pH 6 by sonication with a glass bead for 5 minutes using a Cole-Parmer 8845-4 Ultrasonicator. 5NS was then added followed by more sonication. All

traces of organic solvent were removed from lipid samples by evaporation under nitrogen and then under vacuum for 30 minutes. 0.3-0.5 ml of double-distilled H_2O was added and vesicles formed and labeled in the same manner as for membrane vesicles. In experiments on the effect of cations, vesicles were formed in solutions of 100 mM $CaCl_2$, 100 mM $CaCl_2 2H_2O$ (75.5 mM $CaCl_2$), or 100 mM $MgCl_2$. 2.5 mM $K_3Fe(CN)_6$ was added to prevent signal reduction without altering the experimental results. The spin label concentration was 0.1-0.2% of the lipid weight of the vesicles.

ESR Procedure

ESR spectra were measured with a Varian E112 X-band spectrometer. The temperature was controlled by a Varian variable temperature controller; the quartz cuvette temperature was monitored with a calibrated thermocouple attached to an Omega 250 digital meter. Typical settings for a spectrum were: modulation amplitude = 2.5, magnetic field strength = 3265 Gauss, microwave power = 15 mW (below saturation), and a frequency of 9 GHz. There was no machine-induced broadening. Samples were equilibrated for 5 minutes at each temperature; scans were run at 3°C intervals from low to high temperature in each case.

Whole cells, membrane vesicles and lipid vesicles were labeled with 5NS. The parameters $2T_{\parallel}$ (hyperfine splitting parameter) and $2T_{\perp}$ were measured, and a third parameter, S (order parameter), was calculated (Figure 2). $2T_{\parallel}$ is an accurate measure not of total membrane fluidity

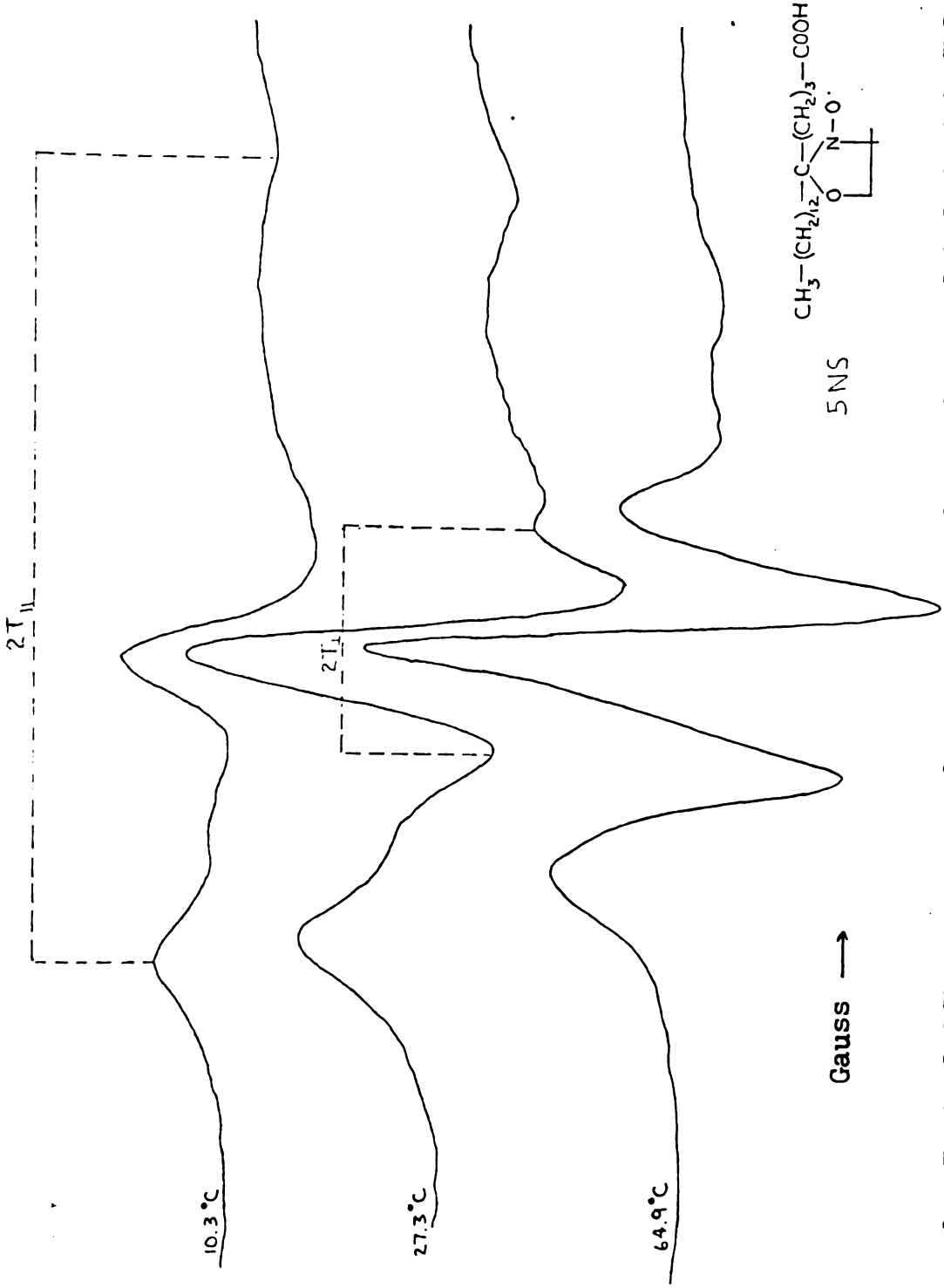


Figure 2: Typical ESR spectra at 3 temperatures for membranes labeled with 5NS. (showing ESR parameters $2T_{||}$ and $2T_{\perp}$, and structure of 5NS)

but of motion in the immediate vicinity of the paramagnetic spin probe. High values of $2T_{\parallel}$ are found at low temperatures, indicating strong immobilization of the probe. $2T_{\perp}$ usually can only be found at temperatures above 20 C. S is calculated by method number two of Griffith and Jost (40), An S value of 1 is considered to represent the most rigid case of lipid acyl chain packing; decreasing S values parallel increasing probe and lipid disordering.

The experimental plots of $2T_{\parallel}$ (or S) vs temperature were analyzed by two methods. Break points were estimated by eye and lines were fit to segments of the experimental data by a linear regression calculation performed on a Varian 620/L-100 computer. The coefficient of determination in each case was greater than 0.95 and in most cases above 0.98. Break point determinations from the intersection of these lines were compared to computer-generated break point determinations made by the method of Brunder, Coughlin and McGroarty (41). The method, which uses normalized β-splines, is based on an interative least squares program. It analyzes the data as a whole, allowing more objective break point determinations.

In all cases the break point determination made by the β-spline program differed only slightly from temperatures at which the linear regression lines intersect. The values reported in Table 1 and Table 2 are averages of several experiments.

Tunicamycin

Stock solutions of 1000 µg/ml of tunicamycin in 0.01 N NaOH were stored at -5°C. The antibiotic was added with the inoculum as cells were subcultured. Growth was monitored by optical density measurements as a function of time using a Gilford 2400 Spectrophotometer. Tunicamycin was the kind gift of Dr. Robert L. Hamill of Eli Lilly Research Laboratories.

RESULTS

Membrane fluidity, as mentioned before, may refer to a regional effect or an overall membrane phenomenon, and may be measured on several different time scales depending on the technique used. One may observe a change in a rotational, diffusional, or translational event and still speak in the same terms. It should be reemphasized that on a nanosecond time scale, spin labels reflect motion in the local environment of the probe and not of the membrane lipids in general (42). 5NS intercalates into lipid domains of the membrane where the nitroxide located on carbon 5 reads a definite stratum of the membrane. For membranes from T. acidophilum, in which acyl chains are especially rigid, 5NS and 12-nitroxy-stearate (12NS) report similar high values of $2T_{||}$ (1). The probe concentration must not perturb the membrane more than necessary; in these experiments a ratio of 0.1-0.2% (w/w) of label to sample is used (42).

