JOURNAL OF BACTERIOLOGY, Nov. 1996, p. 6140–6144 Vol. 178, No. 21
0021-9193/96/$04.00+0

Biosynthesis of Diacylglyceryl-*N*,*N*,*N*-Trimethylhomoserine in *Rhodobacter sphaeroides* and Evidence for Lipid-Linked N Methylation

M. HOFMANN AND W. EICHENBERGER*

Institut für Biochemie der Universität Bern, CH-3012 Bern, Switzerland

Received 1 April 1996/Accepted 20 August 1996

***Rhodobacter sphaeroides*, which produces diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS) under phosphate-limiting conditions, was incubated with L-[1-^{14}C]- and L-[*methyl*-^{14}C]methionine in pulse and pulse-chase experiments. The label was incorporated specifically into the polar part of DGTS and of three other compounds. One of them (compound 3) could be identified as diacylglyceryl-*N*,*N*-dimethylhomoserine by cochromatography with a reference obtained semisynthetically from DGTS. It was labelled when using L-[1-^{14}C]- as well as L-[*methyl*-^{14}C]methionine as a precursor and was converted to DGTS when incubated with the DGTS-forming eukaryotic alga *Ochromonas danica* (*Chrysophyceae*). Of the other two compounds labelled with L-[1-^{14}C]methionine, compound 2 was also labelled with L-[*methyl*-^{14}C]methionine whereas compound 1 was not, suggesting that these two intermediates are the corresponding *N*-methyl and nonmethylated lipids, respectively. The methyltransferase inhibitor 3′-deazaadenosine enhanced the amounts of compounds 1 to 3 but decreased the amount of DGTS. It is concluded that in *R. sphaeroides*, DGTS is synthesized by the same pathway as in eukaryotic organisms and that the N methylation is the terminal step in this process and occurs on the preformed lipid. Since the phosphatidylcholine-deficient mutant CHB20, lacking the phosphatidylcholine-forming *N*-methyltransferase was able to synthesize DGTS, one or several separate *N*-methyltransferases are suggested to be responsible for the synthesis of DGTS.**

Diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (homoserine lipid; DGTS) is a common lipid constituent of ferns, mosses, and most green algae (6). It belongs to the betaine-type lipids, which, together with glycosyl- and phosphoglycerides, represent a third group of widely distributed plant glycerolipids (15). Attempts to elucidate the biosynthetic pathway of DGTS have been undertaken with several eukaryotic organisms (9, 13, 14, 16, 22). These studies revealed methionine to be the precursor of both the C_4 backbone and the *N*-methyl groups of the polar part. Consequently, the biosynthetic pathway of DGTS is suggested to consist of two main steps: (i) attachment of the C_4 part of methionine to the diacylglycerol part, and (ii) *N*-methylation of the amino group. Single steps or intermediates of this process have not been identified so far. Only recently, DGTS was identified in the photosynthetic bacterium *Rhodobacter sphaeroides* grown under phosphate-limiting culture conditions (4). This first report of a betaine lipid in a prokaryotic organism raised the question whether the polar group of this lipid is biosynthesized in the bacterium by the same pathway as in eukaryotic plants. In addition, using *R. sphaeroides*, which promised to be a less complex system than eukaryotic cells, we tried to recognize and identify single steps of the biosynthesis of DGTS.

MATERIALS AND METHODS

Bacterial and algal strains. *R. sphaeroides* 2.4.1 (wild type) and the mutant strain CHB20 (*pmtA* mutant) obtained by chemical mutagenesis (5) were used. This mutant is deficient in the common phospholipid phosphatidylcholine (PC). Both strains were kindly provided by C. Benning, Institut für Genbiologische Forschung, Berlin, Germany. *Ochromonas danica* 933/2b was obtained from the Culture Center of Algae and Protozoa, Cambridge, United Kingdom.

* Corresponding author. Mailing address: Department of Biochemistry, Institut für Biochemie der Universität Bern, Freiestrasse 3, 3012 Bern, Switzerland. Phone: 41 631 43 31. Fax: 41 631 48 87. Electronic mail address: eichenberger@ibc.unibe.ch.

Media and growth conditions. *R. sphaeroides* cells were grown under phosphate-limited conditions in Sistrom's succinate-basal-salt medium (17, 18) containing 100 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 6.8)–KOH and 0.1 mM potassium phosphate as modified by Benning et al. (3). Photoheterotrophic liquid cultures were grown in tightly closed, filled 50-ml bottles under 10,000 lux of continuous fluorescent light with shaking at 30°C. *O. danica* was cultivated in Aaronson and Baker medium as previously described (21).

