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# Biphasic Changes in the Level and Composition of *Dunaliella salina* Plasma Membrane Diacylglycerols following Hypoosmotic Shock†

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ABSTRACT: Hypoosmotic shock has been shown to trigger an immediate and selective increase of plasma membrane diacylglycerols (DAG) in the green alga *Dunaliella salina*, coinciding with an approximately equivalent loss of phosphatidylinositol 4,5-bisphosphate from this membrane [Ha, K. S., & Thompson, G. A., Jr. (1991) *Plant Physiol. 97*, 921-927]. Following a slight decline in amount, DAG levels of the plasma membrane resumed their rise by 2 min after the shock and by 40 min had achieved a maximum concentration equivalent to 230% of DAG levels in unstressed cells. This second, more sustained increase of plasma membrane DAG was matched by a DAG increase in the microsome-enriched cytoplasmic membrane fraction, commencing at 2 min and peaking at 140% of control values. The changing pattern of DAG molecular species produced in the plasma membrane during the early phases of hypoosmotic stress was compatible with their derivation from phospholipase C hydrolysis of inositol phospholipids and phosphatidylcholine. From 8 min following hypoosmotic shock, as relatively larger scale DAG accumulations developed in the cytoplasmic membranes, the molecular species composition changed to reflect a marked increase in de novo synthesis of *sn*-1-oleoyl, *sn*-2-palmitoylglycerol, and dioleoylglycerol. The former molecular species appears to be synthesized in the chloroplast while the latter is produced in the endoplasmic reticulum. The radioisotope labeling data with $Na_2{}^{14}CO_3$ confirmed that the biphasic formation of DAG triggered by hypoosmotic shock culminates in a large-scale de novo synthesis of DAG. This is the first clear evidence for de novo synthesis as a source of DAG following $PIP_2$-mediated signaling. Cells briefly preincubated with [$^{32}$P]$P_i$ sustained a more pronounced labeling of an approximately 29-kDa protein during the first 30 s following hypoosmotic shock than did nonstressed cells during the same time period. The difference in labeling intensity between stressed and nonstressed cells was less marked during the second phase of DAG production.

Transmembrane signaling via the phospholipase C mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PIP_2$)[1] has been implicated in the activation of many cell types by external agonists and environmental stresses (Guy et al., 1989). We have shown this pathway to be triggered in the green alga *Dunaliella salina* by hypoosmotic stress (Einspahr et al., 1988; Ha & Thompson, 1991). A rapid increase in diacylglycerols (DAG), known to act as second messengers in this signaling pathway, occurred selectively in the plasma membrane during the brief period of maximal $PIP_2$ hydrolysis, and the principal molecular species of DAG which increased in amount there were characteristic of those predominating in inositol-containing phospholipids only (Ha & Thompson, 1991). The sudden stress-induced rise in *D. salina* DAG was superimposed on a significantly higher basal level of DAG than is generally found in animal cells. Those higher plants which have been analyzed (Morré et al., 1989; Morse et al., 1987) also contain relatively higher concentrations of DAG, raising the interesting question of how these systems discriminate between "signaling" and "non-signaling" DAG.

When quantitative analysis of the hypoosmotic shock-induced DAG content was extended to longer time periods, it became clear that a second, more pronounced phase of DAG production closely followed the first phase. In this respect, DAG kinetics in *D. salina* closely resemble those observed in a number of animal cell systems (Fukami & Takamura, 1989; Matozaki & Williams, 1989; Cook et al., 1990; Pessin et al., 1990). The present paper examines the composition, the intracellular location, and the metabolic sources of DAG arising

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[1] Abbreviations: DAG, diacylglycerol(s); PC, phosphatidylcholine; PI, phosphatidylinositol; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; PL, phospholipid; PKC, protein kinase C; in the shorthand numbering system used to identify fatty acids, the number preceding the colon indicates the number of carbon atoms and that following the colon the number of double bonds present; pairs of numbers designating fatty acids in this way, when placed before and after a slash, represent the components in the *sn*-1 and *sn*-2 positions, respectively, of a lipid molecular species.

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during the latter part of this biphasic stress response and correlates these DAG changes with concurrent alterations in the pattern of protein phosphorylation.

## Materials and Methods

*Chemicals.* $Na_2{}^{14}CO_3$ (52 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). [$^{32}$P]$H_3PO_4$, carrier-free, was supplied by DuPont–New England Nuclear (Wilmington, DE). *sn*-1,2-Distearoylglycerol and pentadecanoic acid were purchased from Sigma (St. Louis, MO). *Rhizopus arrhizus* lipase was also provided by Sigma.

*Cell Growth and Fractionation.* Cultures of *Dunaliella salina* (UTEX 1644) were grown under continuous light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) in 500 mL of synthetic medium bubbled with 0.5% $CO_2$-enriched air at 28 °C (Lynch & Thompson, 1982). Cells, grown to a cell density of approximately $10^6$ cells/mL as measured using a Coulter counter Model ZB and, in some cases, labeled with $Na_2{}^{14}CO_3$, were harvested and fractionated as described by Peeler et al. (1989). Briefly, following centrifugation at 650*g* for 6 min in a Sorvall GSA rotor, the resulting pellets were suspended in a cell disruption buffer (Peeler et al., 1989) and ruptured in a Parr pressure bomb. The suspension of broken cells was centrifuged at 2640*g* for 3.5 min (Sorvall HB-4 swing-bucket rotor) to pellet chloroplasts. In most experiments, the supernatant (nonchloroplast membrane fraction containing plasma membrane and cytoplasmic organelles) over the chloroplast pellet was then added to a dextran/poly(ethylene glycol) two-phase system, and the mixture was centrifuged at 660*g* for 10 min (Sorvall HB-4 swing-bucket rotor). Lipids were extracted directly from the resulting upper [poly(ethylene glycol)] phase, containing plasma membrane, and the lower (dextran) phase, containing the cytoplasmic membranes. In control experiments, no DAG was found in the supernatant recovered after an additional centrifugation of the upper or lower phases at high speed. Thus, all DAG were membrane-bound.

*Hypoosmotic Shock.* Hypoosmotic shock was induced by adding an equal volume of NaCl-free medium to a cell suspension concentrated to $2 \times 10^6$ cells/mL by gentle centrifugation (Maeda & Thompson, 1986). This reduced the NaCl molarity of the medium from the normal 1.7 M to 0.85 M. The final cell density, $10^6$ cells/mL, was equivalent to that of mid-logarithmic-phase cells normally used for experimentation.