Membrane Vesicles

The temperatures of the discontinuities occurring in plots of $2T_{||}$ vs temperature have been determined for membrane vesicles (1). It was found that the pH at the time of labeling with 5NS is crucial. For vesicles labeled at pH 2, break points were determined at 15°C and 60°C. When label

was added at pH 6, a new break at 45°C was observed, as well as a disappearance of the 60°C break. In another study at pH 4, break points appeared at 15°C and 36°C (15). These discontinuities were interpreted to be the result of a lipid phase transition in the membrane.

These observations have been confirmed by the present experiments. Two break points appear at 15°C and 35°C in the case of vesicles labeled at pH 6, and at 16°C and 55°C for vesicles labeled at pH 2. These values are averages of a fairly wide range of break point determinations. This data, along with that for whole cells, is presented in Table 1.

A calcium effect on membranes labeled at pH 6 with 5NS has been reported to increase the lower break point by several degrees (2). A slight calcium effect has been seen under the present set of experimental conditions for vesicles labeled with 5NS at pH 6. An increase in values of $2T_{\parallel}$ by 1-2% is seen throughout the temperature range in which the experiments were performed.

Whole Cells

In order to provide a comparison between intact and isolated membranes, whole cells were studied at both pH 2 and pH 6. Plots of $2T_{\parallel}$ vs temperature for whole cells labeled with 5NS at pH 6 exhibit three discontinuities at 21°C, 40°C and 56°C. For whole cells labeled at pH 2, two breaks occur at 19°C and 43°C. Attempts to measure $2T_{\parallel}$ above 50°C were experimentally unfeasible due to signal reduction. There is no way of knowing whether any higher

TABLE 1: BREAK POINT DETERMINATIONS FROM ESR PLOTS OF $2T_{\parallel}$ VS TEMPERATURE FOR ISOLATED AND INTACT WHOLE CELL MEMBRANES

Type of Membrane Sample	Break Point Temperatures					
	avg	(range)	avg	(range)	avg	(range)
Membrane Vesicles - pH 2	16	--			55	--
Membrane Vesicles - pH 6	15	(11-20)	35	(28-40)		
Whole Cells - pH 2	19	(16-26)	43	(40-49)		
Whole Cells - pH 6	21	(15-28)	40	(32-44)	56	(54-57)
Whole Cells - pH 6 - 100mM $CaCl_2 \cdot 2H_2O$	19	(18-21)				
Whole Cells - pH 6 - 100mM $MgCl_2$	18	--				

discontinuities in slope occur at pH 2, as one would expect from the data on membrane vesicles. 100 mM $CaCl_2 \cdot 2H_2O$ and 100 mM $MgCl_2$ were added to whole cells labeled at pH 6 without any effect on break point determinations. No increase in $2T_{\parallel}$ was seen (Figure 3). Whole cells were not appreciably more rigid at one pH; plots of $2T_{\parallel}$ vs temperature are presented for whole cells at pH 2 and pH 6 in Figure 4.

Whole cells labeled at pH 2 gave very weak ESR signals. In several experiments the power was increased to 40 mW in order to obtain a sufficiently strong signal. Because such a large increase in microwave power can affect the measured parameters, and since signal reduction occurred above 50°C, no further experiments on the effect of ions on whole cells at pH 2 were performed.

Total Lipid Vesicles

Vesicles formed from total lipid extracts labeled with 5NS proved to be unstable. Above 40°C, the low field trough was distorted by the appearance of a shoulder. Only the low temperature range could be scanned; plots of $2T_{\parallel}$ vs temperature revealed a break point at 25°C (Figure 5). Attempts to label total lipid vesicles with the spin label Tempo were unsuccessful. The break point determinations for total lipid, phospholipid and glycolipid vesicles are presented in Table 2.

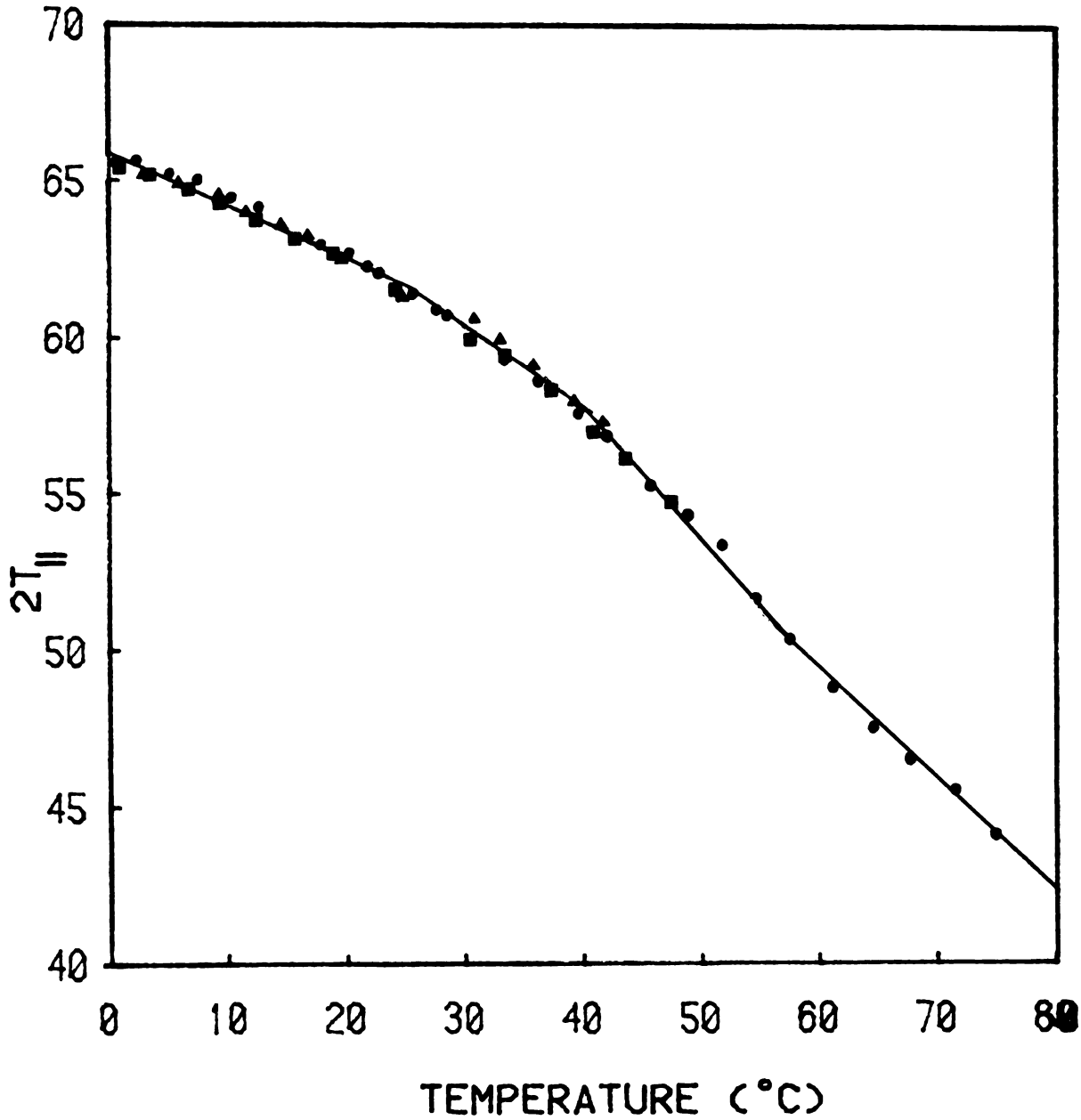


Figure 3: Hyperfine splitting ($2T_{\parallel}$) vs temperature for whole cells at pH 6. (●●● - in H_2O; ▲▲▲ - with 100 mM $CaCl_2 2H_2O$; ■■■ - with 100 mM $MgCl_2$)