Lipid isolation, separation, and analysis. Total lipids were extracted with hot methanol containing 0.05% butyl hydroxytoluene as an antioxidant. The crude extract was dried under a stream of N_2 and further purified by phase partition with 1 volume each of sodium bicarbonate (1%) and diethyl ether. In an additional step, polyhydroxybutyrate was removed by phase partition with 1 volume of methanol and 4 volumes of hexane.

Lipids were separated on precoated silica gel plates (Merck 5715) with a standard solvent system containing chloroform, methanol, and water (65:25:4, by volume) in the first dimension and chloroform, methanol, isopropylamine, and concentrated ammonia (65:35:0.5:5, by volume) in the second dimension. Spots were detected under UV (366-nm) light after being sprayed with 2′,7′-dichlorofluorescein. Betaine lipids, phospholipids, and glycolipids were identified with specific spray reagents (21). Labelled compounds 1 to 3 were detected by autoradiography or with the scanner system. For further analysis, single spots were eluted with chloroform-methanol (2:1). Lysocompounds were obtained by cleaving the fatty acid from position 1 of the glycerol part by incubation with lipase from *Rhizopus arrhizus* (10). Both positions 1 and 2 were deacylated by transesterification with sodium methoxide (19).

Radioactive substrates and chemicals. L-[1-^{14}C]methionine (55.6 mCi/mmol) was purchased from Amersham, and L-[*methyl*-^{14}C]methionine (53.4 mCi/mmol) was purchased from Du Pont. L-α-Dipalmitoylphosphatidyl-*N*,*N*-dimethylethanolamine, L-α-dipalmitoylphosphatidyl-*N*-monomethylethanolamine, and 3′-deazaadenosine (DAA) were obtained from Sigma; 2-hydroxyethylhydrazine was obtained from Fluka; and *N*-methyl-*N*-trimethylsilyltrifluoracetamide was obtained from Macherey-Nagel.

Incubation conditions. In a standard assay, 50 ml of a stationary-phase liquid culture of *R. sphaeroides* was harvested by centrifugation (10,000 × *g* for 10 min in a Sorvall RC 5B centrifuge with an SS34 rotor) and the pellet (10^{10} to 10^{11} cells) was resuspended in 1 to 2 ml of cultivation medium including 5 mM glycerol-*sn*-3-phosphate. The cell suspension was incubated with 1 to 2 μCi of either L-[1-^{14}C]methionine or L-[*methyl*-^{14}C]methionine under the conditions described above. The incubation time was 4 h in pulse experiments and 20 min in pulse-chase experiments. For the latter, at the end of the pulse, the pellet was washed twice with culture medium and resuspended in a total volume of 5 ml of medium. Aliquots were taken after 0, 20, 40, and 120 min of chase.

For inhibition experiments, DAA and 2-hydroxyethylhydrazine were tested in

a concentration range of 0 to 100 μM. Aliquots with 1.74×10^{10} cells of *R. sphaeroides* 2.4.1 were preincubated for 1 h in a total volume of 2 ml of medium with the inhibitor under standard culture conditions. Then, 0.2 μCi of L-[1-^{14}C]methionine was added to each sample, and the mixture was incubated for 2 h. For the transformation of compound 3 into DGTS, it was suspended in 1 ml of Aaronson and Baker medium by sonication; after the addition of 3×10^7 cells from a late-logarithmic-phase culture of *O. danica*, the total volume was adjusted to 3 ml (10^7 cells per ml) and the cell suspension was incubated for 24 h under constant fluorescent light (10,000 lux) with shaking at 22°C. The incubations were stopped by the addition of a 10-fold volume of hot methanol. Lipids were extracted and separated by thin-layer chromatography (TLC), and the radioactivity in single spots was measured.

Radioactive measurements. Radioactive spots on TLC plates were localized either with a System 200 imaging scanner (Bioscan, Washington, D.C.) or by autoradiography with Hyper film MP (Amersham). For the quantification, spots were scraped off and measured on a Betamatic V liquid scintillation counter (Kontron, Switzerland) after addition of 2 ml of methanol and 5 ml of 0.7% (wt/vol) butyl-PBD (Glaser) in toluene. The radioactivity in the polar part of the lipids was measured after transesterification with sodium methoxide (19) and removal of the fatty acid methyl esters with hexane.