*Lipid Extraction and Assay.* Lipids were extracted following the procedure of Bligh and Dyer (1959). Lipid phosphorus was measured according to Duck-Chong (1979) to overcome interference by high levels of poly(ethylene glycol) or dextran. DAG were purified and quantified according to a previous report (Ha & Thompson, 1991). Typically, total lipid extracts, containing a known amount of 1,2-distearoylglycerol added as an internal standard, were applied to a silica gel G plate containing 5.3% (relative to silica gel) boric acid and separated by one-dimensional TLC, using two sequential developments with chloroform/acetone (96:4, v/v) as the first solvent system and petroleum ether/ether/acetic acid (70:30:1, v/v) as the second. Following development, the TLC plate was sprayed with primulin, and the DAG band was identified under ultraviolet light. The DAG extracted from the silica gel with chloroform/mehanol (6:1, v/v) was 96% radiopure upon rechromatography. Fatty acid methyl esters were made by incubating the purified DAG in 5% methanolic HCl at 80 °C for 2 h and quantified by GC (Lynch & Thompson, 1984a).

*Analysis of DAG Molecular Species.* DAG molecular species were analyzed according to the procedures of Ha and Thompson (1991). Briefly, the purified DAG were applied to HPLC, and six separate peaks, representing six different DAG molecular species, were collected using a fraction collector. Following addition of a known amount of pentadecanoic acid as an internal standard, fatty acid methyl esters were made from each collection and quantified by GC. Radioactivity in intact DAG was quantified through the use of a HPLC radioactive flow detector (Ha & Thompson, 1991).

*Analysis of Fatty Acid Positional Distribution.* The positional distribution of fatty acids on DAG was determined using *Rhizopus arrhizus* lipase (Ha & Thompson, 1991). Each DAG sample was emulsified in 0.5 mL of 10 mM Tris buffer (pH 7.2) containing 3 mg of Triton X-100 (Sigma) and mixed with 10 $\mu$L (50 000 units) of the lipase. After 15 min at room temperature, the reaction mixture was concentrated to dryness under $N_2$ gas following the addition of 0.5 mL of ethanol. The lipids were redissolved in chloroform/methanol (1:1) and resolved into fatty acids, monoacylglycerols, and unhydrolyzed DAG by TLC. Each band was visualized and quantified by the above procedures.

*Protein Phosphorylation.* A total of $7 \times 10^6$ cells were washed with phosphate-free medium containing 2 mM MES (pH 6.0) and incubated in 10 mL of the same medium for 2 h (Peeler & Thompson, 1990). Cells were labeled with 5 $\mu$Ci of [$^{32}$P]$P_i$ for 10 min and given hypoosmotic stress for the desired times, namely, 0.5, 4, 8, and 40 min. After the cells were harvested in a microcentrifuge for 1 min, the resulting pellets were suspended in lysis buffer containing 50 mM HEPES (pH 7.4), 400 mM D-mannitol, 5 mM EDTA, 1 mM $MgCl_2$, 67 mM NaCl, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM sodium pyrophosphate, 1 mM phenylmethanesulfonyl fluoride, 1 $\mu$g/mL aprotinin, and 1% Triton X-100. The suspended cells were mixed with an equal volume of double-strength electrophoresis buffer (Laemmli, 1970), and the mixture was boiled for 3 min. After centrifugation in a microcentrifuge at 17000*g* for 15 min, the supernatants were electrophoresed on a 7.5–15% continuous gradient gel. Following electrophoresis, the gels were fixed, dried, and exposed to X-ray film for autoradiography. Autorads were sometimes scanned using an ISCO gel scanner Model 1312, and changes in radioactivity in stressed cells were estimated as percent of control density at each time point.

## Results

*Biphasic Changes in the Plasma Membrane DAG Level during Extended Hypoosmotic Stress.* Hypoosmotic shock was induced by adding an equal volume of NaCl-free medium to cells growing in a 1.7 M NaCl medium. At selected times following hypoosmotic shock, the cells were fractionated into plasma membrane and cytoplasmic membranes (the latter fraction containing all other particulate cell components except chloroplasts) by aqueous two-phase partitioning.

After hypoosmotic shock, the quantity of DAG in the plasma membrane increased in a biphasic manner (Figure 1). As we demonstrated in a previous report (Ha & Thompson, 1991), the early increase of the plasma membrane [DAG] reached a peak at 140% of control values by 30 s. No increase in [DAG] occurred in the cytoplasmic membranes during this early period. The DAG peak in the plasma membrane coincided with the rising phase of a pronounced but short-lived peak of phospholipase C mediated $PIP_2$ hydrolysis in the plasma membrane, and the molecular species of DAG reflected this origin (Ha & Thompson, 1991). After its initial rise, the amount of DAG declined slightly and then, after 2 min, resumed its increase, reaching a new maximum, equivalent to 227% of control values, at 40 min (Figure 1). Following this

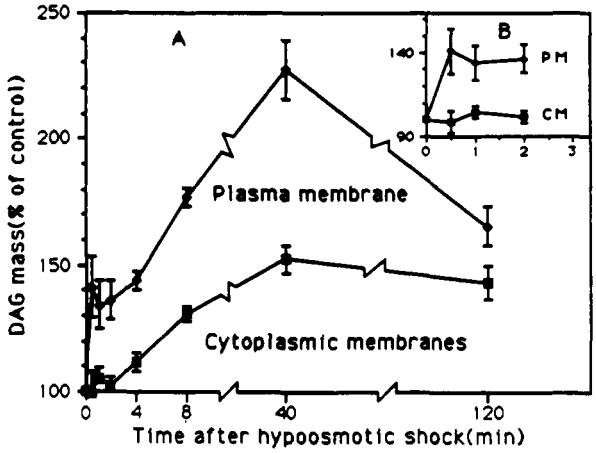


FIGURE 1: Time course of DAG mass changes following hypoosmotic shock. Cells were exposed to hypoosmotic stress for the indicated times and then fractionated (see Materials and Methods) into plasma membrane (closed diamonds) and cytoplasmic membranes (open squares). DAG were purified and quantified from both fractions as detailed under Materials and Methods (A). The inset (B) shows an expanded time scale of the first 2 min of stress. PM and CM represent plasma membrane and cytoplasmic membranes, respectively. Data are means ± SE from three independent experiments. Values are expressed as percent of control values obtained for nonstressed cells at each time point.

