Membrane Properties of *Thermoplasma acidophila*†

Mary J. Ruwart‡ and Alfred Haug*

ABSTRACT: Plasma membranes were isolated from *Thermoplasma acidophila,* a mycoplasma-like organism which grows optimally at pH 2 and 59°. Cells in concentrated suspensions were lysed by titrating to pH 9.3. The membranes were purified by washing at pH 10 and centrifuging in a discontinuous sucrose gradient. Membrane purity was assessed by electron microscopy, determination of deoxyribonucleic acid content, and polyacrylamide gel electrophoretic behavior. Gel patterns and amino acid composition of cells and membranes were found to differ significantly. The lipid contained small amounts of fatty acid esters and larger amounts of branched long-chain alkyl ethers.

Thermoplasma acidophila, a mycoplasma-like organism with no cell wall, grows optimally at pH 2 and 59° (Darland *et al.,* 1970; Belly *et al.,* 1973). Investigations of the lipids of this microorganism showed the presence of large quantities of long-chain alkyl glyceryl ethers but no fatty acid esters (Langworthy *et al.,* 1972). Recently, a lipopolysaccharide was found in lipid extracted with hot aqueous phenol from whole cells of *Thermoplasma acidophila* (Mayberry-Carson *et al.,* 1974). Membrane preparations have been attempted (Smith *et al.,* 1973), but commonly used lytic procedures have not been successful (Belly and Brock, 1972).

In this article we describe properties of purified *Thermoplasma acidophila* membranes obtained in high yield by rupturing the cells at alkaline pH. This membrane may provide a valuable system for future investigations of membrane response to harsh environmental stresses.

Experimental Section

Thermoplasma acidophila cells were obtained from the American Type Culture Collection (ATCC #25 905). The organism was grown in a medium (Darland *et al.,* 1970) containing 0.02% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 0.025% $CaCl_2 \cdot 2H_2O$, 0.3% KH_2PO_4, 1% glucose, and 0.1% yeast extract (Difco). The pH was adjusted to 2 with concentrated H_2SO_4 and the medium was then autoclaved. A 10% (v/v) inoculum from a 22-hr old culture into the same medium gave the best growth. Each culture was continuously aerated for 22 hr with air sterilized by filtration. Cultures (18 l.) were incubated at 56° in 20-l. flasks. Rapid propagation of this organism was enhanced by vigorous aeration, since standing cultures grown in our laboratory have growth characteristics similar to nonaerated cultures as reported by Darland *et al.* (1970). Shaken cultures (Langworthy *et al.,* 1972) appear to grow at rates intermediate between those reported for nonaerated cells (Darland *et al.,* 1970), and those reported herein for vigorously aerated cells.

Depending upon the future use of the cells, two methods of collecting the cells were employed: (a) for intact cells, centrifugation at 4200*g* for 5 min at 25° followed by gentle resuspension in T-buffer (pH 2 medium without glucose and yeast) on an Eberbach shaker at low speed; (b) for membranes, centrifugation at 10,000*g* for 10 min at 4°, resuspension in T-buffer, centrifugation at 34,000*g* for 30 min, followed by resuspension in distilled water, and centrifugation at 34,000*g* for 30 min.

Lysis at high pH was most successful for rupturing cells. Cells were collected and washed with T-buffer (1×) and/or water (1-5×, 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 with stirring. The pH value was determined with a Beckman Zeromatic II pH meter. The lysis was carried out at room temperature; the treatment lasted about 4 min. Immediately after titration, the membranes were centrifuged. 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 buffers at high pH, membranes could no longer be collected. 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*g*. In more dilute cell suspensions (less than 10 mg/ml of protein), lysis was completed between pH 6.5 and 7.5 (Figure 1), indicating that it was concentration dependent. Membrane solubilization as indicated by protein release at high pH (greater than pH 9.5) continued until no sedimentable matter could be collected (pH 12). No other method of membrane preparation was found to give workable yields or a high degree of purity.

Very low yields of lysed *Thermoplasma acidophila* cells were obtained by exposing cells to osmotic shock at pH 2 or 7.4, or sonicating them for 30 min with a Raytheon sonic oscillator, Model DF-101. Denaturants and detergents at high concentrations (5 M urea, 1% Triton X-100, or 0.1% sodium dodecyl sulfate) caused partial solubilization of cellular material, leaving intact cells or amorphous protein aggregates as determined by electron microscopy.

