Proc. NIPR Symp. Polar Biol., 4, 9–16, 1991

EFFECTS OF INCUBATION TEMPERATURE ON DISTRIBUTION OF ^{14}C IN LIPIDS IN A PSYCHROPHILIC MARINE DIATOM

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Abstract: The incorporation of [^{14}C] bicarbonate into cells and the lipid fractions was determined at 6°, 16° and 26°C using the psychrophilic marine diatom, *Navicula* sp. strain *D* grown at 3°C and 13°C. The intramolecular distribution of ^{14}C in both total lipids and three lipid classes (phospholipids, glycolipids and neutral lipids) was also estimated.

The rates of ^{14}C incorporation into cells and the total lipid fraction were highest at 16°C followed in order by those at 6°C and 26°C. The percentage of total incorporated ^{14}C present as total lipids was significantly low at 26°C compared with those at 6°C and 16°C. Among the three lipid classes, neutral lipids were predominantly labeled (50–80%) under the conditions employed. Relative radioactivity of total lipids localized in fatty acid methyl esters was significantly lower at 26°C than at 6°C and 16°C. This decrease at 26°C was attributable to a relative decrease of phospholipids and neutral lipids, the radioactivities of which were heavily presented in acyl moieties. These changes at 26°C were more remarkable with cells grown at 3°C than with those grown at 13°C.

1. Introduction

It has now been well known that diatoms, especially so-called ice algae, are important contributors to primary production in Antarctic marine ecosystems (BUNT, 1963; SMITH and NELSON, 1985). Many investigations have been conducted on the seasonal and/or regional variations in algal abundance and species composition in order to understand polar ecosystems (KOPCZYNSKA *et al.*, 1986). To achieve sufficient growth under low temperatures around 0°C, it is expected that algae should possess special physiological metabolic characteristics which are different from those of mesophilic phytoplankton occurring in temperate regions.

Natural populations are typically composed of large numbers of different species. Consequently, investigations of natural assemblages would involve integration of the different responses of individual species composing the assemblage (RIVKIN, 1985). It may be difficult to grasp the physiological and biochemical characteristics responsible for the low-temperature adaptation of individual species except by measurement using *in situ* single-cell isolation techniques (RIVKIN and SELIGER, 1981; TAGUCHI and LAWS, 1985). Alternatively, controlled laboratory experiments using pure cultures can also be

employed. Clearly there are some important differences between cultured samples and field samples (MORRIS, 1984). Therefore, care should be taken not to oversimplify interpretations of the results of laboratory experiments when applying them to natural communities. Nevertheless, laboratory studies can yield useful information about the responses of certain species to particular physical or chemical changes. So far, very few experiments have been conducted with laboratory-cultured individual Antarctic microalgae except for those by GILLAN *et al.* (1981) and NICHOLS *et al.* (1986).

We previously isolated a psychrophilic diatom, *Navicula* sp. strain D, from the Antarctic Ocean, and established a clonal culture. The optimum temperature for the growth of *Navicula* sp. strain D was between 7° and 10°C. This alga cannot grow at temperatures above 16°C but can grow at 0°C or below even though the growth rate decreased (TOMINAGA, 1990). It is of our particular interest to clarify the possible reason why this alga cannot grow at higher temperatures at which many other mesophilic algae can grow. This also could give us some insight why the alga can grow at low temperatures.

Because of the importance of biomembranes as sites of reactions of membrane-bound enzymes and as transport barriers of several substances indispensable for cell physiology, we considered that the lipid metabolism of psychrophilic diatoms might be unique or different from that of mesophilic diatoms.

In this paper, we describe the distribution of incorporated $NaH^{14}CO_3$ in various lipid fractions in the laboratory-cultured psychrophilic marine diatom, *Navicula* sp. strain D when incubated at suboptimal and higher temperatures for growth.

2. Materials and Methods

Naviucla sp. strain D, a marine psychrophilic diatom, isolated from the Antarctic Ocean by two of the authors (H. T. and N. T.) and grown as an axenic culture, was used as the experimental algal material.

The alga was grown in a incubator (Toyo Scientific Instrument Co., Type IS-3000H) illuminated by fluorescent tubes (National, Type "Homolux" designed for plant cultivation) giving a light intensity of $50\,\mu Einst \cdot m^{-2} \cdot s^{-1}$ at the surface of the culture vessels. The photoperiod was a 12-h light : 12-h dark cycle. The medium for the culture was the slightly modified ASP-2 (PROVASOLI *et al.*, 1957) with a salinity (NaCl, $MgSO_4$) of 1.8 times higher than the original concentration. The stock culture was maintained at 7°C. Prior to the experiment, the cultures were maintained at 3°C or 13°C for at least a month and inoculated once a week, respectively.