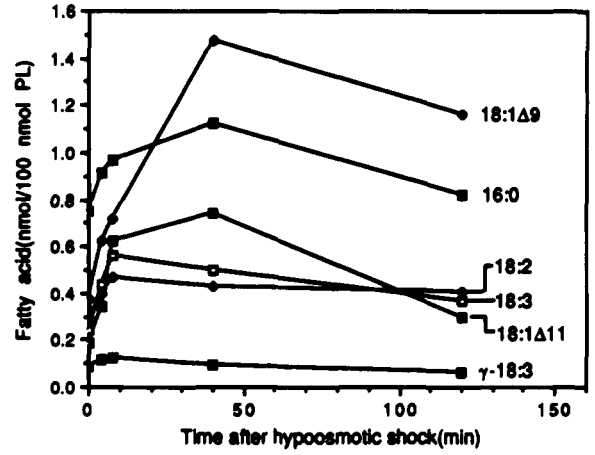


FIGURE 2: Time course of changes in fatty acid composition of plasma membrane DAG following hypoosmotic shock. Plasma membranes were isolated at the indicated times following hypoosmotic shock and utilized for the preparation of DAG. See Materials and Methods for details. Fatty acids were analyzed by GC. Data are means from three independent experiments, with most measurements agreeing to within 10%.

time point, DAG levels declined slowly. The biphasic increase of [DAG] was real and not simply relative to a possibly changing content of phospholipid. We could not detect any significant changes in the amount of individual phospholipids except polyphosphinositides following hypoosmotic shock (data not shown). This extended the observation of Einspahr et al. (1988) that no detectable changes occurred in the mass of major phospholipids such as PC, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylserine in the *D. salina* whole cells.

The fatty acid composition of plasma membrane DAG present at different times after hypoosmotic shock was determined in an effort to identify the metabolic origin of those molecules appearing during the second, prolonged phase of production (Figure 2). At 8 min, major increases in DAG fatty acids were registered in $18:1^{\Delta 9}$, 16:0, $18:1^{\Delta 11}$, 18:3, and 18:2, in decreasing order of magnitude. After 8 min, there was a further large increase in DAG, with the most prominent

Table I: Effect of Hypoosmotic Shock for 40 min on the Fatty Acid Positional Distribution of DAG in Total Nonchloroplast Membranes[a]

| | nmol/100 nmol of PL | | | |
|---|---|---|---|---|
| | control | | hypoosmotic stress for 40 min | |
| fatty acids | *sn*-1 | *sn*-2 | *sn*-1 | *sn*-2 |
| 16:0 | 0.60 ± 0.12 | 3.43 ± 0.13 | 0.90 ± 0.09 | 4.00 ± 0.26 |
| $18:1^{\Delta 9}$ | 1.20 ± 0.11 | 1.34 ± 0.02 | 2.72 ± 0.46 | 3.05 ± 0.35 |
| $18:1^{\Delta 11}$ | 0.65 ± 0.01 | 0.33 ± 0.06 | 1.21 ± 0.14 | 0.50 ± 0.11 |
| 18:2 | 1.82 ± 0.06 | 0.25 ± 0.02 | 1.84 ± 0.11 | 0.29 ± 0.05 |
| γ-18:3 | 0.28 ± 0.05 | 0.05 ± 0.01 | 0.26 ± 0.02 | 0.04 ± 0.00 |
| 18:3 | 0.95 ± 0.02 | 0.12 ± 0.02 | 1.22 ± 0.03 | 0.24 ± 0.04 |
| total | 5.50 ± 0.25 | 5.51 ± 0.25 | 8.14 ± 0.79 | 8.12 ± 0.81 |
| % of control | 100 | | 148 | |

[a] Results are the mean ± SD from three independent experiments.

fatty acids being $18:1^{\Delta 9}$ and 16:0 (up to 379% and 169% of control values, respectively). The data imply a rise in two DAG molecular species, 18:1/18:1 and 18:1/16:0, known to be the primary DAG molecular species synthesized de novo in plants. On the basis of our fatty acid analysis of the principal plasma membrane phospholipids (Peeler et al., 1989), this pattern suggested that PC hydrolysis contributed to the increase of DAG during the initial 8 min but that after 8 min a different source, probably de novo synthesis (see below), became predominant.

*Changes in the DAG Level in Cytoplasmic Membranes during Extended Hypoosmotic Stress.* In the endoplasmic reticulum enriched cytoplasmic membranes, where most lipid biosynthetic enzymes are located, there was no early increase in the DAG level following hypoosmotic shock, but a sharp rise occurred between 4 and 8 min afterward. This rise continued at a slower rate until 40 min, when a level equivalent to 153% of control values was reached. The level declined only slightly during the subsequent 80 min (Figure 1).

Those DAG fatty acid components exhibiting the greatest increases during this 40-min period were $18:1^{\Delta 9}$ and 16:0 (245% and 130% of control, respectively). The rise in these fatty acids was particularly significant because the absolute amount of cytoplasmic membrane DAG per cell was more than 8 times higher than that of plasma membrane DAG (Ha & Thompson, 1991).

*Changes of Fatty Acid Positional Distribution in Total Nonchloroplast DAG.* In an effort to predict the origins of DAG appearing during long-term hypoosmotic stress, the changes of the fatty acid positional distribution in DAG from the combined nonchloroplast membranes (plasma membranes plus cytoplasmic membranes) were determined (Table I). The major fatty acids which were elevated at the *sn*-1 position of DAG following hypoosmotic stress for 40 min were $18:1^{\Delta 9}$, $18:1^{\Delta 11}$, and 16:0 (increases of 1.52, 0.56, and 0.30 nmol/100 nmol of PL, respectively), while $18:1^{\Delta 9}$ and 16:0 (increases of 1.71 and 0.57 nmol/100 nmol of PL, respectively) showed the most pronounced increases at *sn*-2. The results indicated increases of DAG in 18:1/18:1, 18:1/16:0, and also 16:0/18:1, in decreasing order. These proportions were confirmed by direct HPLC analysis of DAG molecular species as described below.