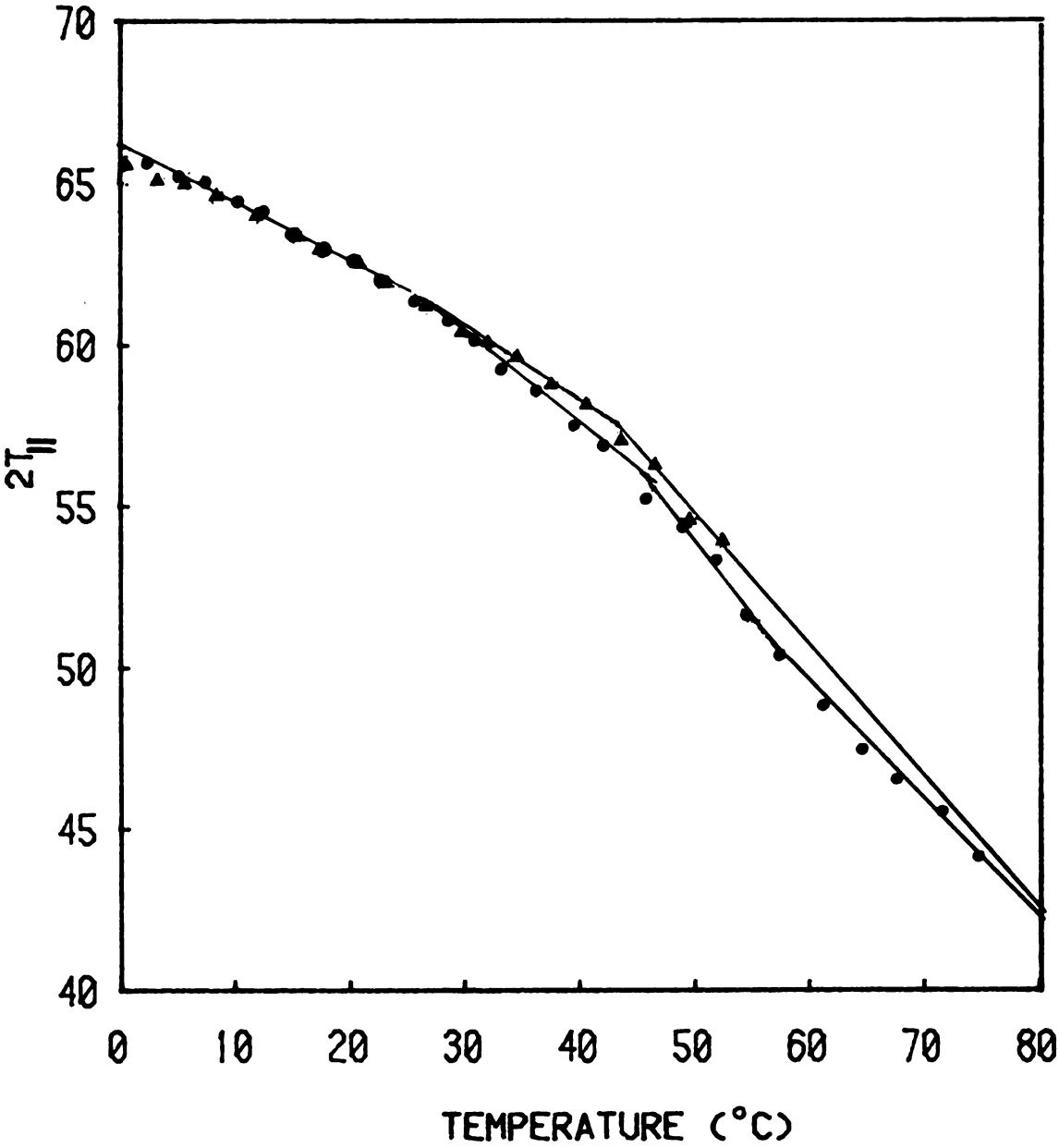


Figure 4: Hyperfine splitting ($2T_{||}$) vs temperature for whole cells. (● ● ● at pH 6; ▲ ▲ ▲ at pH 2)

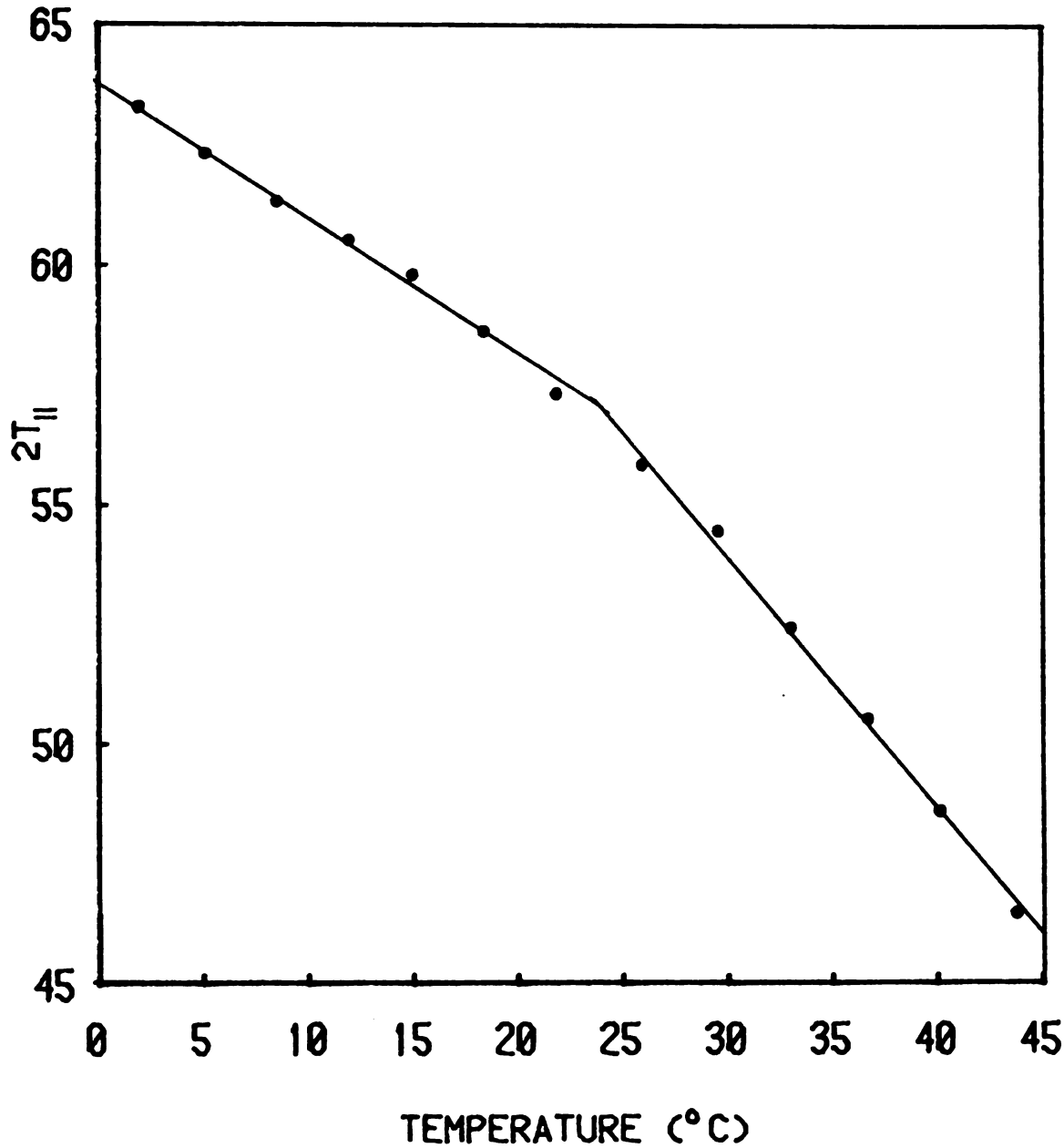


Figure 5: Hyperfine splitting ($2T_{\parallel}$) vs temperature for total lipids at pH 6.