Following lysis at pH 9.3, the membranes were purified as follows. The membranes were first collected at 34,800*g* for 4 hr. The membrane pellet was resuspended in half as much water as was used during lysis and the suspension was adjusted to pH 10 with 2 N NaOH. The membranes were then collected as before, layered onto a discontinuous sucrose gradient (25%/55%, pH 7.4), and then centrifuged on a Beckman L2-65B ultracentrifuge for 2 hr at 40,000 rpm

† From the Department of Biophysics and the MSU/AEC Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824. *Received August 9, 1974.* Work supported by U.S. Atomic Energy Commission Contract No. AT-(11-1)-1338. M.J.R. was supported by U.S. Public Health Service Training Grant No. GM-01422-06 from the National Institutes of Health.

‡ Present address: Department of Surgery, St. Louis University Medical School, St. Louis, Missouri 63110.

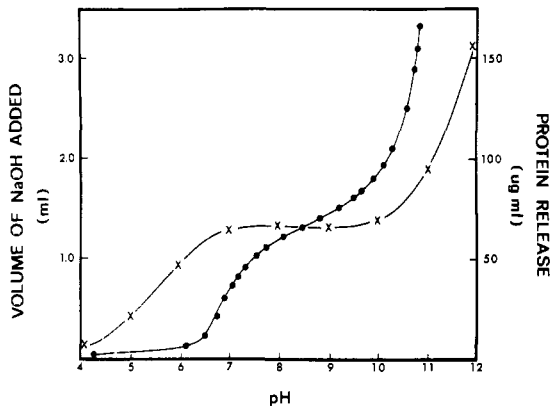

FIGURE 1: Effect of pH on cell lysis of *Thermoplasma acidophila:* (a) pH change (●) in an aqueous cell suspension (11 mg/ml) upon dropwise addition of 0.04 N NaOH; (b) release of protein (×) when cells were suspended in buffers at various pH values, at a concentration of 0.06 mg of protein/ml. All buffers used were 1 M. Buffers at pH 9, 10, and 11 were glycine–sodium chloride; at pH 8 and 4, citrate–NaOH; at pH 7, 6, and 5, phosphate–NaOH.

with a SW 41 rotor. The membranes appeared as a single band at the interface of the sucrose layers and were collected and freed from sucrose by washing with water.

Lipids were extracted from cell or membrane pellets after multiple washings with water and 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, nonlipid contaminants were removed by passing the extract over Sephadex (Rouser *et al.*, 1967), and the lipids were eluted with chloroform–methanol (19:1, v/v) and chloroform–methanol–acetic acid (19:1:4, v/v). Silicic acid chromatography (Anisil, 100–200 mesh; 1.5 × 12 cm^2 column) was employed to separate the lipids into three fractions: (a) neutral (50 ml of chloroform), (b) glycosyl diglycerides (50 ml of acetone), and (c) phospholipids (50 ml of chloroform–methanol, 2:1, v/v; 50 ml of methanol). Quantitative recovery of phosphate from the Sephadex and silicic acid columns was observed. All glassware used in lipid or fatty acid analysis was acid-cleaned prior to use. All solvents were redistilled. All columns were pre-eluted with all solvents to be used.

Fatty acid methyl esters were prepared by incubation of lipid as follows: (1) in 2.5% methanolic H_2SO_4 for 24 hr at 40°; (2) in 10% methanolic H_2SO_4 for 24 hr at 75° (Bowyer *et al.*, 1963); or (3) in 10% methanolic BF_3 and benzene (4:1, v/v) for 48 hr at 75° (Hyun *et al.*, 1965). 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 (Kates *et al.*, 1965). For quantitation by gas chromatography, C_{21} methyl ester or C_{24} alcohol standards were added prior to derivatization.

A Hewlett-Packard gas chromatograph, Model 402, with flame ionization detector and with 6-ft glass columns was employed throughout. Fatty acid methyl esters were determined at 170° 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° or programmed at 8°/min from 200°. Molar response factors were determined empirically from standards obtained from Applied Science. Areas of peaks were measured with a planimeter or by height and width-at-half-height measurements.

Qualitative thin-layer chromatography of the three lipid fractions was performed on Anasil-G (250 μ) plates (Analabs, North Haven, Conn.) as described by Langworthy *et al.* (1972).

All mass spectra were taken with a LKB 9000 gas chromatograph–mass spectrometer equipped with computerized data acquisition (Dr. C. C. Sweeley, Department 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 (2 or 6 ft) column.