*Changes of DAG Molecular Species in Plasma Membrane and Cytoplasmic Membranes.* HPLC revealed that in the plasma membrane molecular species containing 16:0 plus 18:2 and 16:0 plus 18:3 increased markedly within 8 min after hypoosmotic shock (Figure 3A). The positioning of the fatty acids on the glycerol moiety was not determined directly in this instance, but the only combination in potential substrate phospholipids which could account for the observed findings

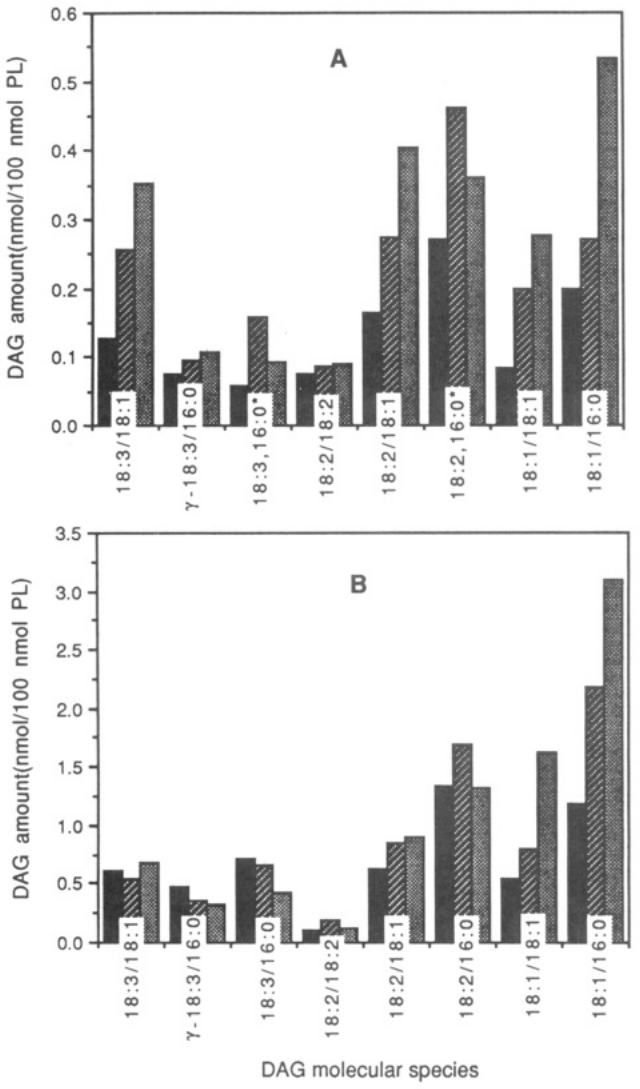


FIGURE 3: Hypoosmotic shock induced changes in the quantities of DAG molecular species. Cells, exposed to hypoosmotic shock for 8 min (hatched bars) and 40 min (stippled bars), were fractionated into plasma membrane (A) and cytoplasmic membranes (B), and then DAG were purified from both membranes. DAG molecular species were quantified by HPLC–GC as described under Materials and Methods. Black bars represent control values. The asterisks represent DAG whose positional distribution was not determined in these experiments. However, major molecular species in control cells were 18:3/16:0 and 18:2/16:0. Data are means from two independent experiments, and almost all values agree to within 10%.

is the 16:0/18:2 and 16:0/18:3 molecular species of PC, together accounting for 70% of that lipid class (Ha & Thompson, 1991). The content of these two molecular species declined in plasma membrane DAG by 40 min (Figure 3A), indicating that there was significant PC hydrolysis only during the first part of the sustained DAG rise. On the other hand, 18:1/16:0 and most molecular species containing two $C_{18}$ fatty acids showed a sizable increase at 8 min and a further large increase after 8 min. Together, these results suggested that both de novo synthesis and phospholipid hydrolysis contributed to the long-term DAG increase following hypoosmotic shock, with de novo synthesis predominating during the later stages.

DAG molecular species changes in the much larger cytoplasmic membrane fraction followed a somewhat different pattern. Only 18:1/18:1 and 18:1/16:0 experienced a pronounced increase, rising to 300% and 261%, respectively, of control values by 40 min (Figure 3B). These sharp increases

Table II: Specific Radioactivity of Supernatant 1,2-DAG Molecular Species from Cells Labeled with $Na_2{}^{14}CO_3$ for 20 min[a]

| molecular species | sp radioact. (dpm/nmol of DAG) | FA/glycerol (dpm/dpm) |
|---|---|---|
| 18:3/18:1 | 464 ± 104 | 2.4 |
| 18:3/16:0 | 274 ± 150 | 0.7 |
| 18:2/18:1 | 582 ± 72 | 1.9 |
| 18:2/16:0 | 329 ± 56 | 0.7 |
| 18:1/18:1 | 3224 ± 1304 | 5.9 |
| 18:1/16:0 and 16:0/18:1 | 1534 ± 33 | 8.2 |

[a] Results are the mean ± SD from three independent experiments.

in the primary products of de novo synthesis led us to suspect that DAG were being synthesized in the endoplasmic reticulum during the course of long-term hypoosmotic stress. The transport of small amounts of these species to the plasma membrane could easily account for the gradual accumulation noted in that fraction (Figure 3A).

*De Novo Synthesis of DAG in Total Nonchloroplast Membranes.* Further efforts were made to determine whether increased de novo synthesis of DAG occurred during long-term hypoosmotic stress. In an initial experiment, the specific radioactivity of the glycerol as well as the fatty acid moieties of DAG molecular species in the combined low-speed supernatant fraction (plasma membrane plus cytoplasmic membranes) was measured after labeling unstressed cells with $Na_2{}^{14}CO_3$ for 20 min. The radioactivity of the individual intact molecular species recovered after HPLC was used to measure specific radioactivity values, because fatty acids as well as glycerol were labeled with $Na_2{}^{14}CO_3$ (Table II). The 18:1/18:1 and 18:1/16:0 plus 16:0/18:1 molecular species showed specific radioactivities much higher than those of other molecular species. Most of the radioactivity in these highly labeled species was in fatty acids rather than in glycerol. The results confirmed that 18:1/18:1 and 18:1/16:0 plus 16:0/18:1 were formed largely from newly synthesized fatty acids.