The algal growth phases were determined by the changes of cell numbers counted using a light microscope (Type BH, Olympus) and a Sedwick-Rafter slide. In the mid-logarithmic phase, cells were harvested by centrifugation at $1600 \times g$ for 10 min at 4°C, washed twice and resuspended in fresh growth medium. After preincubation at 6°, 16° and 26°C for 30 min (MORRIS *et al.*, 1974), $NaH^{14}CO_3$ (ICN Radiochemicals, Irvine, CA) was added with a final concentration of 0.1 mM and a specific activity of $10.8\,\mu Ci \cdot \mu mol^{-1}$. The incubations were carried out at each temperature for 4 h under a light intensity of $160–180\,\mu Einst \cdot m^{-2} \cdot s^{-1}$ where photoinhibition did not occur (TOMINAGA, 1990) and terminated by filtering. To determine the total incorporation, a

portion of the reaction mixture was filtrated onto a GF/C filter, rinsed twice with 3% NaCl solution and placed into a scintillation fluid. Radioactivity was determined using a liquid scintillation spectrophotometer (Packard 3255). The remaining mixture was also filtered onto another GF/C filter and was analyzed for the $NaH^{14}CO_3$ incorporation into total lipids and various lipid classes.

Labeled lipids were extracted from the filtered samples according to the procedure of BLIGH and DYER (1959). An average of 92% of the label was recovered in lipid and residual fractions. An aliquot was transferred to a scintillation vial and the radioactivity was measured. The lipids were separated by high-performance thin-layer chromatography (HPTLC) on an EM pore sheet (Analytichem International, EM pore 3M, 10×10cm). HPTLC sheets loaded with authentic standards and samples were first developed to a distance of 7cm from the origin using acetone : benzene : water (91 : 30 : 8 by volume) as the solvent system according to the method of SATO and MURATA (1982). After evaporation of the solvents under a stream of air, the sheets were fully developed in petroleum ether : chloroform : acetone (4 : 1 : 1). The separated lipid spots on the HPTLC sheets were detected with a fluorescent dye, primuline (LESHEVALIER *et al.*, 1972), and identified by comparison of their R_f values with those of the authentic compounds. Spots of phospholipids, glycolipids and neutral lipids visualized were cut out and their radioactivities were determined. For determining the intramolecular distribution of radioactivity in the lipid molecules, each lipid class separated by HPTLC was treated with HCl/CH_3OH at 90°C for 2h (SATO and MURATA, 1982). The resulting fatty acid methyl esters were extracted with *n*-hexane and the radioactivity of the *n*-hexane phase was estimated. The radioactivity of the remaining methanol phase containing the other parts of the molecules, *i.e.* glycerol, methyl glycosides, phosphoric acid and polar head groups of phospholipids, was also determined. The total lipid fraction was also separated into two phases and their radioactivities were determined.

The protein content of each reaction mixture was determined colorimetrically by the method of LOWRY *et al.* (1951).

3. Results and Discussion

Figure la shows the protein base incorporation of $NaH^{14}CO_3$ into cells incubated at 6°, 16° and 26°C using cells in the logarithmic phase grown at 3°C and 13°C. We have already reported that the total incorporation of $NaH^{14}CO_3$ for 4h by cells grown at 7°C was maximum at around 17°C (KATAHIRA *et al.*, 1990). Above 20°C, the total incorporation declined and the percentage of ^{14}C recovered in the fraction of low molecular weight compounds increased. In this experiment, we intended to examine the possible changes of distribution pattern of ^{14}C within a short time incubation especially under higher temperatures where the *Navicula* sp. strain D cannot grow. HITCHCOCK (1983) reported with some phytoplankton species that total incorporation increased almost linearly with time up to 4 to 6h and a constant proportion of ^{14}C in four fractions was usually achieved until 4h. Total incorporation of $NaH^{14}CO_3$ increased until 4h in the *Navicula* sp. strain D. Therefore, we determined more detailed incorporation of ^{14}C for 4h at the temperatures as follows; 16°C, near the optimum

