Drickamer, L. K. (1976) *J. Biol. Chem. 251*, 5115-5122.
Dzandu, J. K., & Johnson, R. M. (1980) *J. Biol. Chem. 255*, 6382-6386.
Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606-2617.
Furthmayr, H. (1978) *J. Supramol. Struct. 9*, 79-95.
Gratzer, W. B. (1981) *Biochem. J. 198*, 1-8.
Hardy, B., Bensch, K. G., & Schrier, S. L. (1979) *J. Cell Biol. 82*, 654-663.
Hosey, M. M., & Tao, M. (1976) *Biochemistry 15*, 1561-1568.
Hosey, M. M., & Tao, M. (1977) *J. Supramol. Struct. 6*, 61-75.
Laemmli, U. K. (1970) *Nature (London) 227*, 680-685.
Mueller, T. J., & Morrison, M. (1974) *J. Biol. Chem. 249*, 7568-7573.
Owens, J. W., Mueller, T. J., & Morrison, M. (1980) *Arch. Biochem. Biophys. 204*, 247-254.
Plut, D. A., Hosey, M. M., & Tao, M. (1978) *Eur. J. Biochem. 82*, 333-337.
Roses, A. D. (1976) *J. Supramol. Struct. 4*, 481-486.
Shapiro, D. L., & Marchesi, V. T. (1977) *J. Biol. Chem. 252*, 508-517.
Sheetz, M. P., & Singer, S. J. (1977) *J. Cell Biol. 73*, 638-646.
Sheetz, M. P., & Sawyer, D. (1978) *J. Supramol. Struct. 8*, 399-412.
Siegel, D. L., Goodman, S. R., & Branton, D. (1980) *Biochim. Biophys. Acta 598*, 517-527.
Silverberg, M., Furthmayr, H., & Marchesi, V. T. (1976) *Biochemistry 15*, 1448-1454.
Steck, T. L. (1972a) *J. Mol. Biol. 66*, 295-305.
Steck, T. L. (1972b) in *Membrane Research* (Fox, C. F., Ed.) pp 71-93, Academic Press, New York.
Steck, T. L., & Yu, J. (1973) *J. Supramol. Struct. 1*, 220-232.
Tanner, M. J. A. (1978) *Curr. Top. Membr. Transp. 11*, 279-325.
Ungewickell, E., & Gratzer, W. (1978) *Eur. J. Biochem. 88*, 379-385.
Yu, J., Fischman, D. A., & Steck, T. L. (1973) *J. Supramol. Struct. 1*, 233-248.

Phospholipid Molecular Species Alterations in Microsomal Membranes as an Initial Key Step during Cellular Acclimation to Low Temperature†

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ABSTRACT: When *Tetrahymena pyriformis* cells were chilled from 39 to 15 °C, fatty acids of the microsomal membrane phospholipids increased significantly in unsaturation over a 15-h acclimation period. During the initial hour following chilling, only a small fraction of the increase in unsaturation had taken place, yet the fluidity of the lipids, as measured earlier in fluorescence depolarization studies [Dickens, B. F., & Thompson, G. A., Jr. (1981) *Biochim. Biophys. Acta 644*, 211], had already increased almost to the level found in cells fully acclimated to 15 °C. The microsomal lipids from 39 and 15 °C grown cells and cells shifted from 39 to 15 °C for 1 h were analyzed in more detail in an effort to discover what compositional changes might be responsible for the rapid increase in fluidity. The three major phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), and 2-(aminoethyl)phosphonolipid (AEPL), were purified and enzymatically hydrolyzed to diglycerides, which were then converted to *tert*-butyldimethylsilyl (*t*-BDMS) ethers. Coupled gas chromatography–mass spectrometry of the *t*-BDMS derivatives permitted identification of the lipid molecular species and estimation of their relative concentrations under the different temperature conditions. Each phospholipid responded to chilling in a distinctively different way, although there were a few similarities. Thus, PE and PC were alike in showing a marked decrease in combinations of odd chain with even chain fatty acids at low temperature (there were no odd chain fatty acids in AEPL). Likewise, the extent of acyl chain unsaturation increased in combinations of monoalkyl monoacyl species of both PC and AEPL at 15 °C (PE had no alkyl ether side chains). The variety and extent of the changes were in general much greater in PE and PC than in AEPL. The molecular species patterns, particularly in cells exposed to 15 °C for only 1 h, showed very clearly that the extent of fatty acyl group rearrangement by deacylation–reacylation overshadowed acyl chain desaturation as a means of altering lipid structure. In light of these findings, selective phospholipid deacylation–reacylation is indicated as a mechanism which may be of pivotal importance in achieving rapid homeoviscous adaptation.

The protozoan *Tetrahymena pyriformis* has been profitably exploited for studying the mechanism of cellular acclimation to low environmental temperatures (Martin et al., 1976; Fukushima et al., 1977; Dickens & Thompson, 1981). It is widely (Thompson, 1980) but not unanimously (Lands, 1980) believed that a vital response to chilling in *Tetrahymena* and many other organisms is the alteration of membrane lipid composition so as to overcome the rigidifying effect that low temperature invariably has on membrane lipids.

In recent studies (Dickens et al., 1980; Dickens & Thompson, 1980, 1981), our laboratory has begun a detailed correlation of lipid compositional and physical changes in a key *Tetrahymena* membrane system—the endoplasmic reticulum (microsomes). Within as little as 1 h after chilling from 39 to 15 °C (Dickens & Thompson, 1981), we were able to detect (1) a small but significant increase in the unsaturation

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of microsomal phospholipid fatty acids and (2) a dramatic concurrent shift of the microsomal physical properties, as reported by fluorescent probes, so that they became nearly identical with those of microsomes fully acclimated to 15 °C. That such a small modification in phospholipid fatty acid composition could lead to a major change in the membrane's physical state was unexpected. In the present paper, we have examined this relationship in more detail and discovered several hitherto unsuspected changes in lipid composition that are not revealed by the usual lipid analytical procedures.

Experimental Procedures

Materials. Dipalmitoyl-, distearoyl-, and dioleoylphosphatidylcholines were purchased from Serdary (London, Ontario) and dilinoleoyl- and dilinolenoylphosphatidylcholines from Avanti Polar-Lipids Inc. (Birmingham, AL). The purity of these lipids was confirmed by thin-layer and gas–liquid chromatography, and they were used without further purification. β-Amyrin was purchased from Sigma (St. Louis, MO) and its purity confirmed by gas–liquid chromatography. Phospholipase C was in some cases *Bacillus cereus* grade II from Boehringer Mannheim (Indianapolis, IN) and in other cases a phospholipase C, also from *B. cereus*, kindly provided by Dr. Kunihiko Saito, Kansai Medical University, Osaka, Japan. *tert*-Butyldimethylchlorosilane–imidazole reagent was purchased from Applied Science (State College, PA) in sealed 1-mL ampules and was opened just before use. [^{14}C]Palmitate was purchased from New England Nuclear (Boston, MA).