Protein was quantitated by the method of Lowry *et al.* (1951). Phosphate was assayed using the procedure of Bartlett (1959). Ester and ether derivatives were hydrogenated as reported (Appelqvist , 1972). Acyl esters were measured by the ferric hydroxamate method (Rapport and Alonzo, 1955) and by infrared absorption at 5.85 μ (Dittmer and Wells, 1969) on a Perkin-Elmer grating infrared spectrometer, Model 621. Deoxyribonucleic acid content was determined as described by Burton (1956). Assays for the presence of phosphonate (Aalbers and Bieber, 1968) and plasmalogens (Klenk and Friedrichs, 1952; Skidmore and Entenman, 1962; Rietsema, 1954) were performed. After removal of nonlipid contaminants, lipids were quantitated by a colorimetric method (Saito and Sato, 1966). Carbohydrate (hexose) was analyzed colorimetrically (Dubois *et al.*, 1956). Glycerol was liberated by 24-hr incubation of lipid at 25° in BCl_3–chloroform (1:1, v/v) and assayed after periodate oxidation (Wells and Dittmer, 1965).

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis was carried out as follows. Membranes, cells, or cytoplasm (precipitated with hot trichloroacetic acid) were suspended in 0.1% mercaptoethanol, 0.01 M phosphate buffer (pH 7.1), and 1.0% sodium dodecyl sulfate and incubated at 100° for 2 min. Sucrose and Bromophenol Blue were then added. Samples were then layered onto 5 or 10% (8 or 10 cm) polyacrylamide gels containing 0.1 M phosphate buffer (pH 7.1), 0.1% sodium dodecyl sulfate, 0.026 ml of *N,N,N',N'*-tetramethylethylenediamine/100 ml of gel, 0.2% methylene bisacrylamide, and 0.065% ammonium persulfate. The upper and lower buffer reservoirs contained 0.1% sodium dodecyl sulfate and 0.1 M phosphate buffer (pH 7.0). Gels were electrophoresed for 15 min at 10 mA per gel followed by 2 mA per gel for 5 hr. Gels were then stained overnight in 0.15% Coomassie Blue R, 7.5% acetic acid, and 5.0% methanol, shaken in destaining solution (5.0% methanol–7.5% acetic acid) for 6 hr, and electrophoretically destained for approximately 2 hr. They were stored in distilled water at 4°.

Native polyacrylamide gel electrophoresis was performed with 2.2, 4.5, and 9.0% acrylamide as described by Davis (1964) with the following modifications. Sample and spacer gels were omitted, pH was adjusted to 8.6, and 10–20 μl of sample containing 20–200 μg of 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 a constant current of 1 mA/gel for 10 min followed by 2.5 mA/gel per 5 hr. Gels were stained for protein with Xylene Brilliant Cyanin G prepared by a modification of the Malik-Berrie procedure (1972) as described by Blakesly (1974). Gels were stained for 5–8 hr. Stained gels were rinsed twice with distilled water and stored in distilled water at 4° in the dark. The absorbance of the stained gels was monitored at 550 nm with a Gilford spectrophotometer, Model 2400, equipped with a Gilford linear transport.

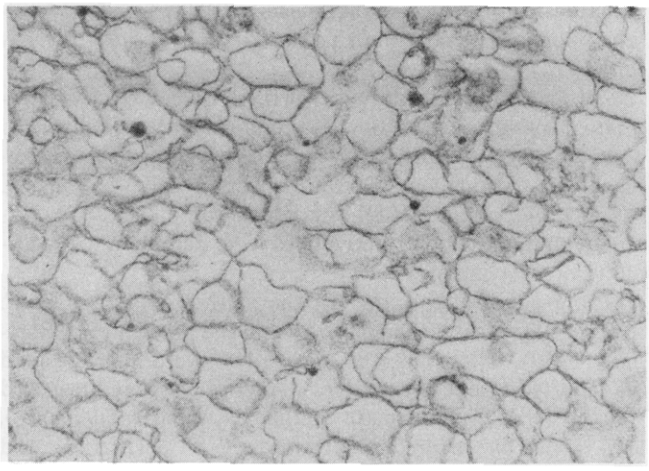

FIGURE 2: Transmission electron micrographs of thin sections of *Thermoplasma acidophila* membranes prepared by high pH lysis, washed once at pH 10, purified by density gradient centrifugation, and washed twice with distilled water. The membranes were stained as described in the Experimental Section.