TABLE 2: BREAK POINT DETERMINATIONS FROM ESR PLOTS OF $2T_{\parallel}$ VS TEMPERATURE FOR LIPID VESICLES

Type of Membrane Sample	Break Point Temperatures			
	avg	(range)	avg	(range)
Total Lipid Vesicles - pH 6	25	--		
Phospholipid Vesicles - pH 2	22	(22-23)	59	(56-61)
Phospholipid Vesicles - pH 2 - 100mM $CaCl_2 \cdot 2H_2O$	22	(21-24)	61	(60-61)
Phospholipid Vesicles - pH 2 - 100mM $MgCl_2$	24	--	66	--
Phospholipid Vesicles - pH 6	22	(20-23)	62	(54-69)
Phospholipid Vesicles - pH 6 - 100mM $CaCl_2 \cdot 2H_2O$	21	--	66	--
Phospholipid Vesicles - pH 6 - 100mM $CaCl_2$	13	--		
Phospholipid Vesicles - pH 6 - 100mM $MgCl_2$	22	--	59	--
Glycolipid Vesicles - pH 2	26	(22-29)	58	--
Glycolipid Vesicles - pH 6	21	--	52	--

Phospholipid Vesicles

Phospholipids readily formed vesicles than, when labeled with 5NS, gave a stable signal over a large temperature range; often up to 80°C. Plots of $2T_{\parallel}$ vs temperature for phospholipid vesicles labeled with 5NS at pH 6 exhibit well-defined breaks at 22°C and 62°C. For vesicles labeled at pH 2, discontinuities appear at comparable values of 22°C and 59°C. A comparison of $2T_{\parallel}$ values shows that phospholipid vesicles are not significantly more rigid at one pH (Figure 6).

Data for phospholipid vesicles to which varying concentrations of divalent cations have been added indicate that neither 100 mM $MgCl_2$, 100 mM $CaCl_2{\cdot}2H_2O$ nor 100 mM $CaCl_2$ result in significantly increased break point determinations at either pH 2 or pH 6. These fairly high ion concentrations were chosen because of a report that the presence of 100 mM $CaCl_2$ causes the appearance of a break in plots of $2T_{\parallel}$ vs temperature at a temperature several degrees higher than in its absence (2). Calcium has been shown to rigidify membranes in several systems, as mentioned in the Introduction. A comparison of plots of $2T_{\parallel}$ vs temperature for phospholipid vesicles with and without divalent cations at either pH indicate a slight overall increase on the order of 2-4% in values of $2T_{\parallel}$ throughout the temperature range of 0°C to 80°C in the case of calcium ions, and of 3-5% in the case of magnesium ions (Figures 7,8).

Other experimental evidence exists for a slight

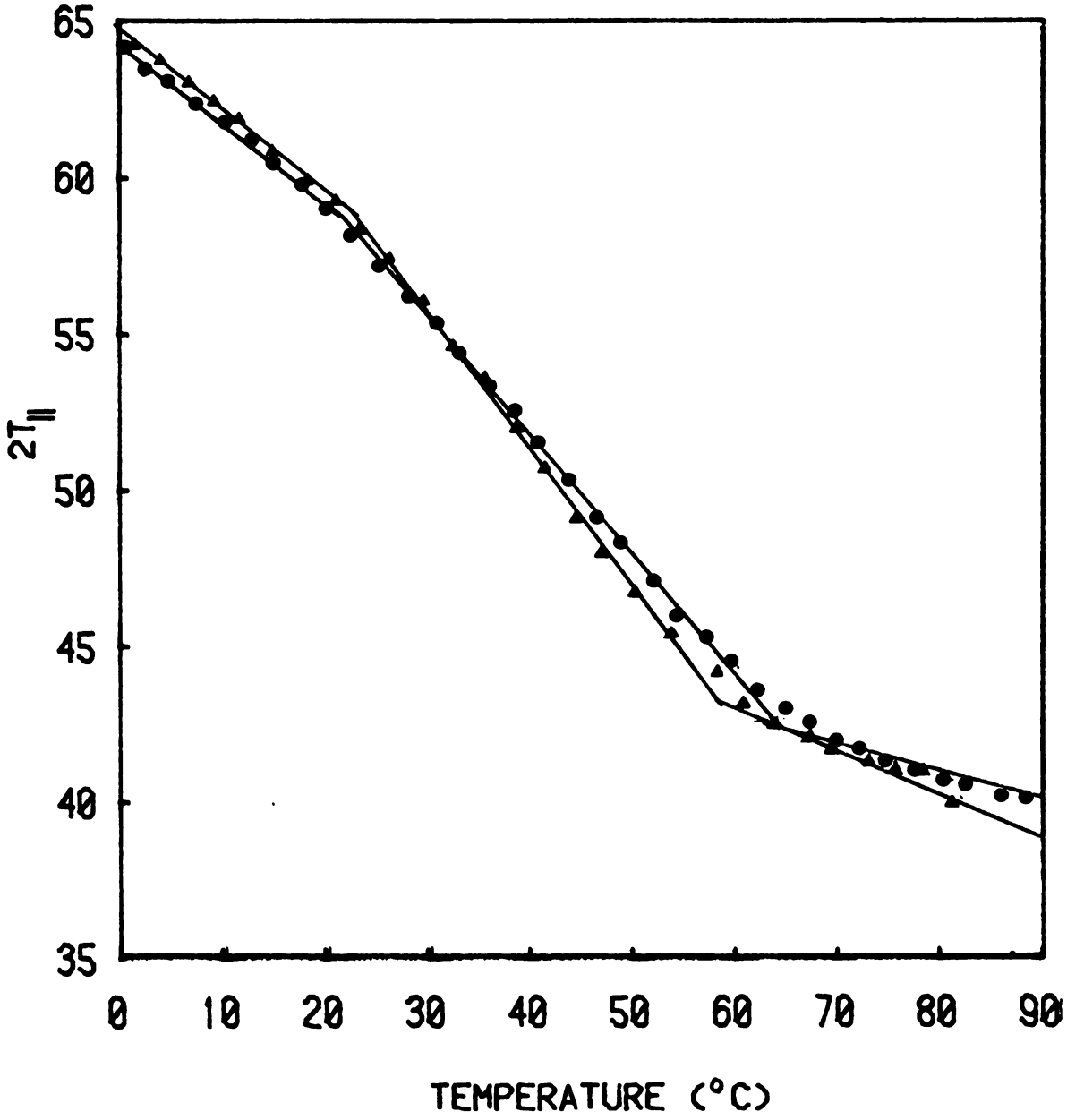


Figure 6: Hyperfine splitting ($2T_{\parallel}$) vs temperature for phospholipid vesicles. (●●● - at pH 6; ▲▲▲ - at pH 2)