Culture Conditions. Tetrahymena pyriformis, strain NT-1, was cultured in the medium previously described by Fukushima et al. (1976). The cells were cultured isothermally at 39 and 15 °C or for 30 or 60 min at 15 °C following a rapid chilling (5 min) to 15 °C from 39 °C as previously described (Dickens & Thompson, 1981), except that the cultures were routinely harvested at a slightly higher cell density [(2.0–2.5) × 10^5 cells/mL].

Lipid Isolation. Microsomes were isolated by using the fractionation procedure of Nozawa & Thompson (1971) except that the length of the 100000*g* centrifugation was increased to 90 min. Microsomal lipids were extracted by the method of Bligh & Dyer (1959). The phospholipid composition was determined by the method of Rouser et al. (1970). Total lipid phosphorus was estimated by the method of Bartlett (1959) as modified by Marinetti (1962). The tetrahymanol content was determined by gas–liquid chromatography with β-amyrin as an internal standard as described earlier (Thompson et al., 1971).

Phospholipid Separation. The isolated microsomal lipids were separated into two major phospholipid classes on silica gel H thin-layer plates. The solvent system $CHCl_3$–HOAc–CH_3OH–H_2O (65:30:5:2 v/v/v/v) gave excellent separation of *Tetrahymena* phospholipids without overlapping of major or minor phospholipid bands. The phospholipid bands were visualized and marked following a very brief exposure to iodine vapor. Control experiments demonstrated that fatty acid peroxidation, as indicated by a decrease in polyunsaturated fatty acid content, did not occur if the phospholipids were scraped from the plates within 2 h of removing the plates from the chromatographic chamber. Routinely, the major phospholipid bands were scraped off the plates and the phospholipids extracted from the silica gel within 30 min after chromatography. Phosphatidylethanolamine (PE) and 2-(aminoethyl)phosphonolipid (AEPL) were extracted with aliquots of $CHCl_3$–MeOH (1:1 v/v) while phosphatidylcholine (PC) had to be extracted with the more polar solvent $CHCl_3$–MeOH–H_2O (1:2:0.4 v/v/v). After the three major phospholipids were scraped from the chromatographic plates, the remainder of the plate was charred to assure that the bands had not run together and that each band had been quantitatively removed. The fatty acyl compositions of the individual phospholipids were determined by gas chromatography as previously described (Fukushima et al., 1976). The percentage of alkyl ether side chains was determined by gas chromatography of *t*-BDMS derivatives as described below.

Molecular Species Determination. Diacylglycerols released from isolated *Tetrahymena* phospholipid classes and synthetic phospholipid standards by phospholipase C treatment were converted to *tert*-butyldimethylsilyl derivatives to analyze the phospholipid molecular species. The removal of the phospholipid head group by phospholipase C was carried out at 37 °C in peroxide-free ethyl ether (1.0 mL) with 0.25 mL of 0.08 M borate buffer, pH 8.0, containing 0.1 mM $ZnCl_2$. Using lipids prelabeled biosynthetically with [^{14}C]palmitic acid, we found the phospholipase C from Boehringer Mannheim to be very active against PE and AEPL. However, only approximately 65% of the PC was converted to the corresponding glyceride by this enzyme.[1] The phospholipase C provided by Dr. Saito, on the other hand, completely converted all *Tetrahymena* phosphoglycerides to diacylglycerides. The Boehringer Mannheim enzyme appears to be inactive on phosphatidylcholines which have an alkyl side chain at the α position. Therefore, we routinely used the phospholipase C provided by Dr. Saito on *Tetrahymena* PC. For the preparation of *t*-BDMS derivatives, we routinely incubated 0.8–3.5 μmol of phospholipid with sufficient phospholipase C to catalyze the total conversion to glycerides within 90 min. A 90–98% conversion of phospholipids to glycerides was routinely obtained with no alteration in the fatty acid composition of the isolated glycerides from that of the phospholipids prior to treatment with phospholipase C.

Following enzymatic treatment, the ether was removed under N_2, and the lipids were reextracted by microapplication of the procedure of Bligh & Dyer (1959). The polar hydroxyl group was then blocked for chromatography by reacting the glycerides with the *t*-BDMS reagent in sealed ampules under N_2 at 160 °C for 60 min. Analysis indicated that this treatment did not alter the fatty acid composition of the lipid samples. The derivatives were then extracted into $CHCl_3$ and used directly or after chromatographic purification by thin-layer chromatography. The *t*-BDMS derivatives were analyzed by gas–liquid chromatography and gas chromatography–mass spectrometry (GC–MS). The gas–liquid chromatography was performed with a Varian Model 3700 chromatograph with a 3% OV-1 packed column (diameter 0.25 in., length 6 ft) at 300 °C, using a flame ionization detector. The GC–MS was carried out by using a Finnigan Model 4000 instrument equipped with a dedicated Data General computer for data accumulation and manipulation. For GC–MS, samples were separated on a packed SP 2100 column at 300 °C and admitted to the mass spectrometer operating at an ionization voltage of 70 eV. Selected ion retrieval was used to identify and quantify the major phospholipid molecular species as described under Results.

Results

Overall Composition of the Three Major Phospholipid Classes. We have confirmed that the relative proportions of the major microsomal phospholipid classes from 39 and 15 °C grown cells differ (Table I). However, no hint of this mod-

[1] Subsequent experiments have revealed that this enzyme will completely convert this PC in dilute phosphate buffers.

Table I: Phospholipid Composition of Microsomal Membranes[a]

phospholipid	growth conditions: 39 °C	39 → 15 °C (1 h)	15 °C
2-(aminoethyl)phosphonate	10.8 ± 1.2	9.8 ± 0.4	26.5 ± 2.1
phosphatidylethanolamine	49.6 ± 2.1	51.0 ± 4.6	39.6 ± 1.9
phosphatidylcholine	30.1 ± 1.9	30.0 ± 2.7	25.3 ± 1.8
others[b]	9.5 ± 1.0	9.2 ± 0.9	8.6 ± 0.6
tetrahymanol/phospholipid	0.062[c]	0.070[c]	0.065[c]

[a] Values expressed as mole percent. [b] Others include cardiolipin, lysophospholipids, and two ceramide-containing lipids. [c] Mole ratio.

ification was apparent in microsomes isolated only 1 h following a 39 to 15 °C shift. Furthermore, the relative proportions of the unique sterol-like triterpenoid tetrahymanol remained nearly constant and extremely low (Table I).