For amino acid analysis an aliquot of cells or membranes was dried under nitrogen. Then 6 N HCl was added and 2.5 $\times 10^{-3}$ μmol of phenol was added per ml of suspension. Hydrolysis was complete after 18 hr at 105°. 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. The results were computed by a modified Autolab System IV.

For electron microscopy, the samples were fixed in 2% glutaraldehyde–0.033 M cacodylate buffer (pH 6.1) for 2 hr at 4°. The sample was collected by 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 (Kellenberger *et al.*, 1958) was carefully added to the pellet without disturbing the surface. After 18 hr at 4°, the preparation was drained and dehydrated through a graded ethanol series. The sample was embedded in Spurr's epoxy resin (Spurr, 1969) and stained with lead citrate and uranyl acetate (Venable and Coggeshall, 1965). A Philips 300 Transmission electron microscope was employed to view the specimens.

Results

Our experiments show that *Thermoplasma acidophila* cells are highly resistant to osmotic shock or sonication when compared with *Acholeplasma laidlawii* microorganisms (Smith, 1971; Huang *et al.*, 1974). *Thermoplasma* cells appear to be more osmotically fragile when grown in an aerated medium.

Complete lysis of cells and solubilization of cytoplasm were achieved by titrating a cell suspension (not less than 10 mg of protein/ml) to pH 9.3 (Figure 1). Similar curves were obtained (Figure 1) when the volume of base added was plotted *vs.* pH in order to observe the release of buffering components (*i.e.*, protein) from cells into the medium. The titration curve indicates that solubilization of membrane protein probably does not occur at a pH value lower than 9.5. Therefore, we would not expect a significant loss of essential membrane components under conditions of our preparation.

When supernatants of cells which had been titrated to pH 9.3 were adjusted to acidic pH values, 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 *Thermoplasma acidophila* is probably in the neutral range unless the cytoplasm is particulate. These data further suggest that the inside and outside of the membrane differ greatly, since neutral pH causes lysis whereas the intracellular pH is physiologically acceptable to this microorganism. A neutral intracellular pH value was also obtained by measuring the distribution of a weak radioactive organic acid (Hsung and Haug, 1974).

One criterion for purity of isolated membranes prepared by high pH lysis was the absence of cytoplasmic components. Electron micrographs (Figure 2) of thin sections showed the presence of membrane vesicles which were approximately the size of intact cells. The interior of the vesicles was free from electron dense cytoplasm (Figure 2). This picture was obtained from a pellet collected at high speed on a Sorvall centrifuge. Similar pictures have been obtained from ultracentrifuge pellets after purification. The sample appears to be homogeneous throughout the pellet. In contrast to membranes from high pH lysis, sonically prepared membranes contained usually smaller vesicles and amorphous debris, in agreement with earlier observations (Smith *et al.*, 1973). The major drawback of this sonication procedure is the cytoplasmic precipitation which occurs at low pH and probably accounts for the amorphous material previously reported (Smith *et al.*, 1973). Failure to remove this cytoplasmic material was probably due to the acidic conditions chosen (pH 5).

Since cytoplasmic contaminants would be expected to penetrate low percentage polyacrylamide (native) gels, as much as 200 μg of membrane protein was loaded onto the gels with no indication of penetration. In contrast, others (Smith *et al.*, 1973) found that their preparations, which contained "amorphous" material, easily penetrated native polyacrylamide gels. Similar gel patterns were obtained in our laboratory on 10% polyacrylamide gels of cytoplasmic components present in the supernatant of cells sonicated at pH 6.5 (Figure 3). The fact that proteins did not penetrate native polyacrylamide gels therefore demonstrates the absence of cytoplasmic proteins—except those adhering perhaps (if at all present) to the membrane.

Thirdly, our *Thermoplasma* membrane preparation was essentially devoid of deoxyribonucleic acid. The membranes contained less than 0.1% μg of DNA/μg of protein. This represents a DNA recovery in the membrane of 1.7% or a purity of 98%. The DNA content in the whole cells is 6% (weight of DNA/weight of protein). Since the DNA of this organism is diffusely distributed (Darland *et al.*, 1970), the DNA loss is considered to be concurrent with the loss of cytoplasmic proteins. Our electron microscopic pictures confirm this interpretation. The small amount of DNA found in our membrane preparation may result from cytoplasm adsorbed to the membrane surface. When comparing our results with those of other laboratories, caution must be exercised, since different strains of *Thermoplasma* may have been selected for (Belly *et al.*, 1973; Freundt, 1972).

