*Biochimica et Biophysica Acta*, 468 (1977) 73—80


BBA 77741

# SELF-REGULATION OF MEMBRANE FLUIDITY

# THE EFFECT OF SATURATED NORMAL AND METHOXY FATTY ACID SUPPLEMENTATION ON *TETRAHYMENA* MEMBRANE PHYSICAL PROPERTIES AND LIPID COMPOSITION

YASUO KITAJIMA and GUY A. THOMPSON, Jr. *

*Department of Botany, The University of Texas, Austin, Texas 78712 (U.S.A.)*

(Received December 29th, 1976)

## Summary

*Tetrahymena* cells elongated and desaturated massive supplements of palmitic or lauric acid at nearly twice the rates employed by unfed cells, thereby maintaining constant the physical properties of their membrane lipids. However, when a mixture of the 9- and 10-monomethoxy derivatives of stearic acid was administered, these compounds were incorporated without further metabolism. The marked fluidizing effect of the phospholipid-bound methoxy-fatty acids elicited an immediate reduction in fatty acid desaturase activity, the pattern of change being very similar to that induced by supplements of polyunsaturated fatty acids. The modulation of fatty acid desaturase activity by methoxy-acids clearly seems to be governed by membrane fluidity rather than by some form of end product inhibition of the type which might have been postulated to explain the similar effect caused by polyunsaturated fatty acids.

## Introduction

A wide variety of organisms ranging from bacteria [1] through higher plant [2] and animal [3] cells, respond to changes in their environmental temperatures by altering their lipid composition. Indeed, lipids are the only structural elements of living cells known to participate in the temperature acclimation phenomenon. Yet the molecular basis for these regulatory processes is still poorly understood.

The principal change with decreasing temperature is an increase in unsaturation of membrane bound fatty acids. Recent work with the protozoan *Tetra-*

* To whom reprint requests should be sent.

*hymena pyriformis* provided evidence that this increase is not simply a direct response of the fatty acid desaturase enzymes to temperature but rather is mediated through a temperature-induced reduction in fluidity of the membrane environment surrounding the desaturases [4,5].

In the above experiments, dietary supplementation with linoleic acid (18 : 2) enabled us to manipulate fluidity, and with it desaturase activity, independently of temperature. It so happened that in both temperature shifted and 18 : 2-fed cells, changes in desaturase activity were proportional not only to membrane fluidity but also to the phospholipid content of 18 : 2 and γ-linolenic acid (18 : 3), the two principal unsaturated fatty acids. While the evidence in both cases was strongly indicative that desaturase activity was controlled by fluidity per se, we were inable to rule out another possible mechanism, namely, end product inhibition of desaturase activity exerted by the increasing levels of polyunsaturated fatty acids. This type of regulation has been demonstrated in rat liver [6,7]. In the present study we report on efforts to vary membrane fluidity isothermally by means other than increasing polyunsaturates.

## Materials and Methods

### *Materials*

Palmitic acid (hexadecanoic acid, referred to hereafter as 16 : 0) was obtained from Eastman Organic Chemicals; lauric acid (dodecanoic acid, 12 : 0) was from Serdary Research Laboratories. [1-$^{14}$C]16 : 0 (56.3 Ci/mol) was from New England Nuclear, and [1-$^{14}$C]12 : 0 (28.8 Ci/mol) was from Amersham.

Mono-methoxy-stearic acid was prepared as described in a later section using oleic acid (*cis*-9-octadecenoic acid, 18 : 1) from Eastman Organic Chemicals and [1-$^{14}$C]18 : 1 (0.25 Ci/mol) from New England Nuclear.

### *Cell growth, lipid isolation and analysis*

Culture conditions for strain NT-1 of *T. pyriformis* were described in a previous paper [8]. Cell densities were measured with a Coulter Counter, Model B. Cells were harvested during mid-logarithmic phase by centrifugation, and cell fractionations were performed by the procedure of Nozawa and Thompson [9]. Lipid extractions were carried out according to Bligh and Dyer [10]. Thin-layer chromatographic separation and analytical determination of phospholipid species, as well as gas-liquid chromatography of fatty acid methyl esters, have been also previously described [8]. In some experiments, lipids were directly extracted from aliquots of the growth medium in order to account for all of exogenously supplied fatty acids.