Synthesis and structural analysis of DGDS. DGTS was isolated from *O. danica* by flash chromatography as previously described (21). Diacylglyceryl-*N*,*N*-dimethylhomoserine (DGDS) was synthesized by demethylation of isolated DGTS as described previously (8). All steps were carried out under nitrogen. The reaction mixture contained 1% (wt/vol) sodium thiophenolate in dioxane previously distilled over $LiAlH_4$. Of this solution, 1 ml was added to 18 mg of DGTS and allowed to react for 45 min at 90°C. The product was isolated on a silica gel plate (Merck 5715) with chloroform-methanol-water (65:25:4, by volume) as a solvent. For identification, the fatty acids were removed by alkaline hydrolysis with methanolic KOH and the polar group was silylated with *N*-methyl-*N*-trimethylsilyltrifluoracetamide. The molecular weight of the trimethylsilyl derivative $(M\text{-}CH_3)^+$ was 422.2210, which was in accordance with the expected elemental composition $C_{17}H_{43}O_5NSi_3$ (calculated value, 422.2214). For gas chromatography-mass spectrometry, a Varian Mat 44S instrument coupled to a Varian 3700 gas chromatograph (OV-1 fused-silica column, 25 m long) was used at 80 to 120°C.

Identification of compound 3 by cochromatography. For the cochromatography of synthetic reference with labelled compound 3 obtained from *R. sphaeroides*, silica gel plates (Merck 5715) with two solvent systems were used. The first system was chloroform-methanol-water (65:25:4, by volume) (first dimension) and chloroform-methanol-isopropylamine-concentrated ammonia (65:35:0.5:5, by volume) (second dimension). R_f values were 0.6 and 0.68 in the first and second solvents, respectively. The second system was acetone-benzene-methanol-water (8:3:2:1, by volume) (first dimension) and chloroform-acetone-methanol-acetic acid-water (10:4:2:3:1, by volume) (second dimension). In this system, R_f values were 0.38 and 0.7. Compound 3 was detected by autoradiography and the reference by spraying with 2′,7′-dichlorofluorescein.

RESULTS

Incorporation of L-[1-^{14}C]- and L-[*methyl*-^{14}C]methionine. Methionine acts as the precursor of the polar group of DGTS in algae (13, 22). To decide whether the same amino acid is also used for DGTS biosynthesis by the bacterium, cells of *R. sphaeroides* cultivated under phosphate-limiting conditions were incubated with 1.3 μCi of L-[1-^{14}C]methionine in a pulse of 4 h. The total lipids, which contained 10.2% of the label, were extracted and separated by two-dimensional (2D)-TLC, and the plate was sprayed with dichlorofluorescein. Monohexosyldiacylglycerol (MHG), DGTS, glucosylgalactosyldiacylglycerol (GGD), PC, phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sulfoquinovosyldiacylglycerol (SQDG), and ornithine lipid (OL) were the major components, as shown in Fig. 1 and reported by Benning et al. (3).

In the autoradiogram, radioactivity was detected in DGTS as well as in three spots (compounds 1 to 3) which were not visible under UV. The radioactivity in single spots was measured by liquid scintillation counting. The total radioactivity of compounds 2 and 3, which were not completely separated on the plate, was measured. Almost 97% of the label was localized in DGTS and in compounds 1 to 3, while glycolipids, phospholipids, and ornithine lipid contained only insignificant amounts of radioactivity. After elution and deacylation, almost 100% of the label was recovered from the polar part of these compounds. This indicates that methionine was incorporated almost specifically into the polar group of DGTS and of compounds 1 to 3.

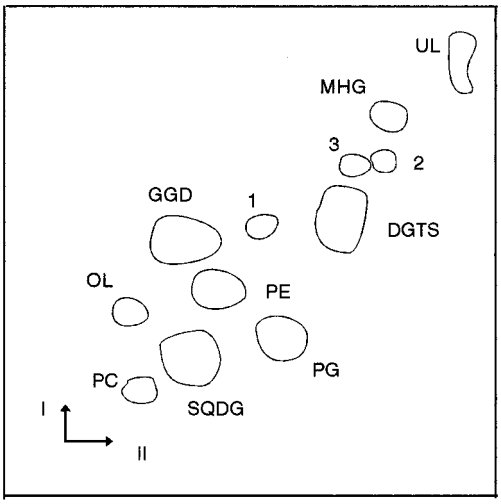

FIG. 1. 2D-TLC of lipids of *R. sphaeroides* grown under phosphate-limiting conditions and incubated with L-[1-^{14}C]methionine. UL, nonpolar lipids; 1, 2, 3, compounds 1, 2, and 3.