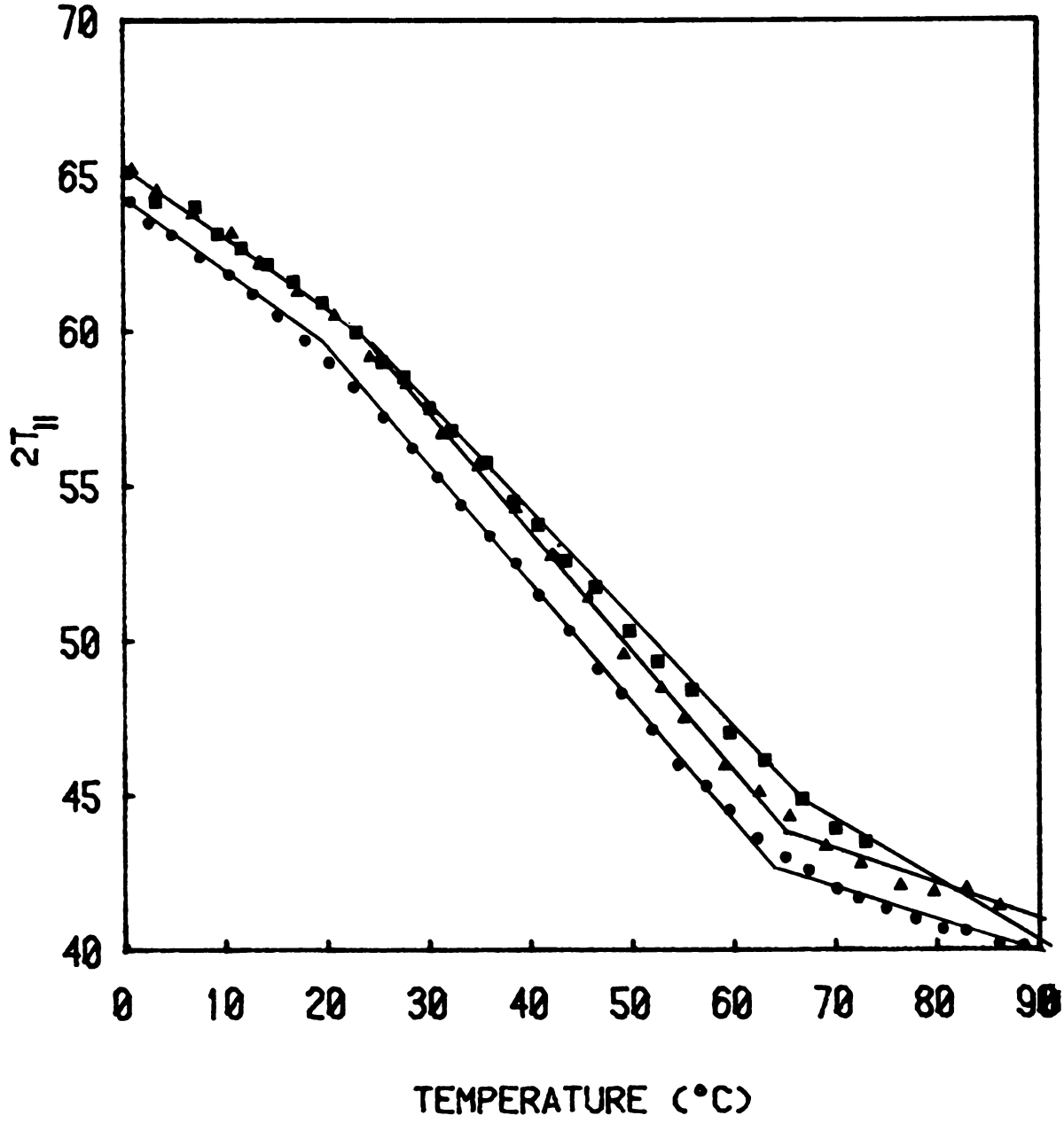


Figure 7: Hyperfine splitting ($2T_{||}$) vs temperature for phospholipid vesicles at pH 6. (● ● ● - in H_2O; ▲ ▲ ▲ - with 100 mM $CaCl_2 \cdot 2H_2O$; ■ ■ ■ -with 100 mM $MgCl_2$)

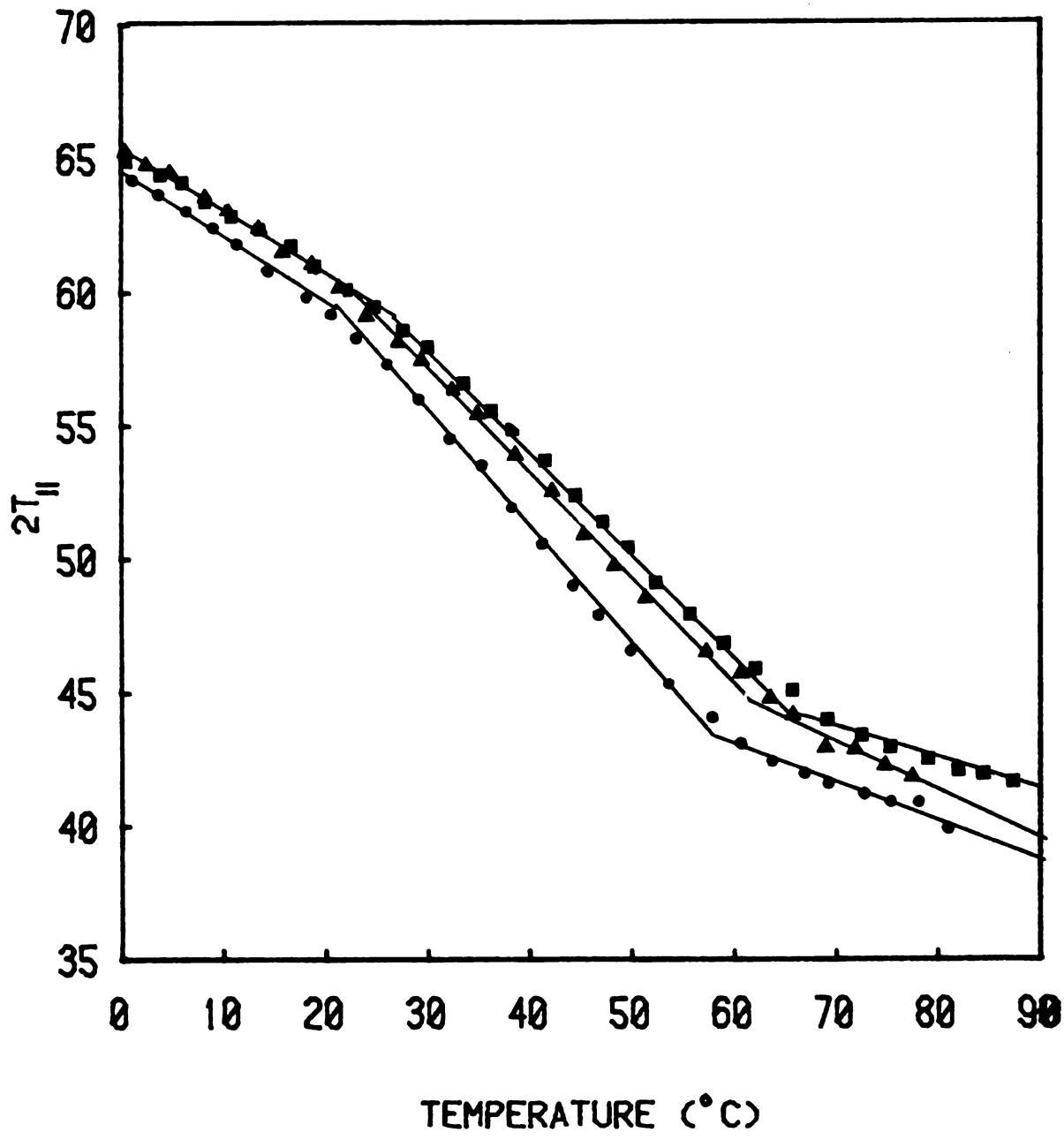


Figure 8: Hyperfine splitting ($2T_{||}$) vs temperature for phospholipid vesicles at pH 2. (● ● ● - in H_2O; ▲ ▲ ▲ - with 100 mM $CaCl_2 \cdot 2H_2O$; ■ ■ ■ -with 100 mM $MgCl_2$)

interaction of calcium with phospholipid vesicles. Readings at higher temperatures than normal may be obtained before signal reduction occurs when calcium is present in the sample. Also, in the presence of calcium some sedimentation is visible in the sample indicating a possible aggregation effect, although normal ESR readings are obtained indicating the presence of lipid bilayers.

Glycolipid Vesicles

Glycolipids in aqueous solution are, to a large extent, immiscible. For this reason, glycolipid vesicles were only occasionally formed, after at least 30 minutes sonication, in sufficient quantities for an ESR experiment. Only temperature scans at pH 2 and pH 6 in the absence of added cations were obtained.