### *Preparation of mono-methoxy-18 : 0*

To prepare mono-methoxy-18 : 0 by the procedure of Plattner et al. [11], 0.5 mmol (141.2 mg) of oleic acid was dissolved in 2 ml of methanol, and 0.5 mmol (149.4 mg) of mercuric acetate was then added. The mixture was refluxed for 30 min. After cooling, solid $NaBH_4$ was added slowly until bubbling stopped, and the mixture was stored overnight. 1 ml of distilled water was added, and the preparation was acidified with 6 M HCl. The methoxy-18 : 0

was obtained by 3 extractions with 5 ml of ether. After washing the combined ether phase with water 3 times, it was evaporated, and the product was dissolved in 10 ml of chloroform/methanol (6 : 1).

One night before each supplementation experiment, a further purification was carried out using Silica Gel G thin-layer chromatography with the solvent system, petroleum ether/ethyl ether/acetic acid (70 : 30 : 1). With this system, methoxy-18 : 0 migrated as a distinct, well separated spot between straight-chain free fatty acids and tetrahymanol. Methoxy-18 : 0 recovered from preparative thin-layer plates showed a single spot on repeated chromatography and a 97—98% purity by gas chromatography. 2—3% of free 18 : 1 was still present.

Although we confirmed by gas chromatography-mass spectrometry that the methoxy-18 : 0 was a mixture of the 9- and 10-methoxy derivatives as reported earlier [11], the methyl ester of the product showed only a single peak on gas chromatographs.

Gas chromatography of the purified [$^{14}$C]methoxy-18 : 0 methyl esters produced no significant radioactivity prior to that peak during the time when 12 : 0—18 : 3 should be eluted. Considerable amounts of radioactivity, but no mass peaks, were gradually eluted following the methoxy-18 : 0 peak, suggesting the continued presence of some contamination.

*Fatty acid supplememtation*

Fatty acid supplements containing 3.2 μmol and 1—2 μCi of either 16 : 0, 12 : 0 or methoxy-18 : 0 were prepared as 3-ml sonicated emulsions in inorganic medium as described in our previous paper [4]. When a 200 ml culture reached a density of 75 000 cells/ml, the supplement was added to the growth medium dropwise with shaking over a 10-min period. Aliquots were removed for lipid extraction after 5, 10, 15, 30, 60, 120 and 240 min (and 600 min in the case of methoxy-18 : 0 supplemented cultures).

*Freeze-fracture electron microscopy*

Freeze-fracture replication was carried out according to the method described in a previous paper [12]. Cells were cooled directly to the appropriate temperature (27, 24, 21°C) over a 4-min period by shaking the flask in cold water. After incubation of the samples for 5 min at the described temperatures, they were fixed for 15—20 min by the addition of 1/3 volume of 4% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.2). The fixative was precooled to the sample temperature, and care was taken to control the temperature to within ± 0.5°C during the fixation. The fixed cells were frozen in liquid Freon 22 and transferred to liquid nitrogen. Replicas were made with Balzers apparatus (Balzers BA, 360M, Fürstentum, Liechenstein) at −110°C and examined with a Hitachi HS-8 electron microscope.

## Results

Our aim in this work was to test the response of cells of manipulation of membrane fluidity by different means than we have used earlier [4,5]. Specifically, we desired to change the phospholipid fatty acid pattern isothermally without administering large amounts of unsaturated fatty acids.

The first experiments involved feeding 39.5°C-grown cells palmitic acid (16 : 0) in amounts equal to 80% of the cells' own total phospholipid fatty acid content. We hoped that the rapid influx of 16 : 0 would raise the level of that acid in phospholipids enough to reduce membrane fluidity. The supplement, including a trace of [$^{14}$C]palmitate, was indeed incorporated into phospholipids quickly, as monitored by thin-layer chromatography (Fig. 1A). Whereas cell growth in unsupplemented cultures produces a 29% increase of phospholipid fatty acids per h, these 16 : 0-fed cells experienced a 53% increase in phospholipid fatty acids from 16 : 0 incorporation alone during the first hour after feeding. There was a sizable enrichment of the phospholipids with 16 : 0 (Table I). But even by 1 h, nearly 50% of the radioactivity found in phospholipids had already been transferred to unsaturated fatty acids. An estimation of the incipient phase separation temperature by freeze-fracture electron microscopy 1 h following 16 : 0 addition indicated no significant lowering of fluidity in any membrane by the increased level of 16 : 0. This was apparently due to the offsetting changes in the levels of other components, e.g. a drop in 14 : 0 and 18 : 0 and an increase in 16 : 1.