The constancy of the above-mentioned parameters for 1 h following a shift to 15 °C suggested that the rapid modification of microsomal physical properties must be due to changes in the phospholipid fatty acid composition, as previously concluded (Dickens et al., 1980, Dickens & Thompson, 1981). The extent of fatty acid change in each of the three major phospholipid classes was therefore carefully assessed (Table II). The primary differences between 39 and 15 °C microsomal phospholipids were a lower amount of odd chain fatty acids, which were especially prominent in phosphatidylethanolamine, and of 16-carbon fatty acids, and a higher amount of 18-carbon fatty acids, particularly 18:3. In cells shifted to 15 °C for 1 h, microsomal phospholipids sustained a slight reduction in 14:0 and 16:0 along with an increase in one or more 18-carbon fatty acids in each phospholipid class. By far the largest change was an increase in the alkyl ether content of PC from 25% to 31%.

Acyl and Alkyl Chain Pairing on the Basis of Chain Length. We analyzed the possibility of intra- and intermolecular fatty acid rearrangements during rapid temperature acclimation. The procedure involved the hydrolysis of each purified phospholipid class by phospholipase C followed by conversion of the resulting diglycerides to their *tert*-butyldimethylsilyl (*t*-BDMS) ethers. These derivatives were then separated and quantified by combined gas chromatography–mass spectrometry (GC–MS).

Figure 1 illustrates typical gas chromatographic elution patterns of *t*-BDMS derivatives prepared by using each microsomal phospholipid class from 39 and 15 °C grown cells and cells chilled from 39 to 15 °C for 1 h. Each peak was identified by its carbon number (C_n = combined number of carbon atoms in the two attached fatty acids), by its mass spectrum, and by its retention time relative to those of authentic diglyceride *t*-BDMS standards.

Several changes in relative peak size were immediately obvious by visual examination of elution profiles. These are quantified in Table III. Most noteworthy in the phosphatidylethanolamine (PE) fraction was the change from a relatively high percentage of phospholipid molecules containing one odd and one even chain fatty acid, e.g., C_{29}, C_{31}, and C_{33}, in 39 °C grown cells to a significantly lower percentage in cells grown at 15 °C. In 1-h-shifted cells, the major alterations in PE were a large decrease in the C_{33} and C_{34} peaks coupled with increases in the C_{30}, C_{31}, and C_{36} peaks.

The pattern of the 2-(aminoethyl)phosphonolipid (AEPL) was much simpler than that of PE and was altered less by changing environmental temperature. The most notable characteristic of this fraction was its very high content of alkyl

Table II: Effects of a Rapid Temperature Shift on the Phospholipid Fatty Acid Composition of Microsomal Membranes[a]

fatty acid	2-(aminoethyl)phosphonate: 39 °C	39 → 15 °C (0.5 h)	39 → 15 °C (1 h)	15 °C	phosphatidylethanolamine: 39 °C	39 → 15 °C (0.5 h)	39 → 15 °C (1 h)	15 °C	phosphatidylcholine: 39 °C	39 → 15 °C (0.5 h)	39 → 15 °C (1 h)	15 °C
12:0				0.7 ± 0.2	1.5 ± 0.2	2.2 ± 0.5	0.9 ± 0.5	1.8 ± 0.5	0.4 ± 0.1			0.7 ± 0.3
14:0	1.7 ± 0.4	4.1 ± 0.3	3.8 ± 0.2	5.3 ± 0.4	19.9 ± 0.3	20.9 ± 0.7	17.8 ± 1.5	16.8 ± 1.2	10.9 ± 0.4	9.5 ± 0.2	8.7 ± 0.6	10.7 ± 0.4
iso 15:0	1.2 ± 0.2	1.5 ± 0.1	1.6 ± 0.2	1.4 ± 1.0	6.4 ± 0.1	7.2 ± 0.3	6.0 ± 0.2	1.8 ± 1.2	4.0 ± 0.2	2.8 ± 0.2	3.1 ± 0.5	2.0 ± 0.3
15:0	1.4 ± 0.1	1.1 ± 0.1	0.9 ± 0.1		3.4 ± 0.1	3.6 ± 0.2	3.6 ± 0.1	1.1 ± 0.6	2.1 ± 0.1	0.8 ± 0.2	2.0 ± 0.2	0.6 ± 0.2
16:0	9.1 ± 0.4	6.4 ± 0.6	6.1 ± 0.4	5.2 ± 0.5	14.4 ± 0.1	12.7 ± 0.2	12.5 ± 0.4	10.9 ± 0.5	11.1 ± 0.2	10.2 ± 0.2	9.7 ± 0.6	9.6 ± 0.5
16:1	9.6 ± 0.6	8.0 ± 0.5	7.5 ± 0.5	7.9 ± 0.3	20.5 ± 0.1	22.2 ± 0.2	22.4 ± 0.6	19.5 ± 0.4	13.2 ± 0.3	14.7 ± 0.1	13.8 ± 0.5	12.3 ± 0.3
16:2[b]	3.6 ± 0.2	3.5 ± 0.2	3.3 ± 0.4	2.7 ± 0.2	7.8 ± 0.3	7.8 ± 0.3	7.9 ± 0.1	6.1 ± 0.9	7.0 ± 0.2	7.4 ± 0.2	6.9 ± 0.4	4.2 ± 0.3
e16:0 + e16:1[c]	42.3	43.0	44.0	44.3					25.7	26.3	31.1	21.5
18:0	1.2 ± 0.4	1.0 ± 0.3	0.8 ± 0.1	0.4 ± 0.1	2.5 ± 0.1	2.3 ± 0.1	2.9 ± 0.2	0.8 ± 0.2	1.8 ± 0.4	2.5 ± 0.0	2.0 ± 0.6	1.3 ± 0.5
18:1	3.9 ± 0.8	3.5 ± 0.2	3.5 ± 0.3	2.7 ± 0.4	4.5 ± 0.4	3.6 ± 0.5	4.3 ± 0.6	8.0 ± 0.8	3.6 ± 0.1	3.6 ± 0.2	2.8 ± 0.3	5.7 ± 0.4
18:2($\Delta^{6,11}$)	2.7 ± 0.3	4.4 ± 0.1	3.7 ± 0.2	7.3 ± 0.9	0.3 ± 0.1	0.2 ± 0.2		1.8 ± 0.3	1.9 ± 0.4	1.7 ± 0.2	1.8 ± 0.3	2.8 ± 0.3
18:2($\Delta^{9,12}$)	6.5 ± 0.1	6.0 ± 0.2	5.4 ± 0.4	5.6 ± 0.8	8.1 ± 0.2	7.7 ± 0.9	9.3 ± 1.0	14.2 ± 0.6	6.0 ± 0.3	6.7 ± 0.1	5.1 ± 0.7	9.0 ± 0.4
18:3	16.9 ± 0.7	17.5 ± 0.3	19.5 ± 0.4	16.1 ± 0.6	9.4 ± 0.2	9.5 ± 0.7	12.4 ± 0.1	17.6 ± 1.6	12.3 ± 0.0	13.9 ± 0.2	13.0 ± 0.6	19.6 ± 0.6