Plots of $2T_{\parallel}$ vs temperature for glycolipid vesicles labeled at pH 2 show discontinuities at 26°C and 58°C; for vesicles labeled at pH 6 breaks appear at 21°C and 52°C. No significant difference in values of $2T_{\parallel}$ was seen (Figure 9). In one experiment in which calcium was added to glycolipid vesicles no increase in values of $2T_{\parallel}$ was observed up to 20°C.

pH Dependence of 5NS

The pH dependence of ESR-derived parameters for membranes and whole cells labeled with 5NS appears in Table 3.

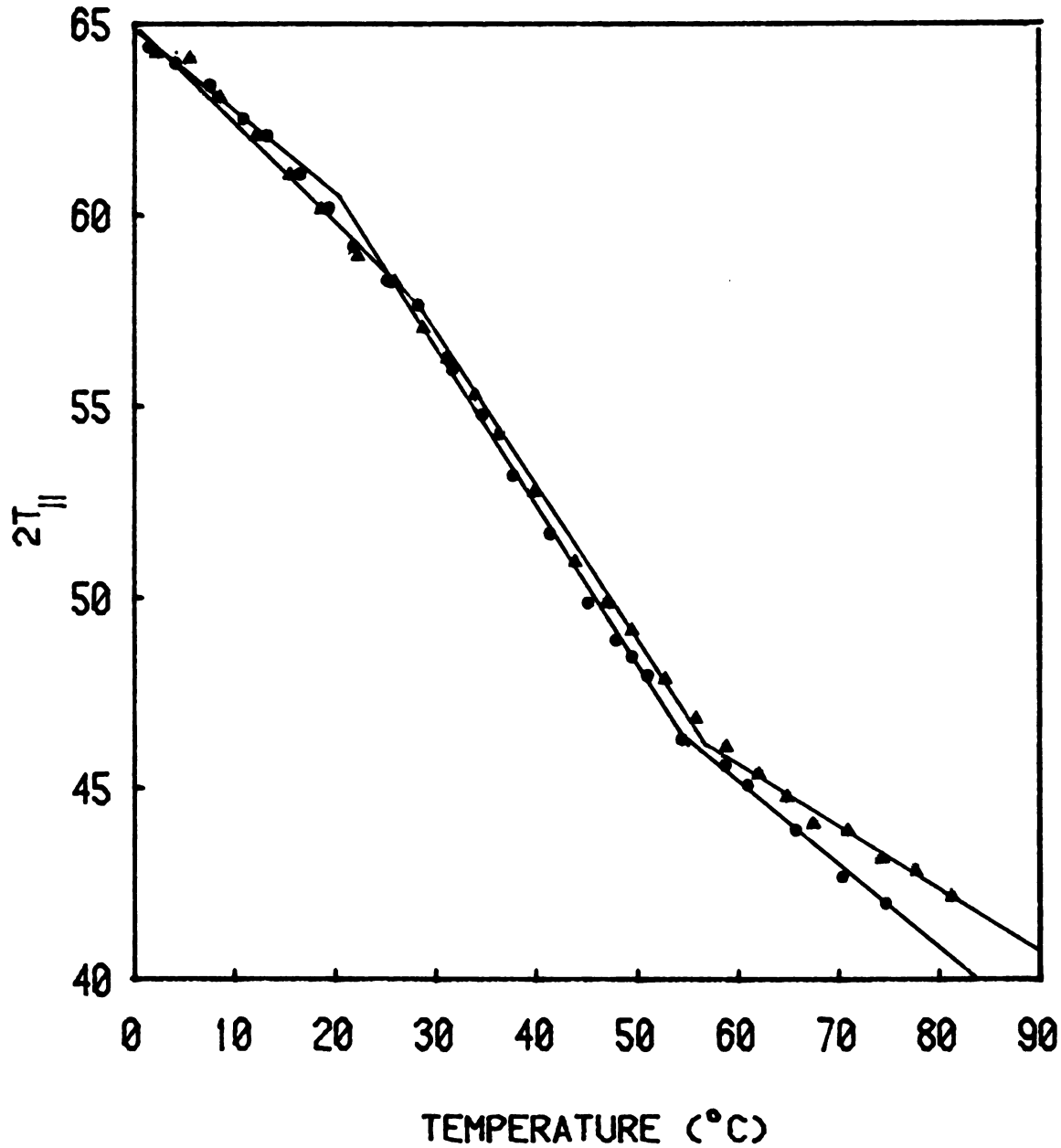


Figure 9: Hyperfine splitting ($2T_{||}$) vs temperature for glycolipid vesicles. (● ● ● - at pH 6; ▲ ▲ ▲ - at pH 2)

Table 3. pH dependence of 5NS

	pH	S
Membrane Vesicles:	2.76	0.687
	3.89	0.714
	5.41	0.699
	5.93	0.675
	7.10	0.703
	10.31	0.707
Whole Cells:	2.34	0.723
	3.23	0.721
	4.34	0.703
	5.55	0.713
	6.23	0.690
	6.80	0.688
	8.44	0.700

Conditions for each measurement were standardized but some degree of experimental variation was inevitable. Despite this fact, a variation of less than 10% was found, discounting the probability that changes in the spin label as a result of pH are responsible for differential effects in membrane vesicles.

Tunicamycin

Growth curves for T. acidophilum grown in the presence of varying concentrations of tunicamycin are presented in Figure 10. Absorbance values are reported as % of control value. Incubation of cells with 1 μg/ml tunicamycin resulted in a 12% growth inhibition after 500 minutes; at 5 μg/ml tunicamycin, the inhibition was 56% after the same time period.

Membranes isolated from cultures grown with 5 μg/ml tunicamycin were labeled with 5NS and plots of $2T_{||}$ vs temperature were determined. The absence of any difference either in $2T_{||}$ values or in the temperatures at which the discontinuities occur is shown in Figure 11.