Since, on the basis of their labelling, compounds 1 to 3 were supposed to be intermediates in the formation of DGTS, pulse-chase experiments were carried out. Cells were incubated with 0.7 μCi of L-[1-^{14}C]methionine for 20 min, centrifuged, washed, and resuspended in nutrient solution. Samples were harvested after 0, 20, 40, and 120 min of chase. After 2D-TLC separation of the lipids, the radioactivity was measured. At the end of the pulse, the label incorporated into total lipids was 172,500 dpm (11.2%). The time course of labelling was different in DGTS and compounds 1 to 3, as shown in Fig. 2a. In compound 1, the label was highest at the end of the pulse and then steadily decreased during the chase period. In compounds 2 and 3, the label first increased and then decreased suggesting a turnover process. In DGTS, the radioactivity accumulated continuously over time.

These results demonstrate that in *R. sphaeroides*, methionine is used for the synthesis of the polar group of DGTS. The carboxyl group of L-[1-^{14}C]methionine is incorporated into DGTS as well as into compounds 1 to 3, which, on the basis of their labelling kinetics, are strongly suggested to be intermediates in the biosynthesis of DGTS.

To find whether compounds 1 to 3 contain *N*-methyl groups, a pulse-chase experiment was carried out with L-[*methyl*-^{14}C]methionine, which acts as the methyl donor in DGTS biosynthesis in algae, as a substrate (13, 14). After a pulse of 20 min, the radioactivity was measured at different times during a chase of 2 h. As demonstrated in Fig. 2b, compounds 2 and 3 and DGTS were labelled while compound 1 was inactive, indicating that no methyl group had been introduced into compound 1.

The radioactivity continuously decreased in compounds 2 and 3 but increased in DGTS, suggesting that the first two are partially N-methylated precursors of DGTS.

Since compound 1 was labelled with L-[1-^{14}C]methionine but not with L-[*methyl*-^{14}C]methionine as a precursor, it is supposed to be a nonmethylated precursor of DGTS biosynthesis.

When the products obtained by the pulse-chase experiment were analyzed by TLC (data not shown), three additional radioactive spots were detected. However, they did not contain

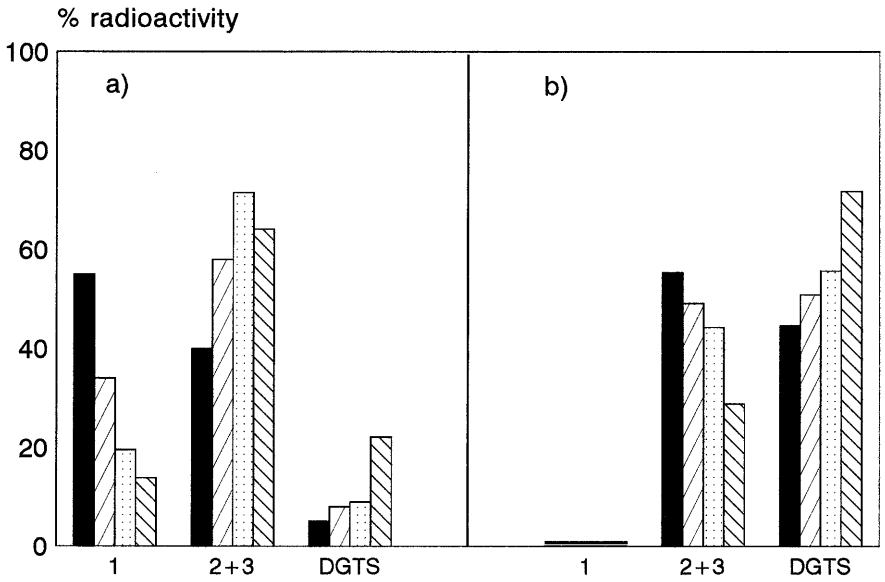

FIG. 2. Incorporation of L-[1-^{14}C]methionine (a) and L-[*methyl*-^{14}C]methionine (b) by *R. sphaeroides* into DGTS and compounds 1 to 3 with a pulse of 20 min and a chase of 120 min. Symbols: ■, no chase; ▨, 20-min chase; ▤, 40-min chase; ▧, 120-min chase.

intermediates of DGTS biosynthesis. Instead, they could be assigned to the phospholipids *N*-methyl- and *N*,*N*-dimethyl-PE and PC. They represent the biosynthetic route leading from PE to PC operating in this organism, as described elsewhere (2, 20).

Transformation of precursor lipids into DGTS by *O. danica*. If compounds 1 to 3 are precursors of DGTS, they should be converted to DGTS during incubation with cells. For these experiments, we used the chrysophycean alga *O. danica*, which easily takes up lipid substrates and which was used previously for studying DGTS biosynthesis (21, 22). Labelled compound 3 was isolated from *R. sphaeroides* cells that had been incubated with either L-[1-^{14}C]- or L-[*methyl*-^{14}C]methionine. It was then suspended in culture medium by sonication and incubated with 3×10^7 cells of *O. danica* for 24 h.