[a] Values are normalized to mole percent. [b] The 16:2 peak coelutes with a 17-carbon peak. The values presented for 16:2 contain an undetermined contribution from this odd chain fatty acid. e indicates an ether-linked hexadecyl group at the *sn*-1 position of the phospholipid. This was quantified by GC of *t*-BDMS derivatives.

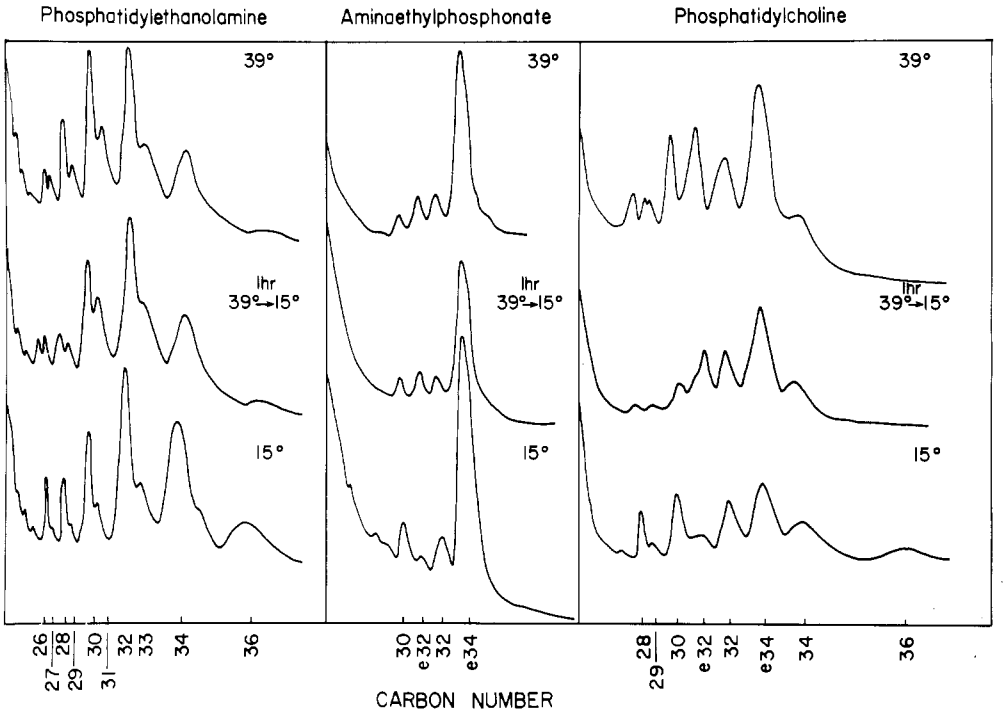

FIGURE 1: Gas chromatographic elution pattern of microsomal *t*-BDMS derivatives. The prefix e appearing before some carbon numbers indicates the presence of an alkyl ether side chain replacing one acyl group.

Table III: Distribution of Carbon Numbers in Microsomal Membrane Phospholipids[a]

carbon no.	2-(aminoethyl)phosphonolipid			phosphatidylethanolamine			phosphatidylcholine		
	39 °C	39 → 15 °C (1 h)	15 °C	39 °C	39 → 15 °C (1 h)	15 °C	39 °C	39 → 15 °C (1 h)	15 °C
26				1.1 ± 0.1	1.7 ± 0.5	4.0 ± 0.9			
27				1.5 ± 0.3	1.2 ± 0.4	tr			
28	0.2 ± 0.6	0.9 ± 0.6	0.8 ± 0.4	5.3 ± 0.6	3.7 ± 0.4	5.8 ± 1.1	1.5 ± 0.2	0.7 ± 0.1	3.7 ± 0.8
29				4.1 ± 0.6	3.4 ± 1.4	tr	2.3 ± 0.1	0.3 ± 0.1	1.0 ± 0.4
30	3.2 ± 0.6	3.1 ± 1.1	2.5 ± 0.4	15.2 ± 3.8	19.9 ± 0.6	12.2 ± 1.0	7.0 ± 0.3	4.8 ± 0.1	6.6 ± 0.6
31				11.1 ± 0.6	16.3 ± 0.1	4.0 ± 1.2	15.0 ± 0.3[c]	18.2 ± 0.8[c]	9.6 ± 1.1[c]
e32[b]	9.2 ± 1.0	9.1 ± 0.3	4.2 ± 1.3						
32	10.4 ± 1.3	8.3 ± 3.9	7.3 ± 0.4	25.3 ± 1.9	26.5 ± 2.5	31.8 ± 1.6	18.4 ± 0.3	18.1 ± 0.8	17.2 ± 0.8
33				12.7 ± 0.4	7.3 ± 1.3	9.4 ± 1.1	*d*	*d*	*d*
e34[b]	75.4 ± 4.0	78.9 ± 4.1	84.3 ± 0.9				42.2 ± 1.0	48.4 ± 1.5	34.3 ± 2.3
34	*d*	*d*	*d*	20.6 ± 2.8	11.5 ± 1.4	26.0 ± 3.8	13.5 ± 1.3	9.6 ± 0.4	23.0 ± 2.9
36				3.2 ± 2.9	7.7 ± 3.3	7.4 ± 1.3	tr	tr	3.0 ± 1.6

[a] Values expressed as mole percent. tr means <1%; a blank means not detected. [b] Small e signifies the presence of an alkyl ether side chain at the *sn*-1 position. [c] This peak contains sizable proportions of both C_{31} and C_{e32}. [d] This combination was detected by selective ion retrieval but was masked under a much larger alkyl ether peak with a similar retention time. The actual content of each of these peaks was approximately 2–4% of the total lipids. They are included in the percentage of the adjacent ether peaks. Additionally, trace amounts of e33 are present in both AEPL and PC.

ether side chains. The ether-linked hydrocarbon moieties consisted almost entirely of hexadecyl (16:0) chains, with a trace of 16:1 also being present. Alkyl ether containing derivatives, e.g., C_{e34} (the designation of C_{en} indicates an ether-linked hexadecyl group at the *sn*-1 position of the phospholipid), were incompletely separated from diacyl species having one less carbon atom but could be easily distinguished from them by mass spectrometry (see below). Cells grown at 15 °C had appreciably more C_{e34} than did 39 °C grown cells, but there were no significant changes in cells transferred to 15 °C for 1 h.