Because 16 : 0 supplementation did not produce a measurable perturbation in membrane physical properties, we turned our attention to a shorter chain species, lauric acid (12 : 0), hoping in this case for a fluidizing effect. This compound, supplied with radioactive labeling to growing cultures in the same proportions as described above for 16 : 0, was also incorporated into phospholipids rapidly (Fig. 1B). However, the massive influx of exogenous 12 : 0 produced only minor changes in the phospholipid fatty acid composition, the most noteworthy after 1 h being an increase in 12 : 0 itself from 1.4 to 5% (Table I). By that time 80% of the phospholipid radioactivity was present in longer chain saturated and unsaturated fatty acids. Not surprisingly, none of the cellular membranes sustained a measurable increase in fluidity as inferred by freeze-fracture electron microscopy 1 h after 12 : 0 supplementation.

From these results it was obvious that *Tetrahymena* is capable of integrating even abnormally large quantities of exogenous fatty acids into its biosynthetic pathways quickly enough to prevent sizable alterations in fatty acid composi-

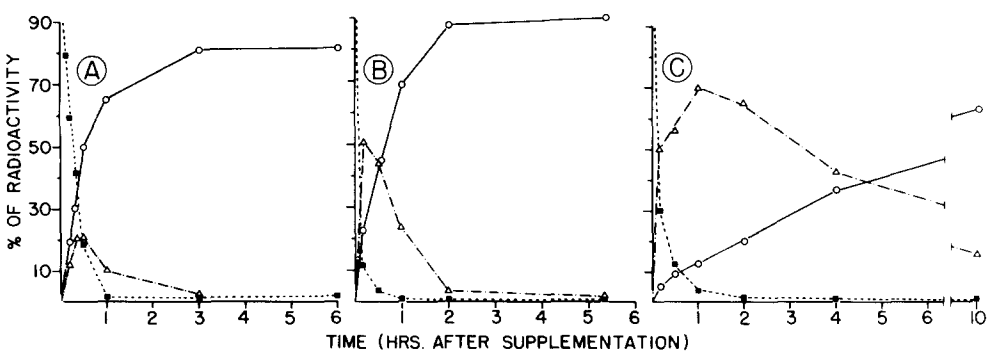


Fig. 1. The rate of incorporation of palmitic acid (A), lauric acid (B), and methoxy-stearic acid (C) into lipids of *Tetrahymena*. Each fatty acid was added in the amount of 3.2 μmol per culture ($1.5 \cdot 10^7$ cells) growing at 39.5°C. Aliquots of the cultures were removed for analysis at the times indicated. ■- - - - -■, free fatty acids; ○———○, phospholipids; △— · —△, triglycerides.

TABLE I

MAJOR FATTY ACIDS OF PHOSPHOLIPIDS FROM 16 : 0 OR 12 : 0-SUPPLEMENTED *TETRAHYMENA*

| Fatty acid | 16 : 0-Supplemented | 12 : 0-Supplemented | Control |
|---|---|---|---|
| 12 : 0 | 0.5 ± 0.3 | 5.0 ± 1.7 | 1.4 ± 0.4 |
| 14 : 0 | 8.8 ± 0.5 | 11.8 ± 0.7 | 10.5 ± 0.7 |
| 16 : 0 | 23.1 ± 2.0 | 15.5 ± 0.5 | 15.1 ± 1.8 |
| 16 : 1 | 14;6 ± 1.4 | 12.0 ± 0.6 | 11.4 ± 1.0 |
| 16 : 2 + 17 : 0 | 4.5 ± 1.0 | 5.6 ± 0.6 | 5.9 ± 0.8 |
| 18 : 0 | 1.8 ± 0.6 | 2.7 ± 1.4 | 2.2 ± 0.2 |
| 18 : 1 | 3.4 ± 0.5 | 4.2 ± 2.0 | 5.2 ± 1.5 |
| 18 : 2 | 11.4 ± 1.3 | 11.8 ± 1.7 | 13.1 ± 1.7 |
| 18 : 3 | 21.6 ± 2.5 | 22.3 ± 1.2 | 24.3 ± 2.5 |
| | $n = 4$ | $n = 4$ | $n = 4$ |

Each fatty acid was added in the amount of 3.2 μmol per culture ($1.5 \cdot 10^7$ cells) growing at 39.5°C. Lipids were extracted 60 min later.

tion and membrane fluidity. In an effort to prevent the retailoring of our supplement, we resorted to an unnatural, and hopefully non-metabolizable fatty acid.