To test the effect of hypoosmotic stress on this labeling pattern, *D. salina* cells were prelabeled with $Na_2{}^{14}CO_3$ for 12 min and then exposed to hypoosmotic stress for 8 min. If de novo synthesis of DAG is stimulated during the hypoosmotic stress period, the specific radioactivity of DAG should be increased over the control level by the production of additional radioactive DAG. The specific radioactivity of DAG was 1770 ± 252 dpm/nmol of DAG in stressed cells vs 755 ± 220 dpm/nmol of DAG in control unstressed cells. This result supports the compositional data indicating an increased de novo synthesis of 18:1/18:1 and 18:1/16:0 plus 16:0/18:1 DAG during long-term hypoosmotic stress.

*De Novo Synthesis of DAG in Chloroplasts.* The positioning of fatty acids on 18:1/16:0, the predominant DAG molecular species enhancing the cytoplasmic membranes following hypoosmotic shock (Table I and Figure 3B), is more typical of DAG formed in chloroplasts than in endoplasmic reticulum (Roughan & Slack, 1984). Only one nonchloroplast source of 18:1/16:0 exists in the cells. This is the inositol lipids, which account for about 15% of microsomal phospholipids (Einspahr et al., 1988) and are unexpectedly rich in this molecular species, with phosphatidylinositol (PI) containing about 50% 18:1/16:0 (unpublished data). The specific radioactivity of nonchloroplast PI from the $Na_2{}^{14}CO_3$-labeled and hypoosmotically stressed cells described above was, at 141 dpm/nmol of PI, less than 10% of the specific radioactivity of free DAG, and PI recovered from the chloroplast fraction was, at 152 dpm/nmol of PI, nearly 30 times less radioactive

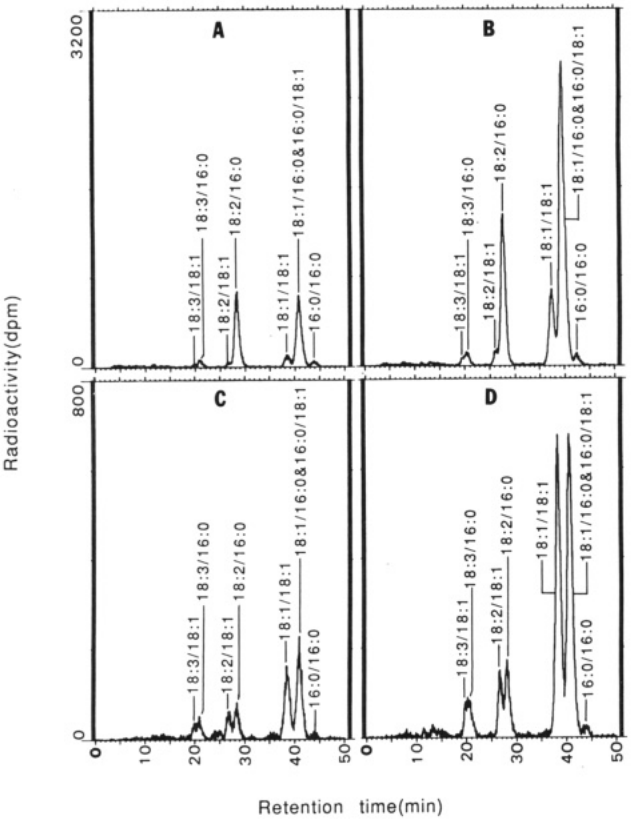


FIGURE 4: Hypoosmotic shock induced changes in radioactivities of DAG molecular species. Cells were prelabeled with $Na_2{}^{14}CO_3$ for 12 min and then exposed to hypoosmotic stress for 8 min. The labeled cells were fractionated into nonchloroplast membranes and chloroplasts. The radioactivity of individual DAG molecular species was measured by HPLC using an on-line radioactivity monitor. (A and B) Control and stressed chloroplasts, respectively; (C and D) control and stressed nonchloroplast membranes, respectively.

than free DAG from that fraction. Therefore, it seems highly unlikely that fatty acids or glycerol in the free DAG reached there by cycling through inositol phospholipids.

Changes of chloroplast DAG, the other potential suppliers to the nonchloroplast DAG pool, were measured to determine whether the 18:1/16:0 DAG of cytoplasmic membranes might originate there during long-term hypoosmotic stress. The specific radioactivity of chloroplast DAG, after labeling unstressed cells with $Na_2{}^{14}CO_3$ for 20 min, was, at 3081 ± 756 dpm/nmol of DAG, 4 times higher than that of DAG in the nonchloroplast membranes. When hypoosmotic shock was given 8 min prior to harvesting, the specific radioactivity of chloroplast DAG, at 4001 ± 587 dpm/nmol of DAG, was elevated by 30% over the value in unstressed cells and was more than twice as high as that of DAG from the companion nonchloroplast fraction. Increased radioactivity observed in nonchloroplast membranes of stressed cells was recovered mainly in two peaks, one containing 18:1/18:1 and the other a mixture of 16:0/18:1 and 18:1/16:0 (Figure 4, panels C and D). In contrast, chloroplast DAG from the same experiment contained a large increment of radioactivity in 18:1/16:0 but relatively low labeling in 18:1/18:1 (Figure 4, panels A and B). Chloroplasts would therefore appear to be a major site of stimulated 18:1/16:0 DAG biosynthesis during long-term hypoosmotic stress.