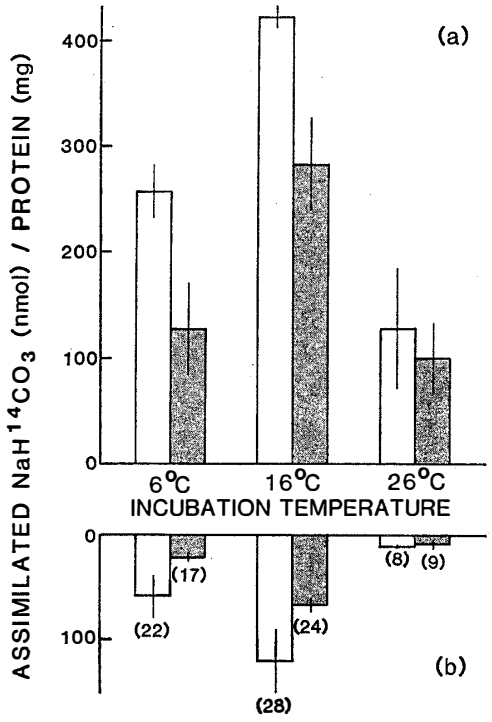

Fig. 1. Effect of incubation temperature on incorporation of $NaH^{14}CO_3$ into a) cells and b) total lipid fraction for cultures grown at 3°C (clear columns) and 13°C (solid columns). Values in parentheses (Fig. 1b) indicate the percentages of total ^{14}C incorporated into the total lipid fraction. Vertical lines indicate the standard deviations of 3 determinations.

temperature of total incorporation of $NaH^{14}CO_3$, 6°C, around the optimum temperature of growth and 26°C, temperature where strain D might be damaged. Total incorporation was highest at 16°C followed by those at 6°C and 26°C with cells grown at both 3°C and 13°C (Fig. 1a) similar to the results obtained for the cells grown at 7°C (KATAHIRA *et al.*, 1990). Higher activity was obtained for cells grown at 3°C compared with those grown at 13°C irrespective of incubation temperatures.

Net incorporation into total lipid fraction and percentage of total ^{14}C incorporated into the fraction are shown in Fig. 1b. Radioactivity in total lipid fraction and the proportion of total incorporation were both highest also at 16°C, followed by those at 6°C and 26°C. Particularly, when incubated at 26°C, incorporation of ^{14}C into total lipids declined to 8% and 9% of total incorporation for cells grown at 3°C and 13°C, respectively. This tendency was consistent with previous results showing that synthesis of biopolymers was inhibited at higher temperatures (KATAHIRA *et al.*, 1990). *Navicula* sp. strain D grown at 3°C incorporated 22% of total ^{14}C into lipids when incubated at 6°C. Our result is almost consistent with that for eastern Canadian arctic phytoplankton in which lipid-^{14}C did not exceed 30% of total ^{14}C fixed within a temperature range of $-1.0°$ to 6.0°C and an irradiance range of 1 to 700 $W \cdot m^{-2}$ (LI and PLATT, 1982). Although the experimental conditions were different from ours, SMITH and MORRIS (1980) reported that phytoplankton from the Antarctic Ocean showed a large percentage (80%) of photosynthetically fixed ^{14}C into lipids under conditions of low temperatures (<0°C) and low light levels (1 $W \cdot m^{-2}$).

Photosynthetic carbon incorporation into phospholipids, glycolipids and neutral lipids expressed as nmol $NaH^{14}CO_3$ per mg protein is shown in Fig. 2. An average of 93% of total label spotted on the EM pore sheet was recovered after fractionation by

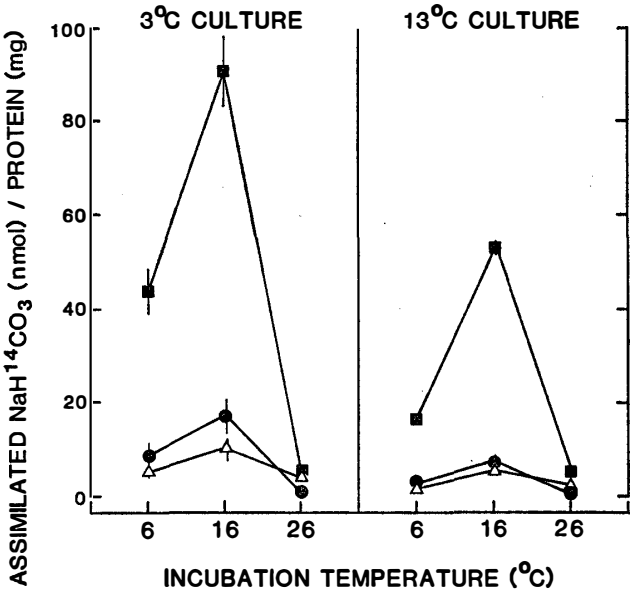

Fig. 2. Photosynthetic incorporation of $NaH^{14}CO_3$ into phospholipids (solid circles), glycolipids (clear triangles) and neutral lipids (solid squares). Vertical lines indicate the standard deviations of 3 determinations.