Phosphatidylcholine (PC), like AEPL, contained sizable amounts of monoalkyl, monoacyl molecular species (C_{e32} and C_{e34}). The C_{30} and C_{e34} peaks decreased at 15 °C, and the C_{36} peak increased. These changes were already quite evident by 1 h following the 39 to 15 °C temperature shift. It is noteworthy that in PE and PC, the two phospholipid classes affected most by chilling, the percentage change in the distribution of combined side-chain pairs (Table III) was much greater than the changes in overall fatty acid and alkyl ether composition (Table II).

Analyses were also made of microsomal membranes isolated only 30 min after the shift to 15 °C (data not shown). In all cases, there was clear evidence for changes similar to but not so pronounced as those noted at 1 h. Since very little net lipid synthesis occurs in the newly shifted cells (Martin et al., 1976), it was necessary to conclude from the gas chromatographic data that retailoring of existing phospholipids by deacylation–reacylation reactions plays an important role in temperature acclimation.

Molecular Species Analysis by Gas Chromatography–Mass Spectrometry. Further information regarding the nature and extent of these changes was obtained by mass spectrometry. Unlike the trimethylsilyl derivatives more commonly used for GC–MS in the past, the *t*-BDMS derivatives yield a prominent ion indicating the mass of the intact diglyceride (Myher et al.,

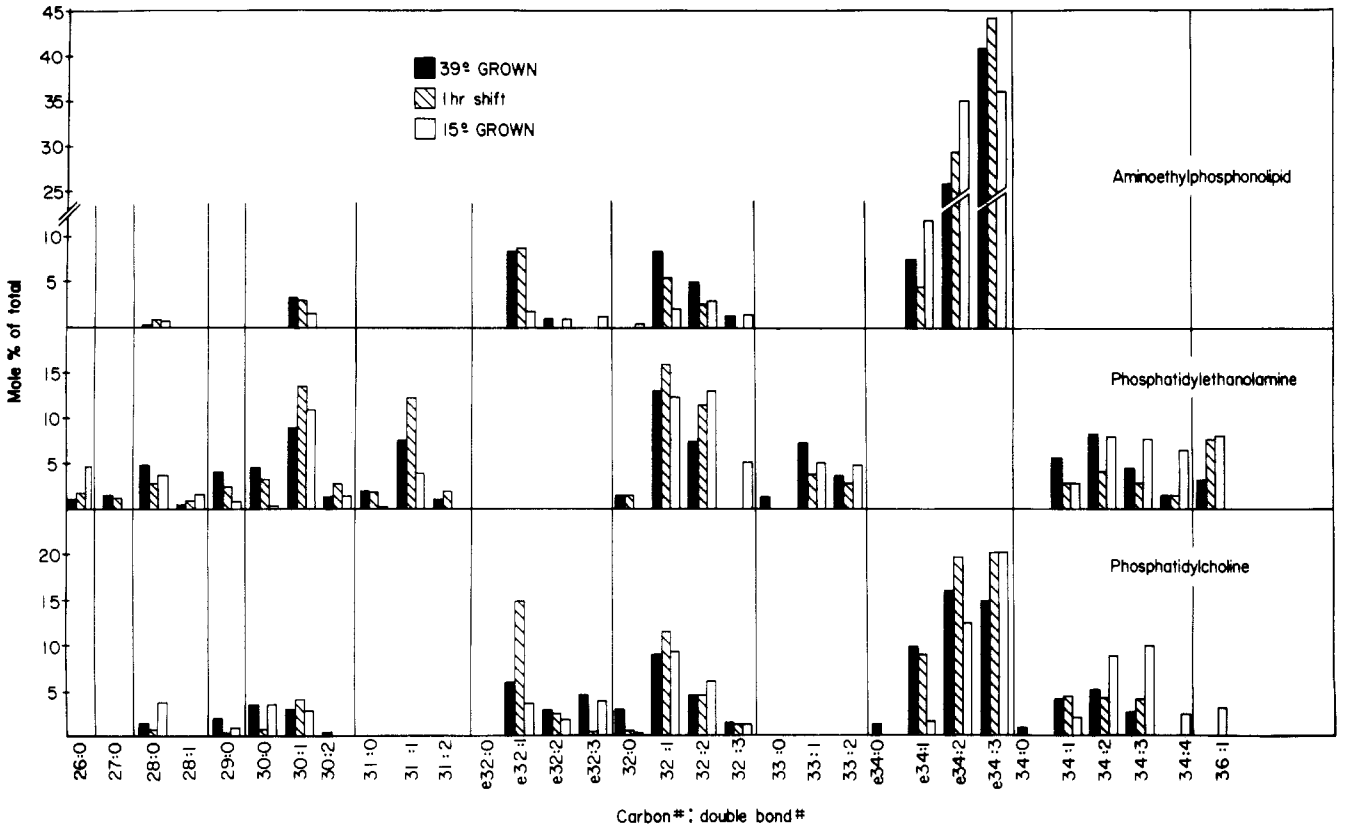

FIGURE 2: Relative abundance of M − 57 ions in *t*-BDMS derivatives of microsomal phospholipids from *Tetrahymena* grown at 39 or 15 °C or shifted from 39 to 15 °C for 1 h.

1978). The identity of M − 57 (mass of the derivative minus the *tert*-butyl group) ions characteristic of each gas chromatographic peak can be determined by using the selected ion retrieval mode of the mass spectrometer. In this way, it is feasible to identify in each chromatographic peak those diglyceride molecular species having 0, 1, 2, 3, or more double bonds, since each double bond reduces the M − 57 ion by 2 mass units. Within a given chromatographic peak, selected ion retrieval reveals a family of partially resolved M − 57 ions, with the more unsaturated components having shorter retention times.

The potential of this technique for quantitative analysis was demonstrated when we recovered two equally large M − 57 values following analysis of equimolar mixtures of synthetic dioleoyl- and distearoylphosphatidylcholine. However, diglycerides containing 18:2 or 18:3 fatty acids yielded less than their true values when analyzed in mixtures, due to the increased tendency for polyunsaturated fatty acids to undergo further fragmentation. Providing the mass spectrometer ionization voltage is held constant, we can correct for underestimation of polyunsaturated fatty acids due to inherent lower ion sensitivity by using factors derived from such analyses of known mixtures (Kuksis et al., 1978).