Fourthly, in our laboratory we have demonstrated that *Thermoplasma acidophila* has malate dehydrogenase activity associated with the cytoplasmic fraction (J. C. Hsung and A. Haug, unpublished results). The isolated membrane has no measurable dehydrogenase activity.

By way of lipid recovery, yields of purified membranes amount to about 75%. This value is significant considering

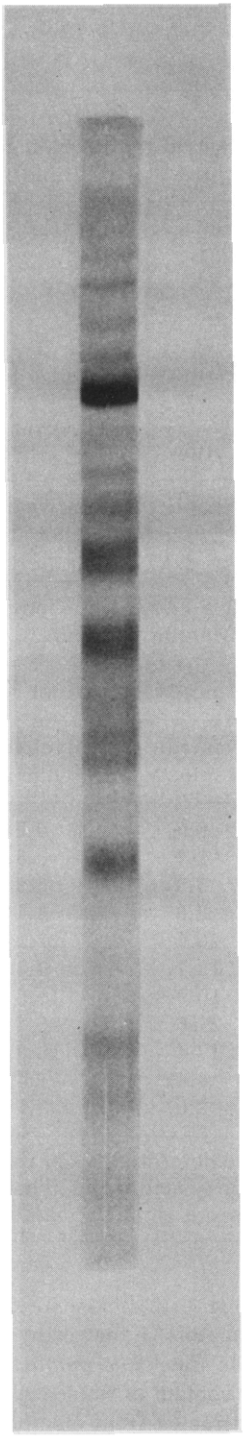

FIGURE 3: Sodium dodecyl sulfate–polyacrylamide gel of proteins from *Thermoplasma acidophila* membranes, precipitated from sonication fluid with 5% trichloroacetic acid in boiling water (10 min). The gel was subjected to electrophoresis and stained with Coomassie Blue.

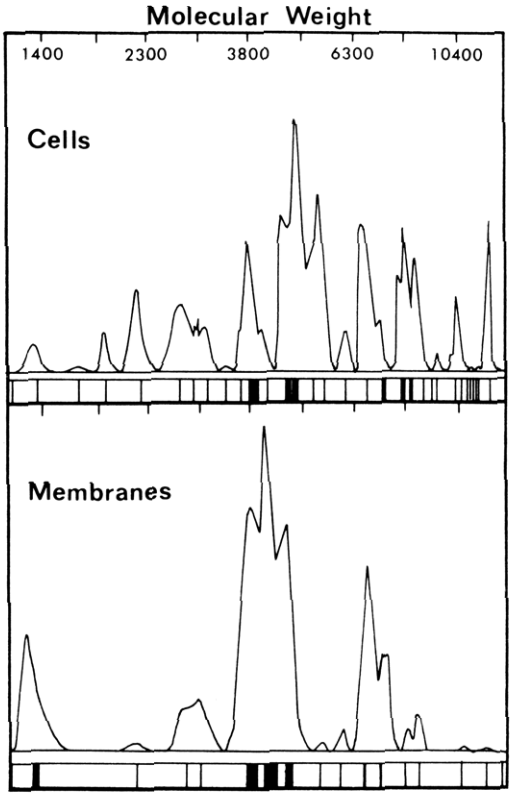

FIGURE 4: Profiles of sodium deodecyl sulfate–polyacrylamide gels of cells and membranes from *Thermoplasma acidophila*. Absorbance was measured at 550 nm. Gels were subjected to electrophoresis and stained.

our finding that the lipid content of whole cells is the same as that of the membrane. Furthermore, practically all of the lipid in *Mycoplasmas* is associated with the plasma membrane, based on total lipid, unsaponifiable lipid, and lipid phosphorus analysis (Smith, 1971). The purified membrane was composed of 19% lipid, 5% carbohydrate, and 76% protein. Chloroform–methanol (2:1) extractions with or without acid (0.1 N HCl) contained large amounts of hydrophobic protein which had to be removed by column chromatography for accurate quantitation of lipids (Nelson, 1968). The exact quantity of protein present in the extract varied with the length and type of homogenization during the extraction and with the total volume of organic solvent employed. These extracts always contained 2 to 15 times as much protein as lipid (w/w). These considerations may account for the higher lipid-to-protein ratio reported by investigators (Smith *et al.*, 1973) who did not remove protein before lipid quantitation.

Membranes solubilized and electrophoresed on sodium dodecyl sulfate–polyacrylamide gels had a pattern strikingly different from solubilized *Thermoplasma acidophila* cells (Figure 4). Cells had approximately 32 protein bands on sodium dodecyl sulfate gels; the molecular weights varied from 10,000 to 125,000. Membranes contained 16 protein bands and four components accounted for about 70% of the membrane protein. Ninety per cent of the membrane proteins had molecular weights of less than 80,000. Belly *et al.* (1973) detected a total of at least 12 different cellular protein bands after electrophoresis. No clear differences could be found among three different *Thermoplasma* isolates.

The amino acid composition of membranes differed from that of whole cells (Table I). The cells contained more basic and acidic amino acids, while the membranes were enriched in tyrosine and cysteine. The amount of hydrophobic amino acids was approximately the same in both membranes and cells. The amino acid composition of our cells was similar to that reported by other investigators (Smith *et al.*, 1973).

Lipids from *Thermoplasma acidophila* were fractionated into neutral lipids, glycosyl diglycerides, and phospholipids; the relative quantities were 17, 8, and 75%, respectively. Thin-layer chromatography patterns of the glycolipids and phospholipids were similar to those previously described (Langworthy *et al.*, 1972). However, in the neutral fraction

Table I: Amino Acid Composition of Cells and Membranes from *Thermoplasma acidophila*.[a]

Amino Acid	Cells (mol %)	Membranes (mol %)
Lys	7.6 ± 0.00	4.05 ± 0.35
His	1.7 ± 0.10	1.15 ± 0.25
Arg	5.2 ± 0.10	3.25 ± 0.05
Asp	11.4 ± 0.40	8.40 ± 0.01
Thr	4.8 ± 0.10	6.40 ± 0.30
Ser	4.9 ± 0.00	6.35 ± 0.35
Glu	9.6 ± 0.00	6.00 ± 0.00
Pro	3.8 ± 0.10	5.25 ± 0.05
Gly	8.9 ± 0.40	7.75 ± 0.55
Ala	7.1 ± 0.10	8.40 ± 0.30
Cys	0.8 ± 0.00	5.75 ± 0.05
Val	8.2 ± 1.10	6.90 ± 0.30
Met	2.7 ± 0.40	2.95 ± 0.05
Ile	7.4 ± 0.20	7.20 ± 0.20
Leu	7.6 ± 0.20	9.60 ± 0.00
Tyr	3.7 ± 0.10	4.35 ± 0.00
Phe	4.5 ± 0.40	6.25 ± 0.05

[a] Average of three determinations.

Table II: Lipid Distribution in *Thermoplasma acidophila*[a] Membranes.

Moiety	Neutral Lipids	Glycolipids (μmol/mg of Lipid)	Phospholipids
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
Approx. wt % accounted for	67	95	83
Pigmentation	Yellow	Yellow-brown	Red-brown

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

Table III: Percentage Fatty Acid Composition of *Thermoplasma acidophila* Membrane Lipids.[a]

	Lipid Fraction (mol % Fatty Acid Ester)		
Fatty Acid Chain Length	Neutral Lipids	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. The fatty acid composition for cells is the same as that of membranes.

we found no detectable amounts of cholesterol and large amounts of vitamin K_2-7 (30% of this fraction). Besides the neutral lipid components designated as Q, R, S, T, V, W, and characterized by Langworthy *et al.* (1972), we observed an additional component halfway between V and W; its retention time approximated that of monoether standards. The reason for these deviations may lie in growth conditions.

The distribution of lipid moieties in *Thermoplasma acidophila* is listed in Table II. One component of the phospholipids, comprising about 10% of the fraction weight, contained no phosphate (Langworthy *et al.*, 1972); it most probably accounted for the high glycerol to phosphate ratio in the fraction. In the glycolipid and phospholipid fractions more ethers were found while the content in fatty acid esters was lower than in the neutral fraction (Table II). Table II also lists the approximate weight per cent in order to give a rough estimate of how much of the total lipid we were able to define by our various moiety checks.

Only 83% of the phospholipids were accounted for by weight (Table II). Langworthy *et al.* (1972) 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.

Besides quantitative gas chromatography–mass spectrometry, the presence of fatty acid esters was also qualitatively established by infrared hydroxamate techniques. Mass spectrometry was employed to identify all methyl ester derivatives of fatty acids (Table III). The amount of total fatty acids was 2.1% (by weight) of total lipids. The ester distribution is similar in all lipid fractions except for the increase in $C_{16:0}$, $C_{20:0}$, and $C_{21:0}$ fatty acids and the decrease in $C_{18:2}$ in the phospholipids. Several methylation methods were attempted in order to ensure complete derivatization of fatty acid esters. Incubation (48 hr) at 75° in the presence of methanolic BF_3 and benzene gave the largest yields and highly reproducible results. For shorter incubation times, hydrolysis was incomplete. Ester determinations were routinely made with lipid extracted from 36-l. cultures. Other investigators (Langworthy *et al.*, 1972) did not detect fatty acids in their *Thermoplasma acidophila* lipid extracts. The ester content of whole cell lipids was the same as that of lipids extracted from membranes. There was no measurable difference in ester composition, when cells were grown in media containing yeast extract, or lipid extracted yeast extract. The cells grew equally well on both types of nutrient medium. In this respect, *Thermoplasma acidophila* differs from most *Mycoplasma* which require fatty acid and/or cholesterol supplementation for growth (Smith, 1971). Oleic acid supplementation (5, 2.5, or 1.2 mg/l.) prevented growth of *Thermoplasma acidophila*.

Distribution of the alkyl ethers in *Thermoplasma acidophila* is listed in Table IV. The molecular weights were determined mass spectrometrically with acetate derivatives. The mass spectra of the acetates and two major ethers were consistent with $C_{40:0}$ and $C_{40:1}$ long-chain compounds in agreement with a previous report (Langworthy *et al.*, 1972). The mass spectra also indicated some isoprenoid branching. Two smaller components appeared to be C_{41} and

Table IV: Distribution of Alkyl Ethers in *Thermoplasma acidophila* Lipids as Determined by Mass Spectrometry.[a]

Carbon No.	Neutral Lipids	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 $C_{40:0}$. The values listed are the same ones for whole cells or membranes.

C_{42} by mass spectrometry studies. The distribution of the ether moieties (Table IV) indicated that the neutral lipids contained predominantly $C_{40:0}$ while phospho- and glycolipids contained an equal amount of $C_{40:1}$. The C_{18} to C_{29} alkanes reported by Langworthy *et al.* (1972) were not found to be present in any significant amounts.

No phosphonates or plasmalogens were detected in the lipids of *Thermoplasma acidophila.*

Discussion

The emphasis of this investigation has been to demonstrate that a purified membrane can be prepared by high pH lysis from *Thermoplasma acidophila* microorganisms. For future studies this membrane can be used as a model to elucidate the influence of a harsh environment upon the structure and function of biological membranes.

The stability of the *Thermoplasma acidophila* membrane to osmotic and mechanical treatment has been reported by various investigators (Langworthy *et al.,* 1972; Belly *et al.,* 1973). This stability may be related to its extreme rigidity as evidenced by spin-labelling experiments, which showed that the *Thermoplasma acidophila* membrane is presently the most rigid biological membrane known (Smith *et al.,* 1974). It is also conceivable that the four major polypeptides (Figure 4) represent "structural" elements in the protein-rich membrane.

Lipids in thermophiles generally have higher melting points than those of mesophiles (Singleton and Amelunxen, 1973); this condition is promoted by increase in length, saturation, and branching of the lipids. The membrane lipids of *Thermoplasma acidophila* also show such a behavior characteristic for thermophilic microorganisms. The high ether and the low ester content also confer additional chemical stability to the lipid matrix of the membrane. Such a lipid composition is perhaps a response of the cell to its acidic habitat (Belly *et al.,* 1973). Moreover, the high ester content of the neutral fraction would be in accordance with a membrane model wherein most nonpolar lipids are relatively shielded from the harsh environment.

The solubilization of *Thermoplasma acidophila* at high pH is probably related to the charge distribution of the amino acids (Smith *et al.,* 1973). At pH values higher than four, carboxyl groups are ionized, resulting in charge repulsion; these electrostatic forces contribute to destabilize and solubilize the membrane. In *Thermoplasma acidophila* these effects are minimized since the organism has only half as many carboxyl and amide groups as mesophilic *Mycoplasma* (Smith *et al.,* 1973). Moreover, the *Thermoplasma acidophila* membrane has fewer carboxyl and amide groups than the total cellular material (Table I). Thus, at high pH, the cytoplasm of *Thermoplasma* will be solubilized before the membrane protein, as we 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 cells may require a high acidic amino acid content; as acidophiles, they require a minimum of ionizable groups. Since hydrophobic interactions have a maximum stability at about 60° (Scheraga, 1963), the highest temperature at which both requirements can met is, to a first approximation, the same one at which hydrophobic bonds are still strong, *i.e.,* around 60°.