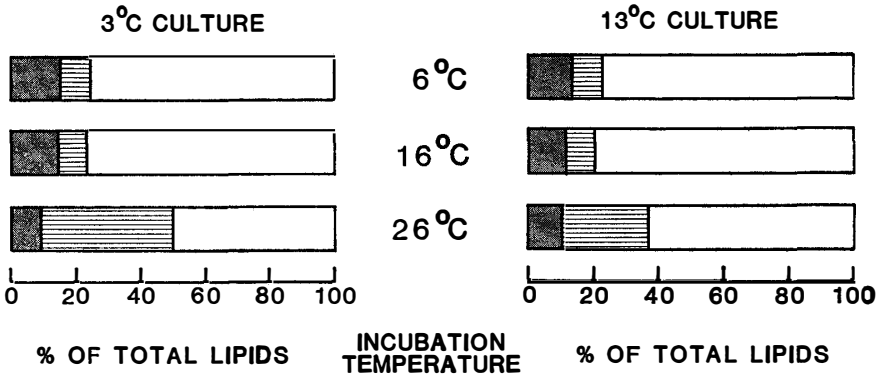

Fig. 3. Effect of temperature on percentage of total labeled lipids in phospholipids (solid columns), glycolipids (hatched columns) and neutral lipids (clear columns).

HPTLC. The incorporation of ^{14}C into neutral lipids was the highest under all experimental conditions examined. At 26°C, the radioactivities incorporated into phospholipids and neutral lipids decreased to 6 to 11% of those at 16°C in both cells grown at 3° and 13°C, whereas that into glycolipids accounted for 40% of that at 16°C.

Figure 3 shows the relative proportions of ^{14}C incorporated into three lipid classes that comprised the total radiolabeled lipid fraction. In Fig. 3, the percentage of each lipid class was calculated by using the sum of the three fractions as 100% and the standard deviations of replicate analysis for phospholipids, glycolipids and neutral lipids were within 5, 4 and 8%, respectively. The patterns of incorporation into three lipid classes at 6°C and 16°C were similar in cells grown at 3°C and 13°C. A distinct one was obtained at 26°C. In both cells grown at 3°C and 13°C, the proportion of ^{14}C incorporated into glycolipids increased and became higher than that into phospholipids. As can be seen from Fig. 2, these relative increases in glycolipids were not due to an absolute increase of incorporation into the glycolipid fraction, but rather to the remarkable decrease of that into neutral lipids and phospholipids.

PALMISANO *et al.* (1988) investigated the variation in synthesis of three lipid classes in Antarctic sea-ice diatoms during a spring bloom. They showed that incorporation of $NaH^{14}CO_3$ in the neutral lipid fraction decreased sharply after a maximal chlorophyll *a* concentration was observed and suggested the occurrence of profound changes in physiological state in sea-ice diatom communities. From our experiments, changes in the distribution of ^{14}C at 26°C at least suggest that the biosynthesis of three lipid classes might be affected by high temperatures in psychrophilic *Navicula* sp. strain D.

Table 1 illustrates the intramolecular distribution of ^{14}C in three lipid classes. The values show the percentage of total ^{14}C recovered in hexane phase after transmethylation of three lipid classes and separation into two phase, hexane phase (fatty acid methyl esters) and methanol phase (other parts of lipid molecules). An average of 91% of the total label was recovered after this procedure. In both phospholipids and neutral lipids, the radioactivity in the hexane phase formed a higher proportion (65–86%), whereas that in glycolipids accounted for only 13–32% under all experimental conditions. In cells grown at 3°C, the percentage at 26°C was lower than those at both 6°C and 16°C irrespective of the lipid classes.