The cells were washed, and the lipids were extracted. After 2D-TLC, the plate was autoradiographed and the spots were measured by liquid scintillation. *methyl*-^{14}C- and *carboxyl*-^{14}C-labelled compound 3 were applied in two separate experiments, as shown in Table 1. Of the substrate used in the incubation mixture, 23.7 and 8.5% were incorporated into lipids by the cells with methyl- and carboxyl-labelled substrate, respectively. Although the yields of DGTS were different in the two experiments, 4.5% of the methyl-labelled and 2.6% of the carboxyl-labelled substrate applied were incorporated into DGTS.

TABLE 1. Transformation of labelled compound 3 from *R. sphaeroides* into DGTS by *O. danica*

Stage	Labelling (dpm) (% of original label) with substrate:	
	methyl-^{14}C	*carboxyl*-^{14}C
Substrate applied (compound 3)	27,000 (100.0)	23,000 (100.0)
Recovered from cells after 24 h	6,400 (23.7)	1,960 (8.5)
Compound 3	4,000 (14.8)	500 (2.2)
Lyso-compound 3	620 (2.3)	833 (3.6)
DGTS	1,215 (4.5)	600 (2.6)
DGTA	550 (2.0)	0 (0)

The concomitant formation of diacylglyceryl-*O*-hydroxymethyl-*N*,*N*,*N*-trimethyl-β-alanine (DGTA) from DGTS by *Ochromonas danica* is in accordance with the earlier work on the transformation of DGTS to DGTA by this organism (22). In both experiments, most of the label was found in unchanged substrate and in an additional spot, migrating much more slowly than the substrate on the plate. The same spot was obtained when compound 3 was treated with lipase from *Rhizopus arrhizus*, indicating that this compound is a partly deacylated compound 3. This again could be further deacylated by alkaline hydrolysis, confirming the presence of two fatty acid residues in compound 3.

Identification of compound 3. The data obtained strongly suggest that compounds 2 and 3 are partially N-methylated intermediates which, by further N methylation, are transformed to DGTS. The structures expected for compounds 2 and 3 were diacylglyceryl-*N*-methylhomoserine and DGDS, respectively. To identify the structures, labelled compounds 2 and 3 were cochromatographed in two different solvent systems by 2D-TLC with reference DGDS obtained from native DGTS by demethylation. Autoradiograms of both plates showed a clear coincidence of the radioactive spot of compound 3 with the fluorescent spot of the reference.

All these results are in accordance with the view that compound 3 is an *N*,*N*-dimethyl precursor of DGTS. Because of the small amount of material, chemical or spectroscopic identification was not possible.

Inhibition of N methylation by DAA. Experiments on the conversion into DGTS of compounds 1 and 2, which are supposed to correspond to a nonmethylated aminolipid and an *N*-methyl lipid, respectively, were impossible because sufficient amounts of radioactive material could not be accumulated. Therefore, we tried to enhance the yield of compound 1 by using DAA, which had been used as a methyltransferase inhibitor for the inhibition of N methylation of PE in rat hepatocytes (11). The results of this experiment are shown in Table 2.

In the presence of DAA at concentrations of more than 20 μM, the formation of DGTS drastically decreased while com-

TABLE 2. Incorporation of L-[1-^{14}C]methionine into DGTS and compounds 1 to 3 in the presence of DAA

DAA concn (μM)	% Radioactivity in[a]:			
	Compound 1	Compound 2	Compound 3	DGTS
20	1.6 (0)	11.8 (12.0)	12.8 (11.6)	73.8 (74.9)
40	5.6 (0)	16.6 (11.6)	17.3 (10.9)	60.5 (77.6)
60	9.8 (0)	25.5 (11.0)	37.4 (12.0)	27.3 (77.1)
80	14.1 (0)	22.0 (11.8)	39.8 (12.2)	24.1 (76.1)
100	7.8 (0)	28.3 (10.9)	43.3 (12.4)	7.8 (76.7)

[a] Values for controls are in parentheses.

pounds 1 to 3 accumulated. For each concentration of DAA, a control experiment was carried out. Despite the inhibitory effect of DAA, it was not possible to accumulate sufficient amounts of radioactive compounds 1 and 2 for further incubation. Nevertheless, the inhibitory effect of DAA is in accordance with the view that compounds 1 to 3 are intermediates of the N-methylation process catalyzed by one or several *N*-methyltransferases. Beside DAA, 2-hydroxyethylhydrazine (12) was also used as a potential inhibitor. This compound, however, did not have any effect on the formation of DGTS in *R. sphaeroides* (results not shown).