Considering that analysis of the M − 57 ions provides at least a semiquantitative measure of the relative abundance of double bonds within a given chromatographic peak, we compiled the data illustrated in Figure 2. So that values could be obtained for this figure, the chromatographically determined percentage of the total *t*-BDMS derivatives constituted by each component peak (Table III) was further subdivided by an M − 57 ion analysis of that peak. Despite some uncertainty regarding the selective lower ion sensitivity of polyunsaturates (Figure 2 is not corrected for such differences), the values are beneficial for making a relative comparison of changes induced by decreasing environmental temperature.

Each family of molecular species responded to temperature change in its own way. In many but not all cases, there was an increase of unsaturation among molecular species having a common carbon number. But the mechanism for achieving this differed widely. For instance, the increase in the C_{e32} family in PC of cells shifted to 15 °C for 1 h was caused entirely by a rise in $C_{e32:1}$, whereas the similar increase in the C_{e34} family of the same fraction involved an elevation of $C_{e34:2}$ and $C_{e34:3}$ but not $C_{e34:1}$. In these and many other cases, the changes occurring within 1 h cannot be explained by a straightforward desaturation of preexisting molecular species because the relative amount of the entire family having a particular carbon number changes significantly. Instead, there must be a rearrangement of phospholipid fatty acids by deacylation–reacylation.

Information concerning the exact nature of these changes may be derived from the mass spectra. Each fatty acid gives rise to several very characteristic fragments in the mass spectrometer (Myher et al., 1978), and these can be used to confirm its presence in certain partially resolved M − 57 peaks and its absence in others.

A compilation of data regarding the distribution of individual fatty acids is presented in Table IV. The values were reproducible in several independent experiments. Compiling the data in this way allowed several general findings to be confirmed or revealed for the first time. Two odd chain fatty acids were never present together in the same phospholipid molecule. Ether-linked alkyl side chains, predominantly 16:0 but with traces of 16:1, were present almost exclusively in PC and AEPL, although traces of them could be found in PE.

The main purpose of our analysis was to identify more accurately the variety and extent of fatty acid rearrangements triggered by low-temperature stress. In order to reach this

Table IV: Phospholipid Molecular Species Composition of Microsomal Membranes[a]

		2-(aminoethyl)phosphonolipid			phosphatidylethanolamine			phosphatidylcholine		
			39 → 15 °C			39 → 15 °C			39 → 15 °C	
carbon no.	molecular species[e]	39 °C	(1 h)	15 °C	39 °C	(1 h)	15 °C	39 °C	(1 h)	15 °C
26	14:0/12:0				1.1	1.7	4.0			
27	13:0/14:0				0.7	0.6	tr			
	15:0/12:0				0.7	0.6	tr			
28	14:0/14:0		tr	tr	2.0	1.0	0.4	0.5	0.5	3.4
	16:0/12:0	tr	0.6	0.5	3.1	1.7	3.5	1.0	tr	tr
	16:1/12:0				tr	1.0	2.0			tr
29	15:0/14:0				2.2	1.0	tr	1.5	tr	tr
	13:0/16:0				1.3	1.2	tr	0.7		tr
	13:0/16:1					tr	tr			
	17:T[b]/12:0				0.6	0.7	tr			0.5
30	18:0/12:0					tr	tr			
	14:0/16:0				1.3	1.6	1.7	2.5	1.0	3.4
	18:1/12:0					0.9	1.1			tr
	14:0/16:1	3.0	2.7	1.6	9.8	11.3	7.5	3.9	3.8	2.4
	18:2/12:0				0.7	1.1	1.2			tr
	14:0/16:2				2.6	3.7	0.9	0.5	tr	tr
	18:3/12:0				0.6	0.9	0.7			tr
31	15:0/16:0				tr	1.4	tr			
	15:0/16:1				5.2	7.5	1.0			
	17:T[b]/14:0				4.9	6.7	2.9			
	15:0/16:2				tr	1.5	tr			
e32[c]	e16:0/16:0	0.9	0.9					1.0	1.6	
	e16:0/16:1	6.2	6.3	3.8				10.0	12.2	7.7
	e16:0/16:2	1.1	0.6	0.7				1.8	2.0	2.1
	e16:1/16:T[d]							2.3	2.4	tr
32	14:0/18:0						0.6	0.7		0.6
	14:0/18:1	0.8	0.6	1.3	4.5	3.9	3.8	4.0	3.1	3.5
	14:0/18:2	1.2	0.8	2.2	7.7	6.0	8.8	5.0	3.6	4.5
	14:0/18:3			1.2	4.1	4.3	6.0	2.3	2.6	2.1
	16:T[d]/16:T[d]	6.1	5.2	2.4	8.8	12.4	12.7	6.3	8.9	6.4
33	17:T[b]/16:0				2.7	1.9	1.7			
	15:0/18:1				3.1	1.1	0.9			
	17:T[b]/16:1				1.5	1.4	2.9			
	15:0/18:2				3.8	1.6	2.1			
	17:T[b]/16:2						tr			
	15:0/18:3				1.5	1.1	1.4			
e34[c]	e16:0/18:1	3.9	2.6	4.6				9.1	4.9	1.6
	e16:0/18:2	14.7	16.3	20.6				13.5	14.7	8.8
	e16:0/18:3	47.5	49.5	48.5				11.7	21.3	19.9
	e16:1/18:T[d]	10.6	11.7	10.2				8.1	7.6	3.9
34	16:0/18:T[d]				12.2	6.7	11.1	10.5	6.6	11.0
	16:1/18:T[d]				7.8	4.8	12.8	2.6	2.9	10.5
	16:2/18:T[d]				0.5	tr	2.2	tr	tr	1.5
36	18:T[d]/18:T[d]				3.2	7.7	7.4	tr	tr	3.0

[a] Values expressed as mole percent. [b] 17:T represents a combination of 17:0 and 17:1 fatty acids. [c] The small e signifies the presence of an alkyl ether linkage at the *sn*-1 position. [d] 16:T and 18:T represent combinations of 16:0, 16:1, and 16:2 or 18:0, 18:1, 18:2, and 18:3 fatty acids, respectively. [e] The fatty acid listed first is that tentatively assigned to the *sn*-1 position. See text for details.

goal by using mass spectrometry, we had to overcome two serious technical problems regarding quantification. Our methods for dealing with the more important of these difficulties are discussed below.