Our choice was a mixture of the 9- and the 10-methoxy-derivatives of stearic acid prepared by methoxymercuration-demercuration of oleic acid [11]. When methoxy-stearate was administered in the same amounts used with the other acids, quite a different result was observed. Although the uptake of the preparation was little different from 16 : 0 and 12 : 0, most of it was incorporated into triglycerides (Fig. 1C). The slow transfer of methoxy-18 : 0 from triglycerides to phospholipids paralleled the growth of the culture, so that the level of methoxy-18 : 0 in phospholipids rose by 0.5 h to approximately 6% of the total fatty acids and climbed only to 8% of the total by 4 h (Table II).

Radioactivity from the methoxy-18 : 0 was found in all the major phospholipids, with the proportions in each class being similar to that found with natural fatty acids [8]. Gas chromatographic analysis of isolated methyl esters indicated very little catabolism of the methoxy-derivative; an average of 16% of the recovered radioactivity was found in the entire column effluent trapped prior to the elution of the methoxy-18 : 0, and 40% of that amount could be attributed to impurities in the fed methoxy-18 : 0. Apart from the methoxy-18 : 0 peak itself, none of the radioactivity was specifically associated with an eluted peak.

The incorporation of methoxy 18 : 0, unlike that of 16 : 0 or 12 : 0, had a significant effect upon membrane physical properties. Using the freeze-fracture electron microscopy technique described earlier [4,12], it was possible to estimate changes in apparent lipid bilayer phase separation by observing the temperature at which particle-free domains first appeared in membranes of various organelles. By 1 h following methoxy-18 : 0 addition to 39.5°C-grown cells, a significant fluidizing effect in the endoplasmic reticulum was evidence by the absence of apparent phase separation at the lowest temperature tested, 21°C, while control cells not treated with methoxy-18 : 0 clearly showed particle-free areas at 24°C. The same change in phase separation was seen in membranes of

TABLE II

MAJOR FATTY ACIDS OF PHOSPHOLIPID FROM WHOLE CELLS AND MICROSOMES OF METHOXY-18: 0-SUPPLEMENTED *TETRAHYMENA*

Supplement added as indicated in Table I.

| Fatty acid | Retention time (min) | Whole cells | | | | | Microsomes | |
|---|---|---|---|---|---|---|---|---|
| | | 30 min | 60 min | 120 min | 240 min | Control | 30 min | Control * |
| 12 : 0 | 1.4 | 2.0 ± 0.9 | 3.0 ± 1.2 | 1.7 ± 0.1 | 2.1 | 1.4 ± 0.4 | 1.3 | 0.6 |
| 14 : 0 | 2.7 | 9.2 ± 2.0 | 13.9 ± 2.2 | 12.8 ± 2.2 | 11.5 | 10.5 ± 0.7 | 10.6 | 7.3 |
| 16 : 0 | 5.3 | 16.4 ± 1.5 | 15.5 ± 1.1 | 14.5 ± 0.1 | 15.6 | 15.1 ± 1.8 | 14.5 | 13.2 |
| 16 : 1 | 6.1 | 7.9 ± 1.5 | 9.3 ± 1.1 | 9.4 ± 0.2 | 8.3 | 11.4 ± 1.0 | 8.9 | 8.7 |
| 16 : 2 + 17 : 0 | 7.3 | 4.8 ± 0.2 | 5.3 ± 1.0 | 5.8 ± 0.1 | 5.3 | 5.9 ± 0.8 | 5.2 | 4.9 |
| 18 : 0 | 10.3 | 3.7 ± 1.4 | 3.2 ± 0.1 | 1.9 ± 0.3 | 1.7 | 2.2 ± 0.2 | 1.5 | 2.2 |
| 18 : 1 | 11.5 | 5.5 ± 3.0 | 4.4 ± 1.8 | 3.2 ± 0.3 | 2.9 | 5.2 ± 1.5 | 3.1 | 13.9 |
| 18 : 2 | 14.1 | 11.3 ± 2.8 | 11.0 ± 0.6 | 10.7 ± 0.2 | 12.9 | 13.1 ± 1.7 | 13.3 | 13.0 |
| 18 : 3 | 16.5 | 18.3 ± 2.0 | 19.4 ± 0.5 | 19.9 ± 0.1 | 19.4 | 24.3 ± 2.5 | 21.8 | 21.4 |
| Methoxy 18 : 0 | 26.1 | 6.2 ± 0.9 | 4.0 ± 1.9 | 7.2 ± 0.6 | 7.9 | — | 9.0 | — |
| | | $n$ =3 | $n$ = 4 | $n$ = 3 | $n$ = 1 | $n$ = 8 | | |

* Data from Fukushima et al. [8].

small vesicles, and outer mitochondrial membranes, whose phase separation was observed in the control cells chilled to 21°C, revealed no sign of change at that temperature in methoxy-18 : 0 fed cells. However, the surface membranes, as represented by the pellicle, had not yet experienced the fluidizing action of the methoxy-18 : 0 by 1 h.

