# The Influence of the Glycerol-3-Phosphate Level in the Stroma Space on Lipid Synthesis of Intact Chloroplasts

Andreas Sauer and Klaus-Peter Heise

Lehrstuhl für Biochemie der Pflanze, Universität Göttingen, Untere Karspüle 2, D-3400 Göttingen

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The G3P level in chloroplasts, rapidly isolated from spinach leaves during a light-dark cycle, oscillated between 2.5 and 4 nmol · $mg^{-1}$ Chl, which corresponds to a concentration of nearly 0.1–0.2 mM. In order to study the role of the stromal G3P level on chloroplast lipid biosynthesis, G3P uptake, measured by silicone oil centrifugation, has been correlated with the lipid synthesizing capacity of intact spinach chloroplasts. The level of G3P in the chloroplast stroma was decreased by high orthophosphate ($P_i$) concentrations in the medium. This decrease was caused by a strong $P_i$ transport into the stroma, which is counterbalanced by a release of phosphorylated metabolites including G3P, mediated by the translocator. But because the reduced stromal G3P concentration exceeded about 3 times that for half saturation of the primary G3P acylation with oleoyl-ACP as preferred fatty acid donor, glycerolipid synthesis was not eliminated. Instead, the lowered G3P level in the stroma space limited the secondary acylation step, indicated by the reduced incorporation of palmitic acid into the diglyceride fraction, and led to an accumulation of free oleic acid. Thus, beside its function as primary acyl acceptor, the stromal G3P level apparently controls the pool size of ACP bound palmitic acid by limitation of the chain elongation step from palmitoyl- to stearoyl-ACP in order to induce the specific palmitic acid channeling into the C-2-position of chloroplast lipids by the secondary G3P acylation.

A similar function may be due to fatty acid consuming reactions from outside the chloroplast like acyl-CoA thioester formation in the outer envelope membrane, stimulated by exogenous CoA and ATP. In contradiction to earlier findings intact Percoll chloroplasts showed a measurable glycerolipid labelling (3–4%) from exogenous [$^{14}$C]oleoyl-CoA in the presence of G3P (0.5 mM), although most of the radioactivity was found in the free fatty acid fraction (7–10%). Incorporation into diglycerides was increased two-fold under fatty acid synthesizing conditions in the medium and the resulting diglycerides were further galactosylated by UDP-galactose addition. The latter observations suggest, that even in spite of the envelope impermeability for physiological concentrations of long-chain acyl-CoA thioesters, fatty acid transfer from these substrates to typical chloroplast lipids cannot be totally excluded.

## Introduction

Sn-glycerol-3-phosphate (G3P) serves as acyl acceptor in the glycerolipid synthesis of chloroplasts [1]. It is synthesized in the cytoplasm of plant leaf cells and is transported into the stroma of the plastid by the phosphate translocator located in the inner envelope membrane [2]. In the chloroplast, G3P is successively acylated with two fatty acid molecules which are synthesized in the stroma. The primary acylation reaction is catalyzed by a soluble stroma enzyme, while the secondary acylation step is bound to the envelope membrane. The probable reaction sequence is G3P → lysophosphatidic acids → phosphatidic acids → diglycerides [3]. In addition to glycerolipid synthesis, the G3P level seems to control also the proportion of oleate: palmitate incorporated into chloroplast lipids [4]. In order to clarify the regulatory influence of G3P on chloroplast lipid biosynthesis we investigated the correