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Effect of Growth Temperature on the Lipid Composition of *Thermus aquaticus*

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The complex lipids of *Thermus aquaticus* include phospholipids, glucolipids, carotenoids, and vitamin K_2 isoprenologues. The phospholipids account for 30% of the total lipids and have been identified as phosphatidylethanolamine (4%), phosphatidylglycerol (3%), phosphatidylinositol (10%), cardiolipin (3%), and phosphatidic acid (1%). The major phospholipid contained three fatty acids, a long-chain unsaturated amine, and one glycerol per phosphate and accounted for 80% of the lipid phosphate. The carotenoids accounted for 60% of the membrane lipid. The majority of the carotenoids were very polar. Mono- and diglucosyldiglyceride and the 35-, 40-, and 45-carbon vitamin K_2 isoprenologues were also identified. All these lipids were localized in the membrane of *T. aquaticus*. When the growth temperature was increased from 50 to 75 C and measured at 5 C intervals, there was a progressive increase in the total lipid content. The phospholipids increased 2-fold, the carotenoids increased 1.8-fold, and the glucolipids increased 4-fold between cells grown at 50 C and 75 C. The vitamin K_2 level did not change. The proportions of the individual lipids within each lipid class remained constant as the temperature of growth was raised. Metabolic studies indicated turnover of the diacyl phospholipids during pulse-chase experiments at rates comparable with mesophilic bacteria. The major phospholipid and the carotenoids did not turn over.

Attempts to explain thermophily have generally been centered around proteins and protein-synthesizing systems (10). Brock (5) proposed that the molecular mechanism of thermophily is more closely associated with the inherent thermostability of the cellular membrane than to the specific macromolecules. This theory is supported by the work of Bodman and Welker (4) concerning the stability of *Bacillus stearothermophilus* protoplasts when exposed to temperatures up to 65 C, and our work (*unpublished data*) concerning the stability of *Thermus aquaticus* spheroplasts to increased temperatures (90 C). In considering the stability of a membrane, one can either investigate the membrane as a whole or study individual components, like proteins or lipids. In this study, the lipids of the membrane of *T. aquaticus* were examined.

Most of the investigations on the lipids of thermophilic bacteria have been concerned with the fatty acid composition. It was shown by Shen et al. (20) that thermophilic bacilli increase the proportions of branched-chain fatty acids and decrease the proportions of monoenoic and heptanoic fatty acids as the temperature of growth is raised. Our work (19) on the fatty acids of *T. aquaticus* has shown a similar sequence of changes as the growth temperature is increased from 50 to 75 C. The phospholipids of one thermophile, *B. stearothermophilus*, have been identified by Card et al. (8) as cardiolipin (23 to 42%), phosphatidylglycerol (22 to 39%), and phosphatidylethanolamine (21 to 32%). They also showed that, in cells grown at 60 C, the cellular lipid content was 8% of the dry weight and that 40% of this was neutral lipid.

In this study, increasing the temperature of growth increased the total lipid content of *T. aquaticus* with corresponding increases in the phospholipids, glycolipids, and carotenoids and only minor changes in the proportions of the individual lipid components.

MATERIALS AND METHODS

T. aquaticus was grown in the basal salts medium described by Brock and Freeze (6) containing 0.2% (w/v) yeast extract (Difco) and 0.2% (w/v) tryptone (BBL). The *p*H was adjusted to 7.6 with 12 N KOH. Cultures were grown in 1-liter flasks containing 400 ml of medium or in Parrot flasks containing 1,500 ml of medium in a Constant Temperature Water Bath Shaker (Fermentation Design, Allentown, Pa.) at the

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temperatures indicated (±1 C) and shaken at 250 rev/min. Growth was measured as the absorbancy at 700 nm with a Beckman DBG spectrophotometer. Dry weights were determined as previously described (24) and were linear with respect to optical density (Fig. 1).

Extraction of the lipids. The total lipids of *T. aquaticus* were extracted by the method of Bligh and Dyer (3) as described previously (19), except that the final two-phase system was made 0.5 M with respect to KCl, to eliminate emulsion formation. Emulsions will occur if more than 0.5 g (dry weight) of cells is extracted in a 1-liter final volume. This extraction removed 92% of the fatty acids in the cells. The total fatty acids were determined by saponification of the cells, extraction, and assay by gas-liquid chromatography (19). No further lipid phosphate or lipid glucose could be extracted by modifying the extraction procedure (19).

Fractionation of the total lipids. The total lipid extract of this organism was highly pigmented, and, without removal of the neutral lipids, the phospholipids could not be resolved by paper or thin-layer chromatography. Two methods were used to separate the phospholipids from the carotenoids: (i) silicic acid column chromatography (Unisil Activated Silicic Acid, Clarkson Chemical Co., Williamsport, Pa.) and (ii) acetone precipitation of the phospholipids. Chromatography of the total lipid extract on silicic acid was used for metabolic studies. In this procedure, less than 15 μmoles of lipid phosphate was added to a 1-g silicic acid column (1 by 15 cm), and the lipids were eluted as described in Table 1. The second method involved precipitation of the phospholipids dissolved in methanol-toluene (1:1) by the addition of 20 volumes of cold acetone followed by centrifugation. The phospholipid pellet was resuspended in methanol-toluene and treated in the same manner three times. After acetone precipitation three times, the acetone supernatant fluid contained more than 90% of the carotenoids, and the pellet contained 86% of the lipid phosphate or ^{32}P.

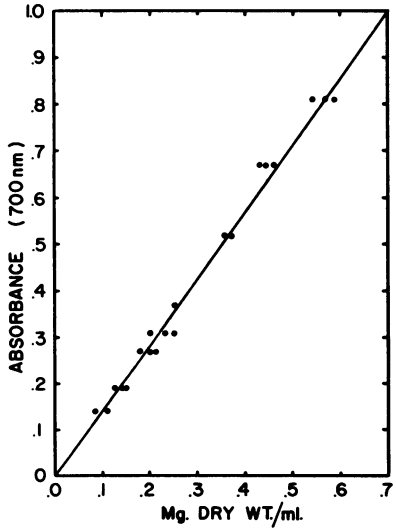

FIG. 1. *Relationship between the absorbance at 700 nm and the dry weight of Thermus aquaticus. Portions of a growing culture were withdrawn, and the absorbancy at 700 nm was measured between 0.2 and 0.6 in a DBG spectrophotometer. The sample was centrifuged, washed, placed in preweighed glass vials, and dried under vacuum at 40 C to a constant weight.*

Paper chromatography of the phospholipids. The phospholipids of *T. aquaticus* were separated on silica gel-impregnated paper (Whatman SG-81) with the following solvents: (A) chloroform-methanol-6.7 N ammonium hydroxide (33:18:2:2.5, v/v) (25), (B) chloroform-methanol-diisobutylketone-acetic acid-water (12:5:23:13.2, v/v) (26), (C) diisobutylketone-acetic acid-water (40:25:5, v/v) (15), and (D) chloroform-methanol-diisobutylketone-pyridine-0.5 M ammonium chloride, *p*H 10.4 (15:9:13:18:3, v/v) (26). For metabolic studies, a two-dimensional system was employed with solvent A in the first dimension and solvent B in the second dimension. A radioautogram of lipids containing ^{32}P and ^{14}C is illustrated in Fig. 2.

Mild alkaline methanolysis. Diacyl phospholipids were deacylated by mild alkaline methanolysis at 0 C (23). The KOH was neutralized with the cation-exchange resin, Biorex 70 (Bio-Rad Corp., Richmond, Calif.; see reference 4). The water-soluble deacylated phospholipids were separated on Chromograms (Eastman 6064 plates, Eastman Organic Chemicals, Rochester, N.Y.) by using a solvent of 3.8 mM ethylenediaminetetraacetic acid-0.7 M ammonium bicarbonate in 90 mM ammonium hydroxide containing 67% (v/v) ethyl alcohol in the first dimension and isobutyric acid-water-14 M ammonium hydroxide (66:33:1) in the second dimension (21). The water-soluble products of mild alkaline methanolysis were also separated on aminocellulose paper (Whatman AE-81), washed with 3 M formic acid, with the solvent systems described by Short and White (21). Column chromatography on a Dowex-1 column, with a gradient of ammonium formate-sodium borate (25), was used to identify the deacylated products. The following abbreviations are used to represent the glycerol phosphate esters resulting from deacylation of the phospholipids: α-glycerol phosphate (GP), derived from phosphatidic acid (PA); glycerolphosphorylethanolamine (GPE), derived from phosphatidylethanolamine (PE); glycerolphosphorylglycerol (GPG), derived from phosphatidylgycerol (PG); glycerolphosphorylinositol (GPI), derived from phosphatidylinositol (PI); and diglycerolphosphorylglycerol (GPGPG), derived from cardiolipin (CL). The diglyceroldiglyceride (DG) and monoglucosyldiglyceride (MG) yielded glyceroldiglucoside (GDG) and glycerolmonoglucoside (GMG) after mild alkaline methanolysis.

Carotenoids. After precipitation of the phospholipids with acetone, the carotenoids of *T. aquaticus* were saponified in methanol containing 5% KOH and 10% water for 2 hr; they were then isolated by adding 0.2 volume of saturated NaCl and extracting the mixture three times with an equal volume of benzene-petroleum ether (1:1) to remove the hydrocarbon carotenoids (13). The more polar carotenoids were removed by adding chloroform and water to the saline-methanol mixture to make the final proportions of chloroform-

methanol-water, 1:1:0.9. The carotenoids were separated on alumina-impregnated paper (SS-288, Schleicher & Schuell, Keene, N.H.) with solvents of methanol-hexane (1:9, v/v) and acetone-hexane (1:1, v/v). The carotenoids were recovered from the paper with methanol-toluene (1:1, v/v), and the absorption spectrum was determined with a Cary 14CM spectrophotometer with hexane or methanol as solvents.

Isolation and identification of vitamin K_2 isoprenologues. Vitamin K_2 isoprenologues were isolated from the neutral lipids by thin-layer chromatography on Silica Gel G plates (Brinkman Instruments, Inc., Westbury, N.Y.) with the solvent system of chloroform isooctane (2:1; see reference 12). The quinone fraction (R_F 0.65) was separated from the phospholipids and polar carotenoids (origin to R_F 0.3) and the nonpolar carotenoids (solvent front) by using ascending chromatography. The absorbancy of the total quinone fraction was determined by using a Cary 15 spectrophotometer at 248 nm ($E = 19.28 \times 10^3$ in isooctane; see reference 12). Individual isoprenologues were separated and co-chromatographed with the isoprenologues isolated from *Staphylococcus aureus* U-71, as described by Hammond and White (12), on hexadecane-impregnated Keiselguhr G plates with a solvent system of acetone-water (95:5) saturated with hexadecane. The quinones were localized by spraying first with 1% $NaBH_4$ in 50% ethyl alcohol and then with 0.2% aqueous neotetrazolium (12).

Turnover of the phospholipids. Cells were grown to an optical density of 0.14, pulsed for 2.3 generations with 750 μCi of $H_3{}^{32}PO_4$ in 400 ml of medium filtered on a 142-mm membrane filter (0.4 μm pore size; Millipore Corp.), washed with 200 ml of nonradioactive medium at the growth temperature, and suspended in 1,500 ml of nonradioactive medium at 70 C. This procedure took 20 min; 250-ml samples were then taken, centrifuged at 0 C, and extracted. After extraction, each sample was fractionated on a silicic acid (1 g) column with the solvents given in Table 1.

Localization of the lipids. Spheroplasts of *T. aquaticus* were induced by the addition of 1 mg of lysozyme per ml directly to the growth medium and incubation at 37 C for 2 hr. After spheroplast formation, the cells were centrifuged, washed twice and with 50 mM phosphate buffer (*p*H 7.6), and subjected to cycles of freezing and thawing. Freezing was done in an acetone-dry ice bath, and thawing was done at 40 C with gentle swirling. After freezing and thawing, the absorbancy was measured and the membrane fragments were centrifuged at 40,000 $\times$ *g* for 30 min.

Chemical assays. Phosphate was determined by the method of Bartlett after digestion with 23% $HClO_4$ for 1 hr at 200 C as adapted for an autoanalyzer (2). Glucose was determined by the method of Radin et al. (17), acyl esters were determined by the method of Rapport and Alonzo (18) and glycerol was determined by the method described by Dittmer and Wells (10). The trimethylsilyl derivatives of glucose were made as described by Sweeley (9) and chromatographed on an SE-30 column by using an F & M gas chromatograph as previously described (19). Deoxyribonucleic acid was measured by the method of Burton (7), with 2-deoxyribose used as the standard.

RESULTS

Identification of the phospholipids. The total lipids of *T. aquaticus* contained 60 to 70% neutral lipid. Resolution of the phospholipid proved impossible without removal of the neutral lipid. For quantitative recovery of the phospholipids, the neutral lipids were removed by chromatography on silicic acid columns (Table 1). An autoradiogram made after chromatography of the phospholipid fraction from the column isolated from cells grown with $H_3{}^{32}PO_4$ and sodium acetate-*1*-^{14}C shows six phospholipids and two glycolipids (Fig. 2). To obtain sufficient quantities of each lipid for identification, the neutral lipids were separated after acetone precipitation. Each phospholipid fraction was separated from the others by chromatography on silica gel-impregnated paper by using the solvents described above. When isolated, each of the six components was homogeneous in each of the solvent systems; each gave the expected reactions with ninhydrin, periodate, and diphenylamine; and each, when deacylated by mild alkaline methanolysis, contained a single glycerol phosphate ester by chromatography on amino-cellulose paper and Dowex-1 column chromatography (25).

Compound 1. Compound 1 (a phospholipid) co-chromatographed with authentic PA. It had a glycerol-phosphate-fatty acid molar ratio of 0.7:1.0:2.0; it was ninhydrin negative and periodate negative. Upon alkaline methanolysis, the resultant water-soluble compound co-chromatographed on the two-dimensional AE-81 system with authentic α-GP and was periodate positive.

Compound 2. Compound 2 (a lipid) was identified as PG and accounted for 2 to 5% of the total lipid phosphate. It had a glycerol-phosphate-fatty acid molar ratio of 2.0:1.1:2.0 and was ninhydrin negative and periodate positive. The deacylated lipid co-chromatographed with authentic GPG from *S. aureus* on both a Dowex-1 column (25) and upon two-dimensional chromatography with Eastman Cellulose Chromograms (21). After hydrolysis with 2 N HCl for 1 hr at 100 C and chromatography on AE-81 paper, two periodate-positive products were obtained which had the chromatographic mobility of α-GP and free glycerol.

Compound 3. Compound 3 has been identified as PI and accounted for 8 to 12% of the total lipid phosphate. It was ninhydrin negative and periodate positive, and reacted with diphenylamine after prolonged heating. The molar ratio of fatty acid to phosphate was 1.8:1.0. The deacylated product co-chromatographed with authentic GPI from yeast on both paper and

TABLE 1. *Fractionation of Thermus aquaticus lipids on a silicic acid column*[a]

Fraction No.	Solvent	Lipid fractionated	Lipid				
			^{32}P (%)	Lipid P[b] (%)	Carbohydrate[c] (%)	Carotenoids[d] (%)	Vitamin K_2[e] (%)
1	$CHCl_3$ (12 ml)	Caratenoids K vitamins	0	0	0	14	100
2	$CHCl_3$ (6 ml) Acetone (6 ml)	Carotenoids	1.2	0	0	79	
3	Acetone (6 ml)	Glycolipid	0	1	5	2	
4	$CHCl_3$ (12 ml): MeOH (49:1)		0	3	2		
5	$CHCl_3$ (12 ml): MeOH (2:1)	P-lipids, glycolipids, carotenoids	97	92	89	4.5	
6	MeOH (12 ml)		1.8	2	0		

[a] The total lipid extract, containing less than 15 μmoles of lipid phosphate, was added to a 1-g column of Unisil silicic acid (1 by 15 cm) that was poured in chloroform. The lipids were then eluted in six fractions for which the recovery of lipid ^{32}P, lipid phosphate, lipid glucose, cartenoid, and vitamin K_2 was determined.

[b] Determined by the method of Bartlett (2).

[c] Determined by the method of Radin et al. (17) with glucose used as the standard.

[d] Measured by absorbancy at 460 nm in methanol-toluene (1:1).

[e] Estimated from the absorbancy at 248 nm in isooctane.

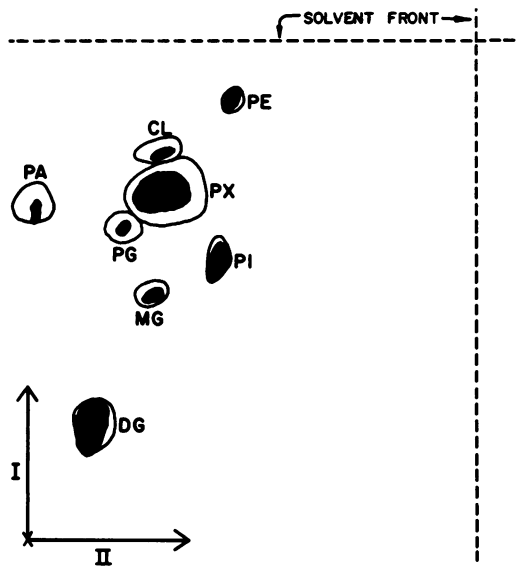

FIG. 2. *Autoradiogram of the lipids of Thermus aquaticus. The lipids were extracted from cells grown in $H_3{}^{32}PO_4$ and ^{14}C-acetate, and the carotenoids were removed by silicic acid column chromatography. The phospholipids and glucolipids were quantiatively eluted in the fifth fraction (see Table 1) and separated by two-dimensional ascending chromatography with SG-81 paper. The first dimension utilized a solvent of chloroform-methanol-6.7 N NH_4OH (33:18:2:2.5, v/v) and the second dimension used a solvent of chloroform-methanol-diisobutylketone-acetic acid-water (12:5:23:13:2, v/v). PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; PE, phosphatidylethanolamine; CL, cardiolipin; PX, the major phospholipid.*

column chromatography. After strong acid hydrolysis (6 N for 48 hr at 100 C) and removal of the acid by repeated evaporation, the products were chromatographed on Whatman no. 1 paper with the solvent system propanol-ethanol-water (5:3:2, v/v). Ascending chromatography revealed two compounds which co-chromatographed with authentic glycerol (R_F = 0.71) and inositol (R_F = 0.19).

Compound 4. Compound 4 (a phospholipid) comprised 75 to 80% of the total lipid phosphate and has not yet been identified. It had a glycerol-fatty acid-phosphate molar ratio of 1.1:2.8:1.0, and was periodate positive and ninhydrin negative. Elemental analysis (Galbraith Laboratories, Knoxville, Tenn.) showed 60.8% carbon, 10.3% hydrogen, 2.0% nitrogen, and 2.5% phosphorous. If the lipid contained 1 mole of phosphate the minimum molecular weight was 1,800, however, evidence suggests more than 1 mole of phosphate. Upon hydrolysis with 2 N methanolic HCl, a long-chain amine was released. Mass spectral analysis suggests that this long chain amine is an unsaturated undecylamine containing an hydroxyl group (*personal communication*, R. K. Hammond, Michigan State University, Lansing, Mich.). Upon alkaline methanolysis, the lipid releases two fatty acid methyl esters; it becomes partially water soluble, but upon chromatography it still behaves as a lipid.

Compound 5. Compound 5 was identified as CL. It had a glycerol-phosphate-fatty acid molar ratio of 1.4:1.0:2.1, and the deacylated product co-chromatographed with authentic GPGPG in two dimensions with the AE-81 paper

system and on the Dowex-1 column.

Compound 6. Compound 6 was identified as PE. It was the only phospholipid which reacted with ninhydrin. It had a glycerol-phosphate-fatty acid molar ratio of 0.9:1.0:1.9, and the deacylated product co-chromatographed with authentic GPE on both the column and the two-dimensional system shown in Fig. 3. Figure 3 illustrates an Eastman Cellulose Chromogram of the water-soluble products of all the lipids after mild alkaline methanolysis. The radioactivity in the upper right-hand corner of Fig. 3 represents the deacylated major phospholipid (compound 4) which becomes partially water soluble but behaves as a lipid.

Identification of the glycolipids. Two glycolipids, DG and MG, were isolated by using silica gel-impregnated paper and solvent C. In this solvent system, the glycolipids remained at the origin and the glycolipids were then resolved chromatographically on SG-81 paper with a solvent of chloroform-methanol-6.7 N ammonium hydroxide (33:18.2:2.5, v/v). Upon hydrolysis with 2 N H_2SO_4 for 2 hr at 100 C and neutralization with Ba $(OH)_2$, the free sugars were chromatographed on Whatman no. 1 paper with the following solvents: isopropanol-acetic acid-water (3:1:1,v/v) and *n*-butanol-acetic acid-water (4:1:5, v/v). Only one sugar could be detected with alkaline $AgNO_3$ reagent (22) with the R_F value of glucose. After hydrolysis with 2 N HCl, the sugar was trimethylsilylated (9), and the trimethylsil derivative was co-chromatographed with pure trimethylsil glucose by gas-liquid chromatography with an SE-30 column. Measurement of the glucose-fatty acid-glycerol molar ratios showed 2.0:1.8:1.0 for the major lipid and 1.0: 2.0:1.0 for the minor glucolipid. After deacylation, GDG and GMG co-chromatographed with these two deacylated glucolipids isolated from *S. aureus* (21).

Identification of the carotenoids. The absorption spectra of the total carotenoids are shown in Fig. 4. Two major components were isolated in the hydrocarbon fraction of the carotenoids. One had the chromatographic mobility (R_F = 0.92) and absorption spectrum (maxima at 277, 285, and 300 nm in hexane) of phytoene. The other had the chromatographic mobility (R_F = 0.73) and absorption spectrum (maxima at 428, 460, and 492 nm in hexane) of Δ-carotene. Minor components were detected at R_F values of 0.23 and 0.05 after chromatography on alumina-im-

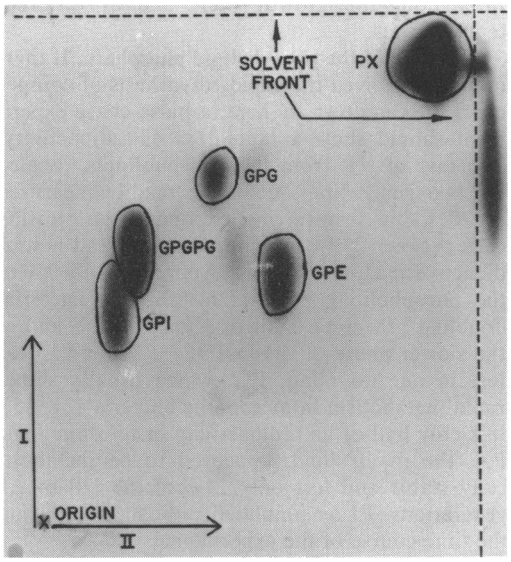

FIG. 3. *Autoradiogram of the deacylated phospholipids of Thermus aquaticus labeled with* $H_3{}^{32}PO_4$. *After separation of the neutral lipid by column chromatography, the phospholipid fraction was deacylated as described and chromatographed two dimensionally on Eastman Cellulose Chromograms with a solvent of 3.8 mM ethylenediaminetetraacetic acid-0.7 M ammonium bicarbonate in 90 mM ammonium containing 67% (v/v) ethyl alcohol in the first dimension and isobutyric acid-water-14 M ammonium hydroxide (66:33:1) in the second dimension (21).*

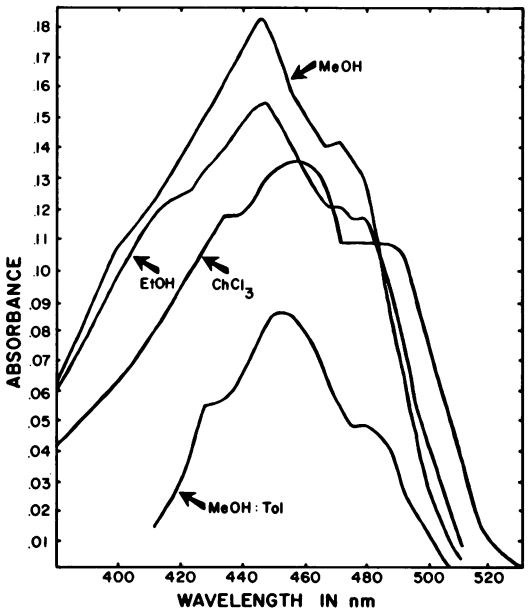

FIG. 4. *Absorption spectra of Thermus aquaticus carotenoids in methanol, ethanol, chloroform, and methanol-toluene (1:1). After extraction of the lipids, the phospholipids were removed by acetone precipitation, and the supernatant fluid containing the carotenoids was dried under nitrogen and suspended in the above solvents. The spectra were measured by use of a Cary 14 spectrophotometer.*

pregnated paper with a solvent of hexane-methanol (9:1, v/v). The polar carotenoids could be separated into two major fractions. One had an R_F value of 0.2 and absorption maxima at 472, 452, and 436 nm in methanol. The other appeared with an R_F value of 0.35 and had absorption maxima at 466, 446, and 426 nm in methanol. The polar carotenoid with absorption maxima of 446 nm represented half the total carotenoid and was present at twice the concentration of the other major polar carotenoid. The polar carotenoids were separated on alumina-impregnated paper with a solvent of acetone-hexane (1:1, v/v). These polar carotenoids were very difficult to recover quantitatively from the alumina-impregnated paper. The polar carotenoid fraction contained less than 1% carbohydrate as determined by the anthrone reaction and are now being identified by mass spectral analysis.

Identification of the vitamin K_2 isoprenologues. The total vitamin K_2 isoprenologues were separated from the lipids by thin-layer chromatography (12). The isoprenologues were identified by their chromatographic mobility compared with those from *S. aureus* (Fig. 5). The 35-, 40-, and 45-carbon isoprenologues comprise 90% of the total vitamin K_2 of *T. aquaticus*.

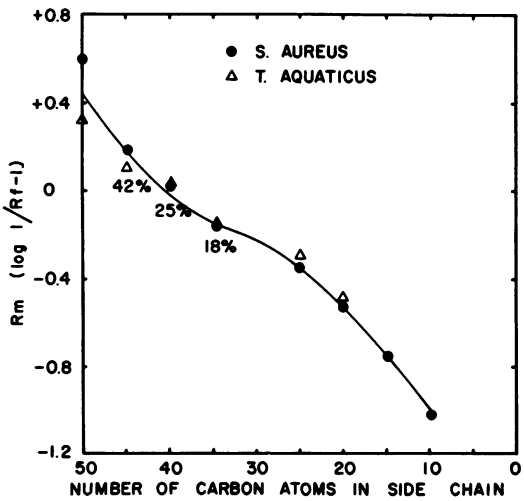

FIG. 5. *R_m values of vitamin K_2 isoprenologues from Staphylococcus aureus and Thermus aquaticus. Total vitamin K_2 was isolated from both S. aureus and T. aquaticus by thin-layer chromatography on Silica Gel G plates with chloroform-isooctane (2:1). The individual isoprenologues were then separated on hexadecane-impregnated Kieselguhr plates by using acetone-water (95:5) saturated with hexadecane. The R_m values were then obtained from the R_F values by using the method of Hammond and White (12).*

Effect of growth temperature on lipid content. The lipid increased from 10 to 26% of the dry weight of the cells as the temperature of growth increased from 50 to 75 C (Table 2). Between 50 and 75 C, the total lipids increased 2.6-fold, the carotenoids increased 1.8-fold, the phospholipids increased 2-fold, and the glucolipids increased 4-fold. The total vitamin K_2 isoprenologues did not change. The proportions of the phospholipids in exponentially growing cells (harvested at 0.35 mg of dry weight per ml) did not change dramatically (Fig. 6), even though the total phospholipid content increased 2-fold. PX increased from 74 to 80%, PI increased from 8 to 12%, PE fell from 8 to 6%, and PG fell from 5 to 3% of the total phospholipid. The CL remained between 2 and 3% and the PA was always less than 1%. There was essentially no change in the phospholipid composition between exponentially growing cells and cells harvested in the stationary growth phase (0.89 mg dry weight per ml) at each temperature. The glucolipids DG and MG maintained a ratio of 2:1 throughout the growth cycle and at different temperatures. The carotenoids maintained approximate proportions of 8% phytoene, 7% Δ-carotene, 50% polar carotenoid 446 nm, and 25% polar carotenoid 452 nm throughout the growth cycle and at different temperatures.

Turnover of the phospholipid phosphate. If thermophily involved the rapid resynthesis of components broken down by heat, a pulse-chase experiment should show a rapid loss of radioactivity. The loss of ^{32}P from the phospholipids, labeled for two generations, could be readily measured at 70 C by transferring exponentially growing cells between ^{32}P-containing medium and nonradioactive medium (Fig. 7). Approximately 7% of the phospholipid ^{32}P was lost in one bacterial doubling (78 min). Half of ^{32}P in the PE and in the slower phase of the loss of ^{32}P from CL was lost in one doubling. PG, which usually shows rapid metabolism in mesophilic bacteria (21, 25), lost only half of its radioactivity in 1.7 doublings. PX, the major lipid, appeared to be metabolically stable and lost only 14% of the ^{32}P in 2.5 generations. PI accumulated radioactivity during the time course of the experiment.

Incorporation experiments indicated that the ^{32}P pool from which PA was synthesized was nearly saturated in about 40 min, which suggested that, if rapid turnover of PX was obscured by resynthesis from ^{32}P containing breakdown products, the rapid synthesis would be in a very restricted compartment of the cells.

Localization of the lipids. Repeated freezing and thawing of lysozyme spheroplasts of *T.*

TABLE 2. *Effect of growth temperature on the total lipids of Thermus aquaticus*[a]

Temp (C)	Doubling time[b] (min)	Lipid[c] (%)	Carotenoid content[d]	Lipid P[e] (μmoles)	Carbohydrate[f] (μmoles)	Vitamin K_2[g] (μmoles)
50	285	10	260	19	4.7	1.9
55	185	10	268	24.0	6.7	1.7
60	150	13.4	200	28	8.1	2.0
65	100	15	294	36	9.6	1.7
70	60	18	407	40	16.4	1.9
75	60	26	415	37	16.4	1.8

[a] Cells were grown to mid-log phase, centrifuged, and washed; the lipids were extracted as described.
[b] Measured at 700 nm.
[c] Measured gravimetrically after extraction and drying under a stream of nitrogen at 40 C; average of three samples.
[d] Measured as the absorbancy of the total carotenoid extract at 460 nm in methanol-toluene (1:1).
[e] Measured by the method of Barlett (2).
[f] Measured by the method of Radin et al. (17) with glucose used as the standard.
[g] Measured at 248 nm in isooctane after chromatography as described previously (12).

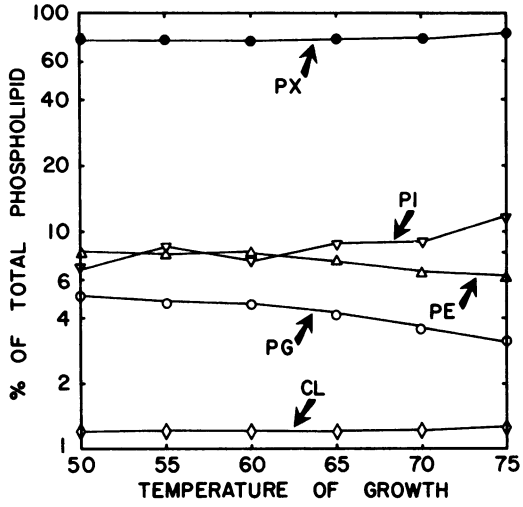

FIG. 6 *Effect of growth temperature on the phospholipid composition of Thermus aquaticus. The cells were grown for at least seven generations in medium containing* $H_3{}^{32}PO_4$ *at the temperatures indicated and harvested in log phase. After extraction of the lipids, the carotenoids were removed by silicic acid column chromatography, and the phospholipids were separated by two-dimensional chromatography as shown in Fig. 2. The individual phospholipids were located by autoradiography and the percentage was calculated from the total radioactivity.*

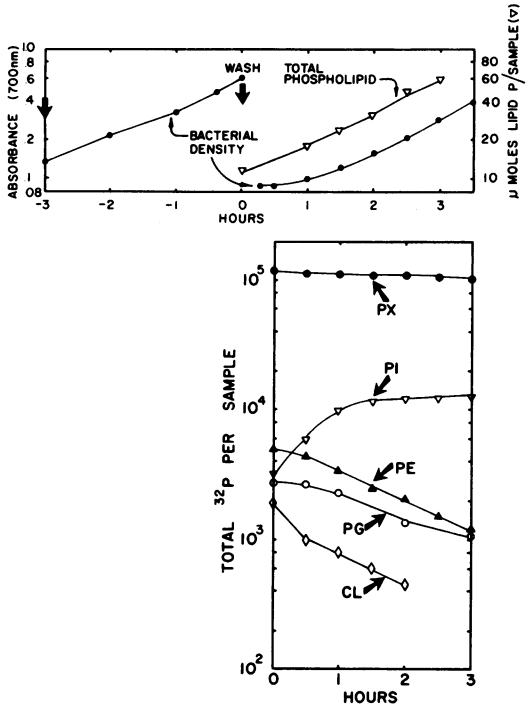

FIG. 7. *Turnover of the phospholipids of Thermus aquaticus. T. aquaticus was grown at 70 C to an absorbancy at 700 nn of 0.14 and pulsed with* $H_3{}^{32}PO_4$ *(750 μCi/400 ml) for two generations. At this time the cells were filtered, washed with prewarmed nonradioactive medium, and suspended in 1,500 ml of nonradioactive medium at 70 C. At the times indicated, 250-ml samples were withdrawn, centrifuged, and washed; the lipids were then extracted. Each lipid sample was chromatographed on a silicic acid column to remove the carotenoids. The phospholipid fraction was separated by two-dimensional chromatography on SG-81 paper (as in Fig. 1). Phospholipids were located by autoradiography, and the radioactivity was determined.*

aquaticus yielded a membrane fraction, sedimented at 40,000 × *g*, that was enriched in carotenoids (Fig. 8). Cell lysis was indicated by the 80% loss in absorbancy at 700 nm and 86% loss of the 260-nm absorbing material which was recovered in the supernatant fluid. After seven cycles of freezing and thawing, the membrane pellet was examined and found to contain 85% of the total lipid phosphate, 92% of the lipid glu-

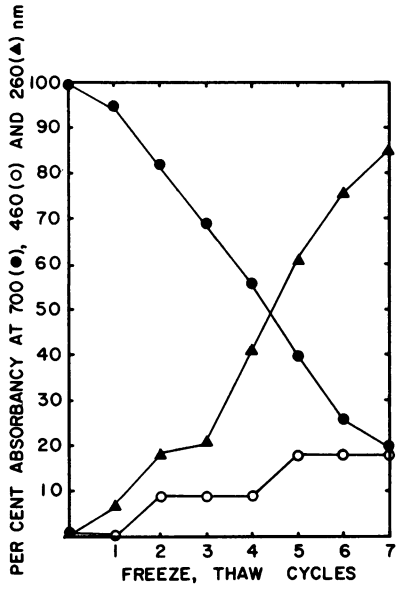

FIG. 8. *Localization of the lipids of Thermus aquaticus. Spheroplasts of T. aquaticus were subjected to cycles of freezing and thawing. Freezing was done at −20 C and thawing at 40 C. After each cycle, samples were withdrawn, the absorbancy at 700 nm (lysis of the spheroplasts) was determined, and the cells were centrifuged at 40,000 × g for 30 min.*

cose, 81% of the carotenoids, and less than 10% of total deoxyribonucleic acid (as measured chemically; see reference 7).

DISCUSSION

The temperature at which cells are grown has been shown to affect the lipid composition in various organisms, such as the fatty acids in *E. coli* and *T. aquaticus* (16, 19) and the total lipids of *Candida* (15), and has been shown to regulate carotenoid synthesis in *S. aureus* (14). If the lipids of *T. aquaticus* play a role in the thermostability of the membrane, growing the cells at various growth temperatures should change the composition or metabolism of the cellular lipids. It was hoped that by increasing or decreasing the growth temperature, there would be a preferential synthesis of some lipid component which would aid in elucidating the control mechanisms responsible for thermophily.

The results (Table 2 and Fig. 6) illustrate the effect of the growth temperature on the total lipids and on the specific phospholipids of *T. aquaticus*. The fact that the total cellular lipids increased from 50 to 75 C suggested that the lipids play a role in the molecular mechanism of thermophily. This was also suggested by the progressive increase in the individual lipid classes as the temperature was increased from 50 to 75 C at 5 C intervals.

All of the phospholipids of *T. aquaticus* have been identified except for the major complex lipid, PX. The phospholipids have been identified as PE, PG, CL, PI, and PA. The major phospholipid, PX, accounted for 80% of the total phospholipid at all growth temperatures. PX is a large molecule containing three fatty acids, a glycerol, a long-chain unsaturated amine per micromole of lipid phosphate, with a minimum molecular weight of 1,800. Although the amount of phospholipid per cell increased at the various growth temperatures, the proportion of each lipid remained constant (Fig. 6). *T. aquaticus* contained an unusually high level of carotenoids which appear to have unique structures. Even so, the proportions of the carotenoids did not change as the temperature of growth was elevated. The most striking change induced by growth at higher temperatures was the four-fold increase in glucolipid. Again, there was no change in the proportions of MG and DG with this increase. An increase in the amount of glycolipids with increased temperature of growth has been detected in the thermophilic algae, *Cyanidium caldarium* (1).

The glycolipids were not separated in this study. The amount of vitamin K_2 per cell remained constant as the temperature growth was increased, suggesting that the electron transport system of this obligate aerobe was not modified by growth at high temperatures. The cell seemed to respond to thermal stress by increasing the total lipid 2.6-fold, with a shift from monoenoic and antiiso heptadecanoic to more thermostable iso-branched and saturated fatty acids in the glucolipids and phospholipids (19). Shifting cells grown at 50 to 75 C resulted in a shift in the growth rate, after an 80-min lag, to one slower than in cells grown at 70 C and a lipid composition that increased only slightly over the 50 C level. Shifting the cells from 75 to 50 C resulted in a 12-hr lag and much cell lysis.

This extreme thermophile showed nothing unusual in the metabolism of the diacyl phospholipids. These phospholipids lost ^{32}P in pulse-chase experiments at rates comparable to mesophilic bacteria. The carotenoids appeared metabolically stable, as in mesophilic bacteria (14). PX, the major phospholipid, lost no ^{32}P in pulse-chase experiments, suggesting great metabolic stability. It proved very difficult to label the glucolipids. There was no evidence that very rapid metabolism of the lipids accounted for the thermal stability of the membrane. The response of the lipids to thermal stress suggests an indirect role for the lipids, because the response of the

cells was an increase of the total without changes in proportions of the lipids in each lipid class. Perhaps the glucolipids increase the thermal stability of the membranes.

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