The most striking feature of the methoxy-18 : 0 effect upon fluidity was that it expressed itself despite an apparent attempt on the part of the cell to restore the normal membrane physical state by reducing the level of polyunsaturated fatty acids, particularly 18 : 3. (Table II). The concentration of 18 : 1 in whole cell phospholipids was also gradually decreased over the 4-h period. This latter decrease could be observed more dramatically in the fatty acids of microsomes isolated 30 min following the supplementation (Table II). It is clear from previous studies [4,12] and the higher microsomal level of methoxy-18 : 0 found here that these membranes are the primary site for processing ingested fatty acids as well as desaturating those acids capable of being so modified.

## Discussion

The results of these studies affirm the rapidity and diversity with which *Tetrahymena* can respond to lipid supplements potentially capable of upsetting its optimum membrane fluidity. When possible, as in the 16 : 0 or 12 : 0 feeding experiments, the cells modified the fed acids quickly enough to prevent a measurable perturbation of membrane properties. This was accomplished despite the fact that fatty acid elongation and desaturation had to be increased significantly over the normal rate to deal with the sudden influx of exogenous components.

The unnatural additive methoxy-18 : 0 could not be enzymatically modified so as to reduce its fluidizing effect. However, the cells were able to divert most of it into triglycerides with no harmful consequences. The cells compensated for that small amount of methoxy-18 : 0 that was incorporated into phospholipids by altering the levels of their other fatty acids.

It is this latter change that answers the original question posed. The response to methoxy-18 : 0 is very similar to that observed after feeding polyunsaturates [5] or raising the environmental temperature [4,8]. In all cases there was an increase in saturated fatty acids and a decrease in unsaturated fatty acids. Thus it is fair to conclude that all three fluidizing perturbations act in the same way, by decreasing desaturation. And in the case of methoxy-18 : 0, it would seem most unlikely that the reduced desaturation was caused by an end-product type inhibition of the pertinent fatty acid desaturase.

We interpret these findings as supporting the concept that fluidity proper regulates the level of fatty acid desaturase activity [4,5,13]. In such a way the enzyme can promote adaptation to temperature and a variety of other factors which lead to non-optimal membrane fluidity.

## Acknowledgments

The authors are grateful for the technical assistance of Mrs. Irene Hrovat. The work was supported by National Institutes of Health Grant GM 20148,

Robert A. Welch Foundation Grant F-350, American Cancer Society Grant BC-110, and National Science Foundation Grant 0IP74-22029.

## References

1 Aibara, S., Kato, M., Ishinaga, M. and Kito, M. (1972) Biochim. Biophys. Acta 270, 301—306
2 Harris, P., and James, A.T. (1969) Biochem. J. 112, 325—330
3 Roots, B.I. (1968) Comp. Biochem. Physiol. 25, 457—466
4 Martin, C.E., Hiramitsu, K., Kitajima, Y., Nozawa, Y., Skriver, L. and Thompson, Jr., G.A. (1976) Biochemistry 15, 5218—5227
5 Kasai, R., Kitajima, Y., Martin, C.E., Nozawa, Y., Skriver, L. and Thompson, Jr., G.A. (1976) Biochemistry 15, 5228—5233
6 Brenner, R.R., Peluffo, R.O., Nervi, A.M. and De Tomas, M.E. (1969) Biochim. Biophys. Acta 176, 420—422
7 Ullman, D. and Sprecher, H. (1971) Biochim. Biophys. Acta 248, 61—70
8 Fukushima, H., Martin, C.E., Iida, H., Kitajima, Y., Thompson, Jr., G.A. and Nozawa, T. (1976) Biochim. Biophys. Acta 431, 165—179
9 Nozawa, Y. and Thompson, Jr., G.A. (1971) J. Cell Biol. 49, 712—721
10 Bligh, E.C. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
11 Plattner, R.D., Spencer, G.F. and Kleiman, R. (1976) Lipids 11, 222—227
12 Kitajima, Y. and Thompson, Jr., G.A. (1977) J. Cell Biol. 72, 744—755
13 Thompson, Jr., G.A. and Nozawa, Y. (1977) Biochim. Biophys. Acta, in the press