Quantitative analysis of DAG following hypoosmotic shock for 40 min was confirmatory, revealing a 241% rise of DAG mass in chloroplasts of the stressed cells (Table III). The fatty acid positional distribution on chloroplast DAG after stress showed large increases in $18:1^{\Delta 9}$ (2.02 nmol/100 nmol of PL)

Table III: Effect of Hypoosmotic Shock for 40 min on the Fatty Acid Positional Distribution of DAG in Chloroplasts[a]

| | nmol/100 nmol of PL | | | |
|---|---|---|---|---|
| | control | | hypoosmotic stress for 40 min | |
| fatty acids | *sn*-1 | *sn*-2 | *sn*-1 | *sn*-2 |
| 16:0 | 0.44 ± 0.15 | 1.31 ± 0.11 | 0.79 ± 0.06 | 3.32 ± 0.16 |
| $18:1^{\Delta 9}$ | 0.60 ± 0.06 | 0.43 ± 0.01 | 2.62 ± 0.19 | 1.31 ± 0.03 |
| $18:1^{\Delta 11}$ | 0.10 ± 0.04 | 0.24 ± 0.02 | 0.12 ± 0.03 | 0.38 ± 0.00 |
| 18:2 | 0.58 ± 0.05 | 0.11 ± 0.05 | 1.10 ± 0.02 | 0.15 ± 0.02 |
| γ-18:3 | 0.08 ± 0.03 | 0.02 ± 0.00 | 0.10 ± 0.01 | 0.03 ± 0.00 |
| 18:3 | 0.42 ± 0.07 | 0.10 ± 0.03 | 0.61 ± 0.04 | 0.13 ± 0.03 |
| total | 2.22 ± 0.06 | 2.21 ± 0.06 | 5.34 ± 0.24 | 5.32 ± 0.16 |
| % of control | 100 | | 241 | |

[a] Results are the mean ± SD from three independent experiments.

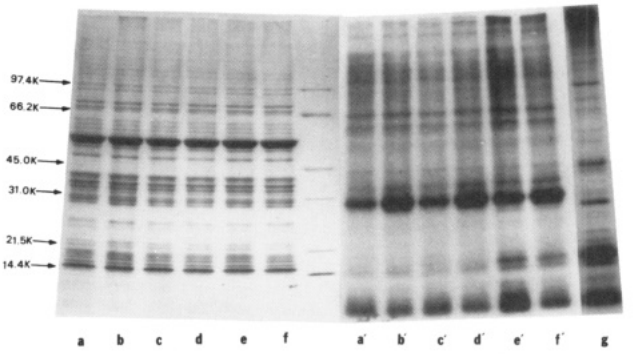


FIGURE 5: Hypoosmotic shock induced changes in protein phosphorylation. Cells were preincubated with $[^{32}P]P_i$ for 10 min and then exposed to hypoosmotic shock. Protein equivalent to $10^6$ cells was applied to each lane of a 7.5–15% continuous gradient SDS–PAGE gel. The Coomassie Blue staining pattern is shown in lanes a–f, and an autoradiogram of the same gel is shown in lanes a′–f′. The lanes represent control and stressed cells, respectively, sampled at 0.5 min (a′ and b′), 8 min (c′ and d′), and 40 min (e′ and f′). For comparison, the labeling pattern of control cells incubated with $[^{32}P]P_i$ for 12 h is shown in lane g.

at *sn*-1, and in 16:0 and $18:1^{\Delta 9}$ (2.01 and 0.88 nmol/100 nmol of PL, respectively) at *sn*-2 (Table III). Taken together, the chloroplast analyses established that 18:1/16:0, long known to be the major DAG molecular species formed in chloroplasts as a precursor of the phospholipids and glycolipids originating there (Roughan & Slack, 1984), increased conspicuously through in situ biosynthesis during 40 min of hypoosmotic stress. The surprising increase in the content of chloroplast 18:1/18:1, which is generally thought to be synthesized mainly in the endoplasmic reticulum, most probably results from importation.

*Hypoosmotic Shock Induced Changes in Protein Phosphorylation.* Both the increasing DAG levels shown above and the rise in inositol trisphosphate concentration (Einspahr et al., 1989) following hypoosmotic shock might be expected to trigger increased protein phosphorylation in *D. salina*. As a first step toward investigating this response, we preincubated cells for 10 min with $[^{32}P]P_i$ and then exposed half of the culture to hypoosmotic shock. Samples of stressed and control nonstressed cells were taken at 0.5-, 8-, and 40-min postshock for protein extraction and SDS–PAGE.

Nonstressed cells (Figure 5, lanes a′, c′, and e′) exhibited a gradually changing pattern of $^{32}P$ incorporation into proteins over the 40-min period. Some of the more prominently labeled bands in these cells were not detected in cells that had been labeled under the same conditions for 12 h (Figure 5, lane g), suggesting that the former proteins undergo relatively rapid turnover.

A marked increase in the radioactivity of a band at approximately 29 kDa was detected at 0.5 and 8 min following hypoosmotic shock (compare lanes a′ to d′, Figure 5). This difference between control and shocked cells declined perceptably with time until at 40 min the labeling patterns were nearly identical. The visual appearance of the autoradiograms (Figure 5, lanes a′–e′) was confirmed by densitometry, and the same trend was confirmed in three additional experiments, which also included a 4-min time point. In all these experiments, only a band of 29 kDa showed significant differences between stressed and nonstressed cells. A plot of labeling differences, as estimated by densitometry, confirmed a sharp initial increase in labeling of the 29–kDa band to 220% of control values at 0.5 min after hypoosmotic shock followed by a decline to 130% of control values at 4 min and another rise to 210% of controls at 8 min. By 40 min, the stressed cell 29-kDa band radioactivity had dropped nearly to the level in nonstressed cells. No short-term differences in labeling between stressed and nonstressed cells were found when cells were prelabeled for 12 h instead of 10 min.

## Discussion

Recent experimentation has shown many animal cells to exhibit a biphasic accumulation of DAG after stimulation, with the second phase being more prolonged and reaching a higher DAG concentration than the first (Matozaki & Williams, 1989; Fukami & Takamura, 1989; Augert et al., 1989; Pessin et al., 1990). The signaling function, generally expressed by the DAG-mediated activation of PKC, was often detected only during the initial peak of DAG production (Leach et al., 1991). The biphasic pattern of DAG increase observed in hypoosmotically shocked *D. salina* (Figure 1) was similar to that found in the above-mentioned animal cell studies and was also accompanied by a short-lived increase in protein phosphorylation (Figure 5).

Our previous work (Ha & Thompson, 1991) analyzed the initial DAG rise, which was confined to the plasma membrane. This change came about in large part through the hydrolysis of $PIP_2$ by a PI-specific phospholipase C localized in the plasma membrane (Einspahr et al., 1989). Some hydrolysis of phosphatidylcholine was also indicated. We have now characterized a second and more sustained rise in cellular DAG levels. This rise occurred in the plasma membrane just following the initial $PIP_2$-derived DAG elevation and in the cytoplasmic membranes as well (Figure 1). Because of their much larger bulk (Ha & Thompson, 1991), the cytoplasmic membranes contributed more than 5 times as much to the net increase in DAG per cell as did the plasma membrane.