Characterization of the *N*-methyltransferase(s) involved in DGTS formation. The results provide strong evidence that the N methylation leading to the trimethylammonium group of DGTS is a multistep process which is very similar to the N methylation of PE leading to PC. This process is catalyzed by a single *N*-methyltransferase (EC 2.1.1.17), which has already been cloned (2).

The formal similarity of the two processes raised the question whether the same enzyme is involved in the formation of both DGTS and PC. An answer could be obtained by using a mutant of *R. sphaeroides* obtained by treatment of the wild-type strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Benning and Somerville (5). This mutant, CHB20, was deficient in the PC-forming *N*-methyltransferase and, hence, also in PC. This had been confirmed by complementation of the mutant with a DNA fragment containing a gene designated *pmtA*, which encodes *N*-methyltransferase (2). Our question was whether the PC-deficient mutant CHB20 was able to synthesize DGTS and its precursors. Cells (2.5×10^{10}) cultivated under phosphate-limiting conditions were incubated with L-[1-^{14}C]methionine in a pulse of 20 min followed by a chase of 2 h. This experiment (results not shown) clearly revealed that DGTS and compounds 1 to 3 were all labelled by the mutant to a similar extent and with the same labelling kinetics as observed with the wild type. From this, we conclude that in the PC-deficient mutant, the biosynthesis of DGTS is operating under phosphate-limiting conditions, indicating that the enzyme(s) for the formation of the trimethylammonium group of DGTS is present whereas the *N*-methyltransferase for the formation of PC is absent.

DISCUSSION

The lipid pattern of the photosynthetic bacterium *R. sphaeroides* greatly depends on the growth conditions. In a high-phosphate medium (1 mM phosphate), PC, PE, PG, SQDG, and ornithine lipid are the major components, with the phospholipids representing approximately 90% of the polar lipids (3). In a low-phosphate medium (0.1 mM phosphate), the amount of phospholipids is reduced to 22%, as demonstrated by Benning et al. (4), while the additional lipids monohexosyldiacylglycerol, glucosylgalactosyldiacylglycerol, and DGTS are produced under these phosphate-limiting conditions. The production of DGTS by *R. sphaeroides* offered the possibility to further investigate the biosynthetic route leading to this lipid.

When L-[1-^{14}C]- or L-[*methyl*-^{14}C]methionine was offered as a precursor to cells grown under phosphate-limiting conditions, radioactivity specifically appeared in the polar group of DGTS. This indicates that methionine acts as a precursor of DGTS, suggesting that for its synthesis, the same biosynthetic pathway is operating in this organism as in algae (9, 13, 14, 22). This process includes a transfer of the four-carbon backbone from methionine (most probably in the form of *S*-adenosylmethionine) to a diglyceride moiety and a three-step N methylation of the amino group. The sequence of these two processes, however, remained unclear, since intermediary compounds could never be detected in eukaryotic cells.

In *R. sphaeroides*, a labelled intermediate (compound 3) was formed, to which the structure of a DGDS could be assigned, on the basis of the following arguments: compound 3 (i) is labelled exclusively in the polar group with either L-[1-^{14}C]- or L-[*methyl*-^{14}C]methionine as a precursor, (ii) is deacylated in two steps and hence contains two fatty acids, (iii) is transitorily labelled during a pulse-chase, (iv) is converted into DGTS by cells of *O. danica*, and (v) comigrates with DGDS in 2D-TLC with two different solvent systems.

The presence of this *N*,*N*-dimethyl lipid and its conversion to DGTS strongly suggest that the N methylation is the last step in the biosynthesis of DGTS. Evidently, this process includes two preceding N-methylation steps with a nonmethylated amino lipid and an *N*-monomethyl lipid as intermediary products. This is in accordance with the presence of two other labelled compounds (compounds 1 and 2). Compound 1 is labelled with L-[1-^{14}C]- but not L-[*methyl*-^{14}C]methionine, as expected for a nonmethylated intermediate. Compound 2, in contrast, is labelled with both L-[1-^{14}C]- and L-[*methyl*-^{14}C]methionine, as expected for an *N*-monomethyl lipid. The labelling kinetics of the two compounds, compared with DGTS, are in accordance with their intermediary role. Final identification, however, was not achieved, because of the small, not chemically detectable amounts and because of the lack of corresponding reference compounds which are not semisynthetically available from DGTS.