Distribution of ^{14}C of total lipids into the hexane and methanol phases is shown in Fig. 4. Most (70–79%) of the ^{14}C was recovered from the hexane phase at 6°C and

Table 1. Relative radioactivity in fatty acyl moiety of each lipid class. All values are mean % ± one standard deviation.

Growth temperature (°C)	Incubation temperature (°C)	Relative radioactivity in fatty acyl moiety (%)		
		Phospholipids	Glycolipids	Neutral lipids
3	6	81± 3	27± 8	83±3
	16	82± 3	32± 6	86±1
	26	70±15	13± 2	65±7
13	6	72± 6	19± 8	78±5
	16	77± 8	24±11	83±5
	26	74± 9	18± 1	72±1

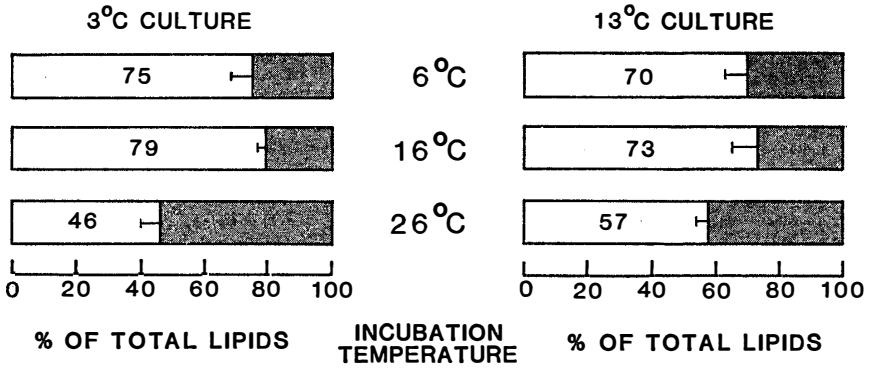

Fig. 4. Intramolecular distribution of ^{14}C in total lipids. The hexane phase (clear columns) after transmethylation contained fatty acid methyl esters and the remaining methanol phase (solid columns) contained other parts of lipid molecules. Bars give the standard deviations.

16°C for cells cultured at both 3°C and 13°C. The proportion of ^{14}C in the hexane phase decreased to 46% and 57% at 26°C in cells grown at 3° and 13°C, respectively. This decrease was largely attributable to the relative decrease of incorporation of ^{14}C into neutral lipids, of which the hexane phase was predominantly labeled and to a lesser extent, to the complementary increase of that into glycolipids, which contained a higher proportion of ^{14}C in the methanol phase (Fig. 3, Table 1). As the magnitude of this change at 26°C for 4h was greater for the 3°C culture than that for the 13°C culture, acclimation of the 13°C culture to somewhat higher temperature might have occurred.

SARGENT *et al.* (1985) reported that during a spring bloom in the northern Norwegian fjords, lipids from phytoplankton were composed almost entirely of polar lipids and the radioactivity present in total lipid was located mainly in the polar lipid fraction (63–86%). At the same time, a relatively low percentage (7–23%) of ^{14}C incorporated in total lipid was present as fatty acids. The lipid composition of the *Navicula* sp. strain D was characterized by high concentration of neutral lipids (50–70 molar%, unpublished data) and high percentage (75 and 80%) of ^{14}C was recovered in neutral lipids incubated at 6°C with cells grown at 3° and 13°C, respectively (Fig. 3). The inconsistency between the results of SARGENT *et al.* (1985) and ours regarding the intramolecular distribution of ^{14}C of total lipid might be due to differences in lipid composition.

From these results, at 26°C, where strain D cannot grow but activity of incorporation of $NaH^{14}CO_3$ still remained, an unfavorable effect of high temperature on the incorporation into the total lipid fraction, was observed (Fig. 1a, b). Consequently, it was suggested that incorporation of ^{14}C into biopolymers such as lipids was inhibited at higher temperature. Furthermore, within the decreased incorporation into total lipids, the carbon flow into three lipid classes changed at 26°C. Although little information is available about the effect of temperature on lipid biosynthesis, particularly fatty acid synthesis, even in mesophilic diatoms, at least two possibilities can be proposed from our results; (1) the activity of fatty acid synthesis of the *Navicula* sp. strain D was decreased at 26°C, (2) once the fatty acids had been synthesized, their transfer to lipid molecules or acylation of glycerol-3-phosphate was not perfomed smoothly at 26°C.

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(Received May 10, 1990; Revised manuscript received October 5, 1990)