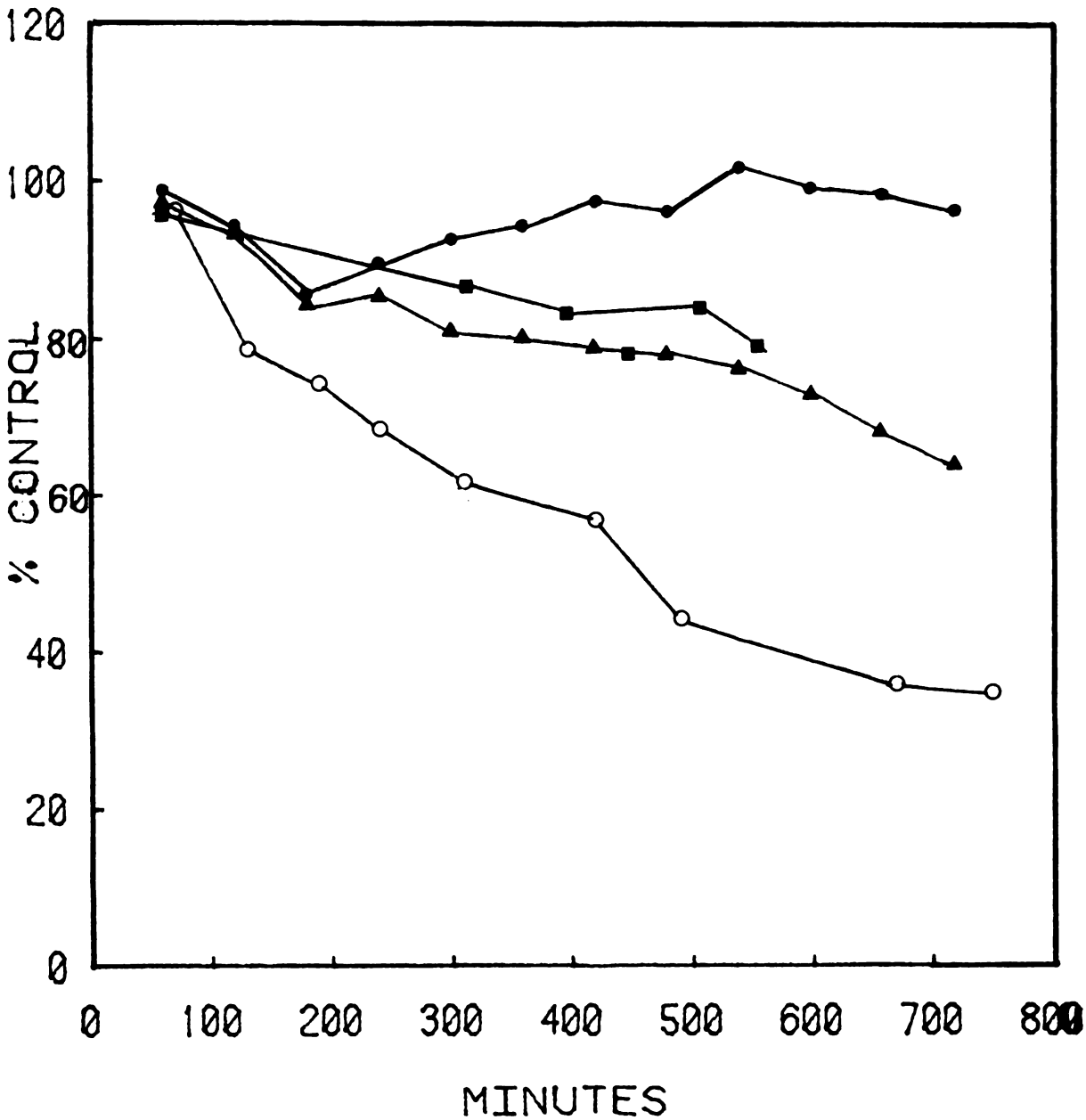


Figure 10: Growth (as % control) as a function of time for cells grown with tunicamycin. (● ● ● - 0.01 ug/ml; ▲ ▲ ▲ - 0.10 ug/ml; ■ ■ ■ - 3.0 ug/ml; ○ ○ ○ - 5.0 ug/ml)

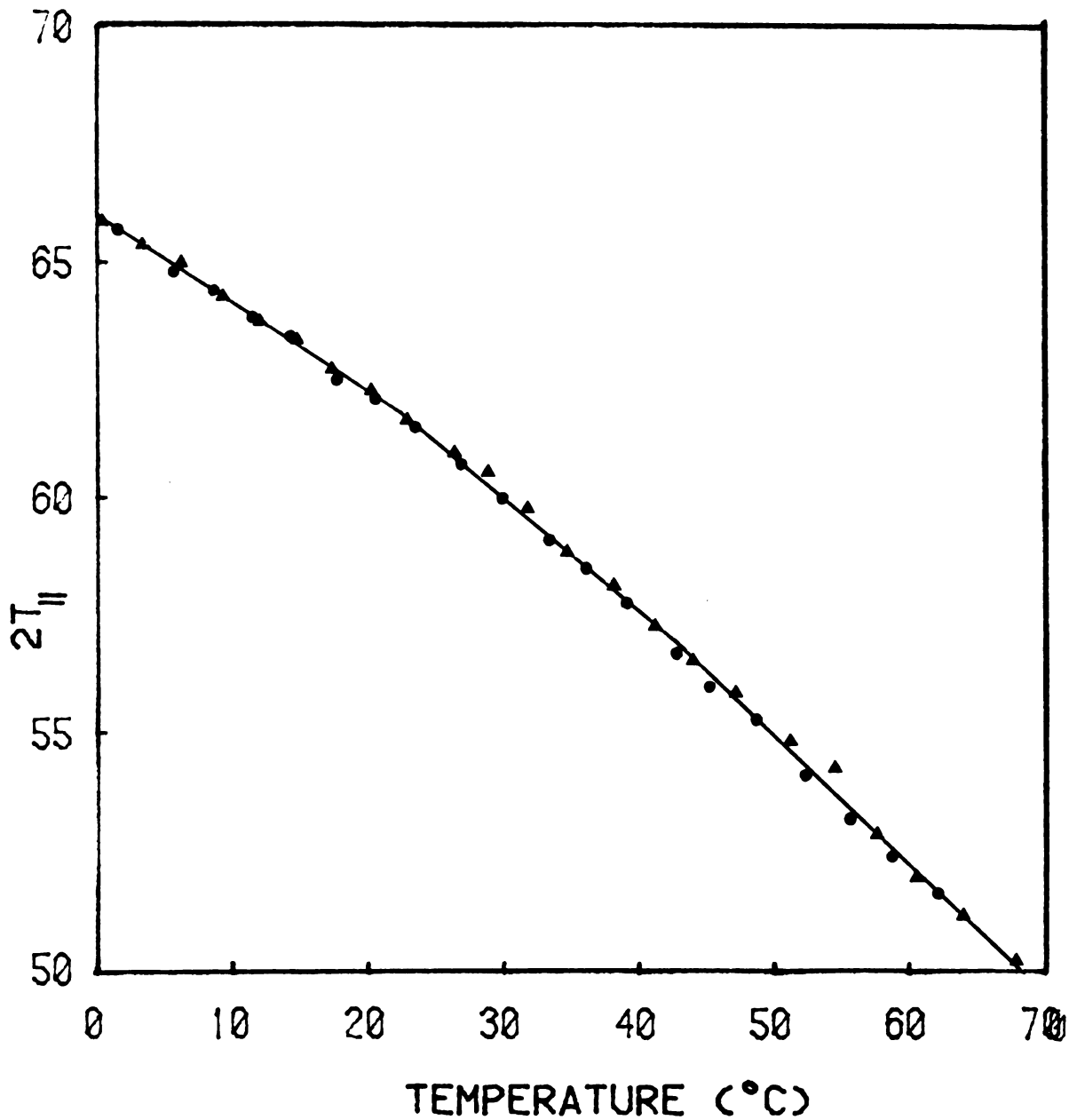


Figure 11: Hyperfine splitting ($2T_{||}$) vs temperature for membrane vesicles (● ● ●) and membrane vesicles from cells grown with 5 ug/ml tunicamycin (▲ ▲ ▲); pH 6.

DISCUSSION AND CONCLUSIONS

ESR Studies

Three discrete slope discontinuities appear and disappear, depending on the experimental conditions. The low temperature discontinuity, occurring anywhere from 15°C to 26°C, is never absent, although under certain conditions of high ionic strength it can be seen at higher temperatures (2,15).

The high temperature discontinuity appeared at values between 56°C and 68°C. Except for membrane vesicles labeled at pH 6 and those cases when signal reduction occurred in the high temperature range, this discontinuity was observed in all membrane samples.

The dominating event in the temperature range between these two discontinuities may be a reorganization of the phospholipids and glycolipids in the membrane due to the induction of a gel-liquid crystalline phase transition among the acyl chains common to both classes of lipids. This interpretation is suggested by the data for phospholipid, glycolipid and total lipid fractions. In each case similar discontinuities are induced regardless of pH. The absence of any protein indicates that a purely lipid phenomenon is being observed.

The term phase transition is often applied to the membrane events which produce discontinuities in plots of $2T_{\parallel}$ vs temperature for membranes labeled with 5NS. A phase transition in the strict sense of the term is an alteration of lipid ordering upon induction of the liquid-crystalline state for pure lipids of a single acyl chain and headgroup type. Those pure lipid phase transitions are sharp, occurr in a cooperative manner over only a few degrees, and are not influenced by lipid-protein interactions.

None of the membrane systems used in these experiments, whether intact whole cells, isolated membranes, or lipid vesicles, will produce pure lipid phase transitions. For example, the phospholipids used for these experiments, while isolated on the basis of their common phosphate group, may contain several different headgroup types or chain derivatizations. This causes packing irregularities which may partially explain why the transitions occur over such a broad temperature range. In the case of membrane vesicles and whole cells, protein-lipid interactions may regulate a complex melting sequence which is reflected in the broad discontinuity.