On the basis of these results, we propose for DGTS a biosynthetic pathway which starts with a transfer of the C_4-α-amino acid moiety from methionine (probably *S*-adenosylmethionine), to a free or activated diacylglycerol, giving a nonmethylated amino lipid, as shown in Fig. 3. This, in turn, is used as a substrate for a three-step N methylation leading through *N*-methyl- and *N*,*N*-dimethylamino lipid to DGTS as the end product. This means that the N methylation occurs as the last step on the preformed lipid molecule and is therefore a lipid-linked process. In a very similar manner, PC is synthesized in microorganisms by a stepwise N methylation of PE through *N*-methyl- and *N*,*N*-dimethyl-PE (20). In *R. sphaeroides*, one single *N*-methyltransferase (EC 2.1.1.17) is responsible for the introduction of all three *N*-methyl groups into PC (2). On the other hand, two different enzymes are involved in the same process in yeasts (12). The similarity of the N-methylation process in PC and DGTS raised the question whether in *R. sphaeroides* the same enzyme is responsible for both reactions. Our experiments demonstrate that a PC-deficient mutant CHB20, lacking the corresponding *N*-methyltransferase, is able to produce DGTS under phosphate-limiting conditions. From this, it is concluded that one or several separate *N*-methyltransferases are responsible for the introduction of *N*-methyl groups into DGTS. Thus, the biosynthesis of

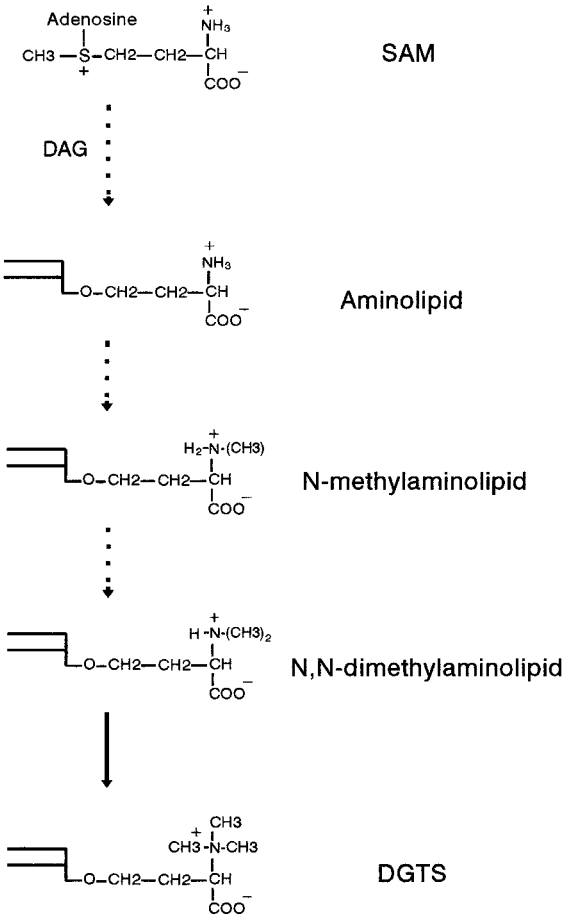

FIG. 3. Tentative pathway of DGTS biosynthesis. SAM, *S*-adenosylmethionine; DAG = diacylglycerol.

DGTS appears to be completely independent of the production of the phospholipid PC. Since the formation of DGTS in *R. sphaeroides* takes place under phosphate-limiting conditions which reduce the production of phospholipids, the betaine lipid is strongly suggested to substitute for the zwitterionic PC (4). This view is also favored by the observation that in many algae, betaine lipids and PC are complementary (1, 6, 7). A similar substitution of the anionic PG by SQDG has been discussed previously (3). However, the mechanism by which the production of additional lipids in *R. sphaeroides* is triggered under phosphate-limiting conditions, as well as the physiological role of this process, remains to be investigated further.

ACKNOWLEDGMENTS

This work has been supported by the Swiss National Science Foundation (grant 31-36078.92).

We are indebted to C. Benning, Institut für Genbiologische Forschung, Berlin, Germany, for the gift of the bacterial strains.