It was mentioned earlier that the values reported by GC–MS for polyunsaturated fatty acids are below those predicted by the fatty acid analysis because of their inherent lower specific ion sensitivity. After a careful study of known lipid mixtures and hydrogenated samples, we determined that under our operating conditions the observed values for 18:2 and 18:3 could be corrected by multiplying them by factors of 1.8 and 6.1, respectively. The use of multiplication factors for correction purposes has been reported by other investigators (Kuksis et al., 1978).

The second source of error lay in the tendency of a fatty acid bound at the *sn*-1 position of the derivative's glycerol moiety to be cleaved in the mass spectrometer more readily than that bound at the *sn*-2 position (Myher et al., 1978). In order to correct for this behavior, we made the assumption that the sum of fragment ions for each molecular species was proportional to the amount of that species present. Thus, in the quantification of the components of *t*-BDMS derivative class $C_{32:1}$, the total ions representing 16:0 plus 16:1 were related to the sum of those representing 14:0 plus 18:1.

Employing the corrections described above, we proceeded to estimate the quantitative molecular species composition of the three major phospholipids under the temperature conditions of this study (Table IV). The values in this table were obtained by using GC to quantify the carbon numbers and GC–MS for quantification of combinations of fatty acids within a given C_n peak. The GC–MS technique does not at this stage of development permit an unequivocal assignment of fatty acids to the *sn*-1 or *sn*-2 positions. Fortunately, several careful studies (Pieringer & Conner, 1979; Watanabe et al., 1980, 1981; Kasai et al., 1981) have dealt effectively with the question of fatty acid positional specificity in *Tetrahymena*. We have used the positional assignments reported by these workers, despite the fact that they were made by using whole cell phospholipids, rather than those of microsomal membranes. In some cases, namely, when pairings of two 16-carbon

fatty acids, two 18-carbon fatty acids, or a 16-carbon with an 18-carbon fatty acid occurred, specific pairing assignments could not be reliably made. This is indicated in Table IV.

Although the values in Table IV have been refined by applying the correction factors described above, they remain in substantial agreement with the other, uncorrected data. They clearly reveal marked differences in the molecular species compositions of the three major phosphoglyceride classes. The significance of these differences as they bear on microsomal membrane physical properties will be considered under Discussion.

Discussion

The challenge of this study was to explore how rapid, but seemingly small, changes in the microsomal lipid composition of chilled *Tetrahymena* cells produce within 1 h a virtually complete alteration of fluidity-related physical properties from values characteristic of 39 °C grown cells to the very different values of 15 °C grown cells (Dickens & Thompson, 1981). Analyses of the polar head group distribution and of the ratio of phospholipids to the sterol-like triterpenoid tetrahymanol confirmed that under our precisely controlled conditions of chilling these parameters remained constant. Therefore, we directed our attention toward further characterizing the changes in phospholipid fatty acid composition detected earlier.

Through the use of GC–MS techniques, we have now been able to show that the comparatively small increase in the overall degree of fatty acid unsaturation within 1 h after chilling (Table II) is accompanied by an extensive redistribution of the preexisting as well as the newly modified fatty acids into different molecular species (Figure 2, Table IV). It is obvious from these findings that the relatively slight changes in fatty acid composition detected after 1 h at 15 °C by a conventional fatty acid analysis (Table II) masked a much more significant retailoring of the phospholipid molecular species. The reality of those alterations was confirmed by gas chromatography, which is basically artifact free, and by mass spectrometry, which is less quantitative because of selective losses but provides much more detailed qualitative information concerning lipid composition.

The potential for altering membrane physical properties through intra- and intermolecular fatty acid rearrangement is considerable. A lipid bilayer composed of dipalmitoylphosphatidylcholine and dilauroylphosphatidylcholine in an equimolar mixture has physical properties dramatically different from those of a bilayer of 1-palmitoyl-2-lauroylphosphatidylcholine (De Kruyff et al., 1974; Dickens et al., 1980), belying the simplistic view that fatty acid composition per se determines fluidity. It has been shown that merely reversing fatty acids between the *sn*-1 and the *sn*-2 positions of a mixed-acid phospholipid molecule markedly affects the physical properties of the lipid in bilayers (De Bony & Dennis, 1981; Roberts et al., 1978), and substituting an alkyl ether side chain at the *sn*-1 position of phospholipids can alter the phase transition temperature (Lee & Fitzgerald, 1980; Boggs et al., 1981) as well as the lamellar phase to hexagonal phase transition temperature (Boggs et al., 1981). Alterations of this sort in a natural membrane would be expected to have a similar effect on membrane structure and membrane enzyme activity (Baldassare et al., 1977), with the magnitude being dependent upon the precise nature of the fatty acids involved. It is difficult to predict the quantitative impact of such changes in natural phospholipids, because little definitive work has been done with model unsaturated phospholipids. But there can be no doubt that these previously unrecognized phospholipid molecular species rearrangements must be largely responsible for the physical changes we have observed in microsomal lipids and membranes.

Although it is quite clear that major changes in the positional specificity of certain *Tetrahymena* fatty acids do occur at low temperature, in this study we have assumed the more conventional positional arrangement (Table IV). When accurate determination of reverse isomer ratios becomes possible, an even better base of information will become available for evaluating the causes of fluidity change.

The changes observed in the major microsomal phospholipids were numerous and complex. Only one, AEPL, remained relatively unaltered in all respects. The resistance of AEPL to temperature-induced modification is perhaps less surprising if its consistently sluggish rate of biosynthesis and metabolic turnover (Thompson, 1969) is taken into account. On the other hand, its close structural analogue, PE, was quite different, with regard to both the types of molecular species present and the sensitivity to temperature-induced change (Tables III and IV). Unlike AEPL, PE contained no significant amount of alkyl ether linked side chains. The most notable changes in PE composition following a 1-h exposure to 15 °C included increases in the fatty acid combinations C_{30}, C_{31}, C_{32} and C_{36} (Table III), mainly at the expense of C_{34} (combinations of 16- and 18-carbon fatty acids).

In contrast, PC exhibited none of the low temperature induced changes found in PE after 1 h except for the fall in C_{34}, particularly combinations of 16:0 plus one or another 18-carbon fatty acid. Instead, the alteration within the PC class involved mainly an elevation of selected alkyl ether containing species, notably combinations of 16:0 alkyl groups with 16- and 18-carbon fatty acids, principally 18:3.

It is impossible at this time to interpret the mechanism by which these changes occurred. Apart from the fatty acid desaturation taking place at an enhanced rate in the microsomal membranes, metabolic pathways capable of transforming one class of phospholipids into another are also active in the microsomes (Thompson, 1980). The general similarity between the molecular species composition of AEPL and PC observed in this study is compatible with the previously proposed utilization of the PC diglyceride moiety in the enzymatic synthesis of AEPL (Thompson, 1969).