It is often possible to identify a likely metabolic source of DAG on the basis of the composition and positioning of their fatty acids. *D. salina* is ideal for such an exercise because of the diverse and characteristic molecular species found in various potential donor phospholipids (Lynch & Thompson, 1984a,b; Peeler et al., 1989). By combining this type of DAG molecular species analysis with cell fractionation procedures, we have been able to reconstruct with a high degree of confidence the probable pathways of DAG synthesis and intracellular trafficking during stress.

As recently described (Ha & Thompson, 1991), the initial hypoosmotic shock induced rise in plasma membrane DAG concentration consisted mainly of the 18:1/16:0 molecular species. This species was atypical of the plasma membrane and was found in no other lipid of that fraction except the inositol phospholipids, which were observed to undergo rapid PI-specific phospholipase C mediated hydrolysis (Einspahr et al., 1988). During this early period, small amounts of 16:0/18:2 and 16:0/18:3 appeared, and our present studies show that these molecular species accumulated in larger amounts in the plasma membrane by 4 min of stress. Among all the *D. salina* phospholipids, these latter two molecular species are characteristic only of PC (Lynch & Thompson, 1984) and probably arise from that source, as has been reported in animal systems (Augert et al., 1989; Musch & Goldstein, 1990; Leach et al., 1991). While the hydrolysis of PC is normally attributed to phospholipase C action, DAG could also arise via the combined action of phospholipase D and phosphatidate phosphatase. In *D. salina*, the amount of whole cell phosphatidic acid increased by 40% of control values within 2 min after hypoosmotic shock, and then decreased nearly to control levels by 10 min (Einspahr et al., 1988). However, the present studies of DAG changes showed a maximal increase of DAG from PC turnover at 8 min following hypoosmotic shock. These results suggest that in *D. salina*, PC turnover following hypoosmotic shock occurs mainly through hydrolysis by phospholipase C rather than phospholipase D.

Table IV: Patterns of Major Molecular Species Increases in Nonchloroplast and Chloroplast Membranes following 40 min of Hypoosmotic Stress[a]

| molecular species | nmol/100 nmol of PL: nonchloroplast membranes | nmol/100 nmol of PL: chloroplast membranes |
|---|---|---|
| 18:1/18:1 | 1.51 | 0.74 |
| 18:1/16:0 | 0.57 | 1.30 |
| 16:0/18:1 | 0.30 | 0.35 |
| 18:2/16:0 | | 0.52 |
| sum of changes[b] | 2.62 | 3.12 |

[a] Data are means of three independent experiments. [b] Including unlisted molecular species increasing less than 0.30 nmol/100 nmol of PL.

As hypoosmotic stress was extended past 4 min, a sharp rise in the content of DAG was measured. Calculations based on data in Table I revealed that the major DAG molecular species increasing in the combined nonchloroplast membranes (plasma membranes and cytoplasmic membranes) during 40 min of hypoosmotic stress were 18:1/18:1, followed by 18:1/16:0 and 16:0/18:1 (Table IV). During the same period, chloroplast DAG concentration increased even more (Table III), with the largest change involving 18:1/16:0 and smaller changes occurring in 18:1/18:1, 18:2/16:0, and 16:0/18:1 (Table IV). Considering that 70% of the cellular phospholipid is in chloroplast membranes (Lynch & Thompson, 1982), the stress-induced DAG increase in that organelle, expressed on a nmol/100 nmol of whole cell phospholipid basis, was 2.7 times higher than in the combined nonchloroplast membranes.

Pulse radiolabeling data showed that the molecular species exhibiting the largest increases were much more highly labeled than the other molecular species present (Table II). As it is known that 16:0 and 18:1 are the initial products of fatty acid biosynthesis in plants (Harwood, 1988), we conclude that the rapid increases of 18:1/18:1, 16:0/18:1, and 18:1/16:0 DAG resulted mainly from de novo synthesis. The data from $Na_2{}^{14}CO_3$ labeling experiments support this conclusion. A surprising aspect of this study was the accumulation in the cytoplasmic membranes of approximately equal proportions of the two reverse positional isomers of the 16:0- and 18:1-containing DAG. Extensive research with both plants and animals has established that 16:0/18:1 is a primary DAG formed de novo in the endoplasmic reticulum while 18:1/16:0 is the major DAG formed de novo in the chloroplast (Roughan & Slack, 1984). We previously reported a surprisingly large

amount of the presumed chloroplast product, 18:1/16:0, in other membranes of unstressed *D. salina* (Ha & Thompson, 1991), and the present report not only confirms that finding but also reveals a substantial additional buildup of this molecular species in extrachloroplast compartments of the cell during prolonged hypoosmotic stress (Figure 3). During 40 min of stress, the cellular net increase of the 18:1/16:0 molecular species alone was more than 3 times greater, on a molar basis, than the entire $PIP_2$ content of the cells, suggesting that it did not arise through the same plasma membrane localized $PIP_2$ hydrolysis responsible for the first phase of 18:1/16:0-enriched DAG production. In theory, substantial amounts of 18:1/16:0 DAG could accumulate through a rapid turnover of the more abundant 18:1/16:0 PI, but this origin is not compatible with the results of our radioisotope labeling experiments (Einspahr et al., 1988).

The probable origin of the prominent 18:1/16:0 species was revealed when it was discovered that hypoosmotic stress induced a pronounced de novo synthesis of DAG within the chloroplast (Figure 4). Considering the amount of 18:1/16:0 generated in the chloroplast plus that accumulating outside the chloroplast during the period up to 8 min following hypoosmotic shock, it is clear that this molecular species was the chief contributor to the observed rise in [DAG]. This is the first report of stress-induced DAG synthesis in chloroplasts. The sudden 135% elevation of 18:1/16:0 DAG in the chloroplasts may well have led to its leakage into other cellular compartments. This could account for the observed increase of 18:1/16:0 in cytoplasmic membranes, which are normally expected to synthesize the reverse isomer, 16:0/18:1. Other chloroplast DAG molecular species, including 18:2/16:0 and two eukaryotic types (16:0/18:1 and 18:1/18:1), also increased in amount during 40 min of stress (Table IV). It has not been possible to establish the site of origin of these molecular species.