A similar case occurs in multilayer membranes from Tetrahymena, a poikilothermic eukaryote (43). Plots of fluoresence polarization vs temperature show discrete slope discontinuities which depend on the lipid composition of the membrane and, as in T. acidophilum, represent the onset of phase transitions in subpopulations of lipid species. By

the criteria of that study, for T. acidophilum with four 40-carbon chain types (discounting the possible rearrangements of ester-linked lipids) and possibly three major phospholipid headgroups, 4X4X3 or 48 molecular species of phospholipids alone may be present in the membrane. Any one of these subpopulations may contribute to the general phase transition. In T. acidophilum these complex melting events may occur approximately between 22°C and 60°C. This region of mixed phase coexistence may be characterized as a separation of the several discrete populations of lipids.

If a gel to liquid-crystalline phase transition is the event responsible for the discontinuities evolved in plots of $2T_{\|}$ vs temperature, then the breadth of the transition is surprising. It extends beyond even the growth range of the organism. In general the width of a transition is a function of the cooperativity among the acyl chains (44). As mentioned before, if only one type of lipid is present the transition can occur over less than one degree Centigrade. If several subpopulations of lipids are present, cooperativity is decreased by irregular packing and discrete non-cooperating units of lipids. Compared to other systems, for example, Tetrahymena, T. acidophilum has relatively few types of lipids; this should encourage a sharper cooperativity in the gel-liquid crystalline transition. Of the four 40-carbon chains present in the membrane, only two appear in large quantities. Only a small amount of diverse types of ester-linked lipids are present (11). This uniformity may

be related to the need for a heat-stable, rigid membrane, capable of excluding protons (9). However, the highly methyl-branched nature of the lipids may serve to push apart the acyl chains and lower the melting point of the chains (3). The rigid, trans-membrane monolayer nature of the lipids may also be responsible for the extremely broad, noncooperative phase separation, in addition to some steric factor or diversity related to the alkyl chains.

The appearance of a discrete slope discontinuity at approximately 40°C, near the lower limit of growth for T. acidophilum, is a response to a protein-mediated event in the membrane. The break point does not appear in experiments with total lipid, phospholipid or glycolipid vesicles under any conditions of pH or ionic strength examined so far. The 40°C discontinuity is present in whole cells at either pH and in membrane vesicles at pH 6. It is not observed when membranes are labeled with 12NS at either pH 2 or pH 6 or with 5NS at pH 2 (1).

The nonappearance of the 40°C discontinuity in membrane vesicles labeled at pH 2 with 5NS, or with 12NS at any pH, may be the result of several factors:

1. Isolated membranes may not be an accurate representation of lipid-protein interactions in intact whole cells.
2. Differential location of 5NS on one side or another of the membrane may occur depending on pH. A differential response may in some cases result from the smaller curvature of the inner monolayer. In distearyol phosphatidylcholine

vesicles a lipid event monitored by ^{1}H-NMR affecting the inner monolayer headgroup orientation leaves the outer layer headgroups undisturbed (45).

3. Membrane vesicles may be formed right-side-out or inside-out, while intact whole cell membranes have only one orientation.

4. 12NS measures a stratum of the membrane seven carbons closer to the interior than 5NS. One may speculate that, if membrane vesicles are an accurate reconstruction of the intact cell membrane, the location of the 40°C event in membrane vesicles may be close to the headgroup region; possibly an interaction with protein or surface carbohydrate.

5. The internal pH of membrane vesicles is also a consideration; membrane vesicles labeled at pH 2 may not have the internal pH near neutrality found in intact cells.

Alternatively, 6. The 40°C discontinuity is an artifact in intact whole cells.

Cation Effect

In none of the cases where it was possible to study the effect of divalent cations such as calcium was there a shift in the position of the low temperature discontinuity, as reported for membrane vesicles at pH 6 (2). A slight increase of 1-2% in values of $2T_{\parallel}$ for membrane vesicles, and of 2-4% and 3-5% for phospholipid vesicles in the presence of calcium and magnesium respectively, as well as a precipitation effect for both whole cells and phospholipid

vesicles indicates some degree of divalent cation binding. This supports the results of cell electrophoresis measurements in which calcium binding decreased the mobility of whole cells by surface charge neutralization (12).

The membrane of T. acidophilum is so rigid that a reorganization of membrane lipids due to calcium cross-linking, or a large increase in $2T_{||}$ is unlikely. The observed calcium binding and the strong effect of aluminum on break point determinations (2,15) are more likely to be a response to alterations in headgroup interaction and surface charge.

Either glycolipids, for which calcium experiments were not performed, or more likely some special organization of lipids and protein peculiar to membrane vesicles at pH 6 is responsible for the previously observed shift in the low temperature break point.

Correlation of Growth Temperature and Membrane Fluidity

In several systems, including some species which experience constant environmental stress, the temperature range over which the organism can survive has been correlated with the existence of its membrane in a mixed lipid state. This has been observed in membranes of the halotolerant alga, Dunaliella primolecta (46) and Blastocladiella emersonii (47), as well as in the thermophilic bacterium LEH-1 (48). Cells can also adapt their lipid composition when forced to grow at an altered temperature, to ensure that its membranes are sufficiently

fluid for proper functioning (49). That a proper membrane fluidity is required has been observed in A. laidlawii (49) membranes and the outer membrane of E. coli (50).

The limits of growth for T. acidophilum are near 37°C and 65°C (2). In several of the present experiments, the upper limit of the phase transition occurs close to the upper limit of growth; in other cases it appears nearer to the optimum for growth— 56°C. This high temperature discontinuity, as mentioned before, may be representative of membrane events that render the cell susceptible to deterioration, such as the absence of any lipids in the gel state and a concommitant loss of protein regulation.

The lower limit of growth for A. laidlawii cells can be elevated by altering the lipid composition to include lipids whose phase transitions occur above the normal growth temperature minimum. Due to a rate-limiting protein factor, however, the lower limit cannot be depressed in a similar manner (49). A similar situation may occur in the case of T. acidophilum. It is well known that protein conformation and function can be affected by the physical state of membrane lipids (51,52). A critical requirement for growth may be met at 40°C by a protein-mediated event in the membrane. This could be a direct conformational change induced in a protein or surface carbohydrate, or the result of an alteration in a lipid-controlled protein at that temperature. In cells adapted to growth at 37°C the 40°C discontinuity is shifted downward by 10°C, indicating that

either the protein or lipid has adapted its composition to extend its growth range at the new temperature. One such function that may be related to membrane fluidity is ATPase activity. A discontinuity in Arrhenius plots of enzyme activity vs temperature (-1)(°K) appears at 45°C (3).

Role of Membrane Lipids

The implication of long chain ether-linked lipids in membrane stability seems to be supported by the present study. As mentioned in the Introduction, long chain ether-linked lipids are thought to confer heat and acid stability on membranes. From this data it is apparent that the lipids provide the stability necessary to maintain membrane structure and fluidity under these harsh conditions.

In some systems, the properties of galactolipids have been found to resemble those of phospholipids, including the existence of gel to liquid-crystalline phase transitions (23). This acyl-chain-induced similarity has been demonstrated in these studies. The high glycolipid content suggests a role of the headgroups in conferring heat and acid stability on the membrane (9).

Tunicamycin

It was hoped that a clue to membrane function of the glycoprotein that constitutes the major portion of membrane proteins might be evident if cells with defective glycoproteins were produced. The addition of tunicamycin at a concentration of 5 μg/ml was sufficient to cause a

substantial inhibition of growth. The events responsible for this inhibition may be the specific effects on protein glycosylation observed in many systems or some other effect on cell function. If tunicamycin caused the production of abnormal glycoproteins, the amount was insufficient to cause a difference in membrane fluidity as measured by ESR.

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