In addition to the metabolic flux taking place within the microsomes per se, there is a rapid exchange of phospholipids between microsomal membranes and other cellular membranes (Martin & Thompson, 1978; Iida et al., 1978). This exchange undoubtedly serves to deplete the microsomal membranes of some of their newly desaturated molecular species.

In view of the above considerations, it is of course unrealistic to consider the *Tetrahymena* microsomal membranes as a closed system, even for very short time intervals. Accordingly, it would be desirable to obtain a more detailed measure of phospholipid dissemination to other cellular sites. We have in earlier work (Fukushima et al., 1976) identified a number of major lipid differences between *Tetrahymena* microsomal membranes and the surface membrane enclosing the cell's many cilia. In the following papers (Ramesha & Thompson, 1982; Ramesha et al., 1982), we present a thorough characterization of the ciliary membrane lipids and describe the rate at which low temperature induced changes in microsomes make their appearance at the cell surface.

Rationalizing the lipid compositional changes with the observed alterations in physical behavior remains a goal for the future. We still know too little about the properties of simple mixtures of synthetic phospholipids, especially those containing unsaturated fatty acids. The lipid changes observed in 1-h-

shifted cells and even in the more extensively altered membranes of cells fully acclimated to 15 °C are presently inexplicable in physical terms. Microsomal PE from 15 °C grown cells contain much less of almost all the disaturated diglyceride species than 39 °C preparations and increased levels of several diglycerides having two and three double bonds. On the other hand, PC from the same membranes shows an entirely different pattern of change, involving a rather more straightforward rise in certain species having 18:2 and 18:3 in combination with 16-carbon acyl and alkyl side chains.

Our data are all sufficiently reliable to detect trends of change, but some values, e.g., those in Table IV, are relatively crude estimates. There is every reason to believe that improvements of the GC–MS technique will soon eliminate the need for assumptions and correction factors. We have recently achieved much better chromatographic resolution of *t*-BDMS derivatives through the use of OV-101 capillary columns. Preliminary experiments (B. F. Dickens, C. S. Ramesha, and G. A. Thompson, Jr., unpublished results) utilizing natural lipid mixtures reduced catalytically with deuterium gas show promise for overcoming the problem of differential fragmentation sensitivities of saturated and polyunsaturated fatty acids in the mass spectrometer. This will make GC–MS the method of choice for phospholipid molecular species analysis.

Added in Proof

Very recently published data (Maruyama et al., 1982) on the fatty acid positional distribution in *Tetrahymena* microsomal phospholipids usefully complement the findings reported here.

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References

Baldassare, J. J., Benckle, G. M., Hoffman, M., & Silbert, D. F. (1977) *J. Biol. Chem. 252*, 8797.

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

Bligh, E. C., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911.

Boggs, J. M., Stamp, D., Hughes, D. W., & Deber, C. M. (1981) *Biochemistry 20*, 5728.

De Bony, J., & Dennis, E. A. (1981) *Biochemistry 20*, 5256.

De Kruyff, B., van Dijck, P. W. M., Demel, R. A., Schuijff, A., Brants, F., & van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 356*, 1.

Dickens, B. F., & Thompson, G. A., Jr. (1980) *Biochemistry 19*, 5029.

Dickens, B. F., & Thompson, G. A., Jr. (1981) *Biochim. Biophys. Acta 644*, 211.

Dickens, B. F., Martin, C. E., King, G. P., Turner, J. S., & Thompson, G. A., Jr. (1980) *Biochim. Biophys. Acta 598*, 217.

Fukushima, H., Martin, C. E., Iida, H., Kitajima, Y., Thompson, G. A., Jr., & Nozawa, Y. (1976) *Biochim. Biophys. Acta 431*, 165.

Fukushima, H., Nagao, S., Okano, Y., & Nozawa, Y. (1977) *Biochim. Biophys. Acta 488*, 442.

Iida, H., Maeda, T., Ohki, K., Nozawa, Y., & Ohnishi, S. (1978) *Biochim. Biophys. Acta 508*, 55.

Kasai, R., Watanabe, T., Fukushima, H., Iida, H., & Nozawa, Y. (1981) *Biochim. Biophys. Acta 666*, 36.

Kuksis, A., Breckenridge, W. C., Myher, J. J., & Kakis, G. (1978) *Can. J. Biochem. 56*, 630.

Lands, W. E. M. (1980) *Biochem. Soc. Trans. 8*, 25.

Lee, T. C., & Fitzgerald, V. (1980) *Biochim. Biophys. Acta 598*, 189.

Marinetti, G. V. (1962) *J. Lipid Res. 3*, 1.

Martin, C. E., & Thompson, G. A., Jr. (1978) *Biochemistry 17*, 3581.

Martin, C. E., Hiramitsu, K., Kitajima, Y., Nozawa, Y., Skriver, L., & Thompson, G. A., Jr. (1976) *Biochemistry 15*, 5219.

Maruyama, H., Banno, Y., Watanabe, T., & Nozawa, Y. (1982) *Biochim. Biophys. Acta 711*, 229.

Myher, J. J., Kuksis, A., Marai, L., & Yeung, S. K. F. (1978) *Anal. Chem. 50*, 557.

Nozawa, Y., & Thompson, G. A., Jr. (1971) *J. Cell Biol. 49*, 712.

Pieringer, J., & Conner, R. L. (1979) *J. Lipid Res. 20*, 363.

Ramesha, C. S., & Thompson, G. A., Jr. (1982) *Biochemistry* (second of three papers in this issue).

Ramesha, C. S., Dickens, B. F., & Thompson, G. A., Jr. (1982) *Biochemistry* (third of three papers in this issue).

Roberts, M. F., Bothner-By, A. A., & Dennis, E. A. (1978) *Biochemistry 17*, 935.

Rouser, G. S., Fleischer, S., & Yamamoto, A. (1970) *Lipids 5*, 494.

Thompson, G. A., Jr. (1969) *Biochim. Biophys. Acta 176*, 330.

Thompson, G. A., Jr. (1980) *The Regulation of Membrane Lipid Metabolism*, CRC Press, Boca Raton, FL.

Thompson, G. A., Jr., Bambery, R. J., & Nozawa, Y. (1971) *Biochemistry 10*, 4441.

Watanabe, T., Fukushima, H., & Nozawa, Y. (1980) *Biochim. Biophys. Acta 620*, 133.

Watanabe, T., Fukushima, H., Kasai, R., & Nozawa, Y. (1981) *Biochim. Biophys. Acta 665*, 66.