REFERENCES

1. **Araki, S., W. Eichenberger, T. Sakurai, and N. Sato.** 1991. Distribution of diacylglycerylhydroxymethyl-β-alanine (DGTA) and phosphatidylcholine in brown algae. Plant Cell Physiol. **32:**623–628.
2. **Arondel, V., C. Benning, and C. R. Somerville.** 1993. Isolation and functional expression in *Escherichia coli* of a gene encoding phosphatidylethanolamine methyltransferase (EC 2.1.1.17) from *R. sphaeroides*. J. Biol. Chem. **268:** 16002–16008.
3. **Benning, C., J. T. Beatty, and C. R. Somerville.** 1993. The sulfolipid sulfoquinovosyldiacylglycerol is not required for photosynthetic electron transport in *R. sphaeroides* but enhances growth under phosphate limitation. Proc. Natl. Acad. Sci. USA **90:**1561–1565.
4. **Benning, C., Z. H. Huang, D. A. Gage, and C. R. Somerville.** 1995. Accumulation of a novel glycolipid and a betaine lipid in cells of *Rhodobacter sphaeroides* grown under phosphate limitation. Arch. Biochem. Biophys. **327:**103–111.
5. **Benning, C., and C. R. Somerville.** 1992. Isolation and genetic complementation of a sulfolipid-deficient mutant of *Rhodobacter sphaeroides*. J. Bacteriol. **174:**2352–2360.
6. **Eichenberger, W.** 1993. Betaine lipids in lower plants. Distribution of DGTS, DGTA and phospholipids, and the intracellular localization and site of biosynthesis of DGTS. Plant Physiol. Biochem. **31:**213–221.
7. **Eichenberger, W., S. Araki, and D. G. Müller.** 1993. Lipids in brown algae: survey on the occurrence of betaine- and phospholipids in brown algae (Phaeophyceae). Phytochemistry **34:**1323–1333.
8. **Eichenberger, W., and A. Boschetti.** 1978. Occurrence of 1(3),2-diacylglyceryl-(3)-O-4′-(*N*,*N*,*N*)-trimethyl)-homoserine in *Chlamydomonas reinhardtii*. FEBS Lett. **88:**201–204.
9. **Eichenberger, W., and M. Hofmann.** 1992. Metabolism and distribution of betaine lipids in algae, p. 18–21. *In* A. Cherif et al. (ed.), Metabolism, structure and utilization of plant lipids. Centre National Pédagogique, Montfleury, Tunesie.
10. **Fischer, W., E. Heinz, and M. Zeus.** 1973. The suitability of lipase from *Rhizopus arrhizus* for analysis of fatty acid distribution in dihexosyl diglycerides, phospholipids and plant sulfolipid. Hoppe-Seyler's Z. Physiol. Chem. **354:**1115–1123.
11. **Haydn Pritchard, P., P. K. Chiang, G. L. Cantoni, and D. E. Vance.** 1982. Inhibition of phosphatidylethanolamine *N*-methylation by 3′-deazaadenosine stimulates the synthesis of phosphatidylcholine via the CDP-choline pathway. J. Biol. Chem. **257:**6362–6367.
12. **Nikawa, J., and S. Yamashita.** 1983. 2-Hydroxyethylhydrazine as a potent inhibitor of phospholipid methylation in yeast. Biochim. Biophys. Acta **751:** 201–209.
13. **Sato, N.** 1988. Dual role of methionine in the biosynthesis of diacylglyceryltrimethylhomoserine in *Chlamydomonas reinhardtii*. Plant Physiol. **86:**931–934.
14. **Sato, N.** 1991. Lipids in *Cryptomonas* CR-1. II. Biosynthesis of betaine lipids and galactolipids. Plant Cell Physiol. **32:**845–851.
15. **Sato, N.** 1992. Betaine lipids. Bot. Mag. Tokyo **105:**185–197.
16. **Sato, N., and K. Kato.** 1988. Analysis and biosynthesis of diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine in the cells of *Marchantia* in suspension culture. Plant Sci. **55:**21–25.
17. **Sistrom, W. R.** 1960. A requirement of sodium in the growth of *R. sphaeroides*. J. Gen. Microbiol. **22:**718–785.
18. **Sistrom, W. R.** 1962. The kinetics of the synthesis of photopigments in *R. sphaeroides*. J. Gen. Microbiol. **28:**607–616.
19. **Thies, W.** 1971. Schnelle und einfache Analyse der Fettsäurezusammensetzung in einzelnen Raps-Kotyledonen. Z. Pflanzenzücht. **65:**181–202.
20. **Vance, D. E., and N. D. Ridgway.** 1988. The methylation of phosphatidylethanolamine. Prog. Lipid Res. **27:**61–79.
21. **Vogel, G., and W. Eichenberger.** 1990. Biosynthesis and metabolism of betaine lipids in *Ochromonas danica* (Chrysophyceae), p. 235–237. *In* P. J. Quinn and J. L. Harwood (ed.), Plant lipid biochemistry, structure and utilization. Portland Press, London.
22. **Vogel, G., and W. Eichenberger.** 1992. Betaine lipids in lower plants. Biosynthesis of DGTS and DGTA in *O. danica* (Chrysophyceae) and the possible role of DGTS in lipid metabolism. Plant Cell Physiol. **33:**427–436.