PC has been widely considered to serve as the principal source of DAG appearing during the second phase of accumulation in animal cells. In instances where fatty acid or molecular species analyses were performed, PC and DAG exhibited similar patterns. However, in at least some of these cases, a contribution of DAG via de novo synthesis would also be compatible with the findings. Thus, 16:0/18:1, a principal DAG molecular species observed to increase in thrombin-stimulated fibroblasts (Pessin et al., 1990), was presumed to arise mainly from PC hydrolysis because it was an abundant molecular species in that phospholipid class. De novo synthesis of this and other DAG molecular species was discussed, but the methods of analysis utilized were not adequate to judge the quantitative contribution of freshly made DAG.

In light of the evidence reported here favoring de novo synthesis as the major source of second-phase DAG accumulation in *D. salina*, it would seem desirable to test this possible origin in other experimental systems. The pattern we observe may be unique to plants and expressed solely in species having two independent and very active DAG-producing systems. It will be informative to examine DAG metabolism in some of the many plant species which have only a rudimentary DAG-producing system in their chloroplasts (Roughan & Slack, 1984).

What is the function of the elevated DAG accumulating in the cell over a period of more than 40 min? Martin et al. (1990) reported that the sustained second phase of thyrotropin releasing hormone induced DAG accumulation in cultured animal cells does not activate PKC as does the first phase. Since all the common naturally occurring DAG molecular species are almost equally effective in activating PKC in vitro (Kishimoto et al., 1980; Ebeling et al., 1985; Go et al., 1987), one must assume that the ineffectiveness of second-phase DAG is due either to a down-regulation of the kinase or to an inaccessibility of that DAG to the kinase. In the work by Martin et al. (1990), there was no down-regulation of PKC, but the molecular species composition and the intracellular localization of DAG were altered during the course of the response. Notably, DAG levels in the plasma membrane fell as those in intracellular membranes rose. In most systems under study (Augert et al., 1989; Leach et al., 1991), temporal changes in the size and location of expanding intracellular DAG pools have not been determined. It is interesting to note from our findings that while the initial hypoosmotic shock induced DAG increase in *D. salina* was confined to the plasma membrane, the second phase was localized mainly in the cytoplasmic membranes and the chloroplast. Plasma membrane DAG did ultimately rise to a new and higher peak during the second phase, but based upon the changing molecular pattern, we believe that a very substantial portion of these DAG originated elsewhere in the cell. It is not known whether DAG imported into the plasma membrane in this manner are distributed within the lipid bilayer differently from DAG generated in situ.

In our system, as in others, little is understood regarding the relative sensitivity of PKC to activation in vivo by DAG from diverse origins. Our initial studies of hypoosmotic shock induced protein kinase action (Figure 5) suggest that kinase activation is more pronounced during the initial phase of DAG production than during the second phase. However, we have not shown that the 29-kDa protein is phosphorylated by PKC, and typical PKC activity has been reported from only one plant source, *Amaranthus tricolor* (Elliott et al., 1988). It is quite possible that the phosphorylation we describe is accomplished by a different $Ca^{2+}$-requiring protein kinase such as the one described in *D. salina* by Guo and Roux (1990). The nature of the 29-kDa phosphorylated protein is also unknown. Phosphoproteins of the photosystem II light-harvesting complex fall within this molecular weight range, but the appropriate 28.5-kDa protein of *Dunaliella tertiolecta* appears not to have the requisite serine or threonine residue needed for kinase attack (LaRoche et al., 1990). A second interesting possibility is a 29-kDa GTP binding protein recently detected in the *D. salina* plasma membrane (M. P. Rodriguez Rosales, D. H. Herrin, and G. A. Thompson, Jr., unpublished observations). Phosphorylation of closely related small GTP binding proteins of HeLa cells has been implicated in their function during secretion (Bailly et al., 1991), and it is known that a massive fusion of vesicles with the plasma membrane accompanies hypoosmotic shock in *D. salina* (Maeda & Thompson, 1986). Work is currently underway to further characterize the substrate for shock-induced phosphorylation in this alga.

**Registry No.** 18:1/16:0 DAG, 29541-66-0; 18:1/18:1 DAG, 2442-61-7.

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# Enzymatic Synthesis and Structure of Precorrin-3, a Trimethyldipyrrocorphin Intermediate in Vitamin $B_{12}$ Biosynthesis†

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ABSTRACT: The trimethylated intermediate of vitamin $B_{12}$ (corrin) biosynthesis, precorrin-3, was produced from various $^{13}C$-enriched isotopomers of 5-aminolevulinic acid (ALA), using a multiple-enzyme system containing ALA dehydratase, porphobilinogen deaminase, uro'gen III synthetase, and the *S*-adenosyl-L-methionine- (SAM)-dependent uro'gen III methyltransferase (M-1) and precorrin-2 methyltransferase (M-2) in the presence of [$^{13}C$]SAM. Structural analysis of the resulting product, precorrin-3, reveals a close similarity to precorrin-2 but with several subtle differences in the conjugated array of C=C and C=N bonds which reflect the presence of the new *C*-methyl group at C20 and its influence on the electronic distribution in the dipyrrocorphin chromophore. The implications of this structure for corrin biosynthesis are discussed.

For the past 20 years, the elucidation of the biosynthetic pathway to vitamin $B_{12}$ has proven to be a major experimental challenge to chemists and biochemists alike. The reasons for this are manifold but include the small number of known biosynthetic intermediates, which are mostly oxygen sensitive, and the complexity of their structures which had to be determined at the submilligram level. However, the recent localization of the genes required for the synthesis of $B_{12}$ (Jeter & Roth, 1987; Crouzet et al., 1990a,b) has now paved the way for a more thorough understanding of approximately 20 enzyme-catalyzed steps involved in the formation of the corrinoids, which are among the most complex of natural products.

Cobyrinic acid (**6**), the simplest corrinoid precursor of $B_{12}$, is synthesized from 5-aminolevulinic acid (ALA)[1] by the shared pathway outlined in Scheme I via the first common

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[1] Abbreviations: ALA, 5-aminolevulinic acid; PBG, porphobilinogen; SAM, *S*-adenosyl-L-methionine; M-1 (or SUMT), uroporphyrinogen (uro'gen) III methyltransferase; M-2 (or $SP_2MT$), precorrin-2 methyltransferase; DTT, dithiothreitol; NMR, nuclear magnetic resonance.

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