The question whether *Thermoplasma acidophila* has to be classified as a *Mycoplasma* or a stable L-form is not yet settled (Belly *et al.,* 1973). If *Thermoplasma* should be classified as a *Mycoplasma, Thermoplasma* would belong to the genus *Acholeplasma,* since we did not detect any cholesterol in the membrane lipids. Contrary to *Acholeplasma* cell such as *Acholeplasma laidlawii* (Tully, 1973; Huang *et al.,* 1974), *Thermoplasma* grows on a medium containing no fatty acids.

References

Aalbers, J., and Bieber, L. (1968), *Anal. Biochem. 24,* 443.

Appelqvist, L. (1972), *J. Lipid Res. 13,* 146.

Bartlett, G. (1959), *J. Biol. Chem. 234,* 466.

Belly, R. T., Bohlool, B. B., and Brock, T. D. (1973), *Ann. N.Y. Acad. Sci. 225,* 94.

Belly, R. T., and Brock, T. D. (1972), *J. Gen. Microbiol. 73,* 465.

Blakesly, R. (1974), Thesis, Biochemistry Department, Michigan State University, East Lansing, Mich.

Bowyer, D., Leat, W., Howard, A., and Gresham, G. (1963), *Biochim. Biophys. Acta 70,* 423.

Burton, K. (1956), *Biochem. J. 62,* 315.

Darland, G., Brock, T. D., Samsonoff, W., and Conti, S. (1970), *Science 170,* 1416.

Davis, B. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Dittmer, J., and Wells, M. (1969), *Methods Enzymol. 14,* 482.

Dubois, M., Gilles, K., Hamilton, J., Rebers, P., and Smith, F. (1956), *Anal. Chem. 28,* 350.

Freundt, S. (1972), *in* Pathogenic Mycoplasma, New York, N.Y., Associate Science Publishing Co., Ciba Foundation, pp 9–11.

Hsung, J. C., and Haug, A. (1974), *Fed. Proc. Fed. Amer. Soc. Exp. Biol. 33,* 274.

Huang, L., Jaquet, D. D., and Haug, A. (1974), *Can. J. Biochem. 52,* 483.

Hyun, S., Vanhouny, G., Treadwell, C. (1965), *Anal. Biochem. 10,* 193.

Kates, M., Yengoyan, L., and Sastry, P. (1965), *Biochim. Biophys. Acta 98,* 252.

Kellenberger, E., Ryter, A., and Sechaud, J. (1958), *J. Biophys. Biochem. Cytol. 4,* 671.

Klenk, E., and Friedrichs, E. (1952), *Z. Physiol. Chem. 290,* 169.

Langworthy, T., Smith, P. F., and Mayberry, W. (1972), *J. Bacteriol. 112,* 1193.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951), *J. Biol. Chem. 193,* 265.

Malik, N., and Berrie, A. (1972), *Anal. Biochem. 49,* 173.

Mayberry-Carson, K. T., Langworthy, T. A., Mayberry,

W. R., and Smith, P. F. (1974), *Biochim. Biophys. Acta 360,* 217.
Nelson, G. (1968), *Lipids 3,* 267.
Rapport, M., and Alonzo, N. (1955), *J. Biol. Chem. 217,* 199.
Rietsema, R. (1954), *Anal. Chem. 26,* 960.
Rouser, G., Kritchevsky, G., and Yamamoto, A. (1967), *in* Lipid Chromatographic Analysis, Vol. 1, Marinetti, G., Ed., New York, N.Y., Marcel Dekker, pp 113–166.
Saito, K., and Sato, K. (1966), *J. Biochem. 59,* 619.
Scheraga, H. (1963), *Proteins 1,* 477.
Singleton, R., and Amelunxen, R. (1973), *Bacteriol. Rev. 37,* 320.
Skidmore, W., and Entenman, C. (1962), *J. Lipid Res. 3,* 471.
Smith, G. G., Ruwart, M. J., and Haug, A. (1974), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 45,* 96.
Smith, P. F. (1971), *in* The Biology of Mycoplasmas, New York, N.Y., Academic Press.
Smith, P. F., Langworthy, T., Mayberry, W., and Hougland, A. (1973), *J. Bacteriol.* 116, 1019.
Spurr, A. (1969), *J. Ultrastruct. Res. 26,* 31.
Tully, J. G. (1973), *Ann. N.Y. Acad. Sci. 225,* 74.
Venable, J., and Coggeshall, R. (1965), *J. Cell. Biol. 25,* 407.
Wells, M., and Dittmer, J. (1965), *Biochemistry 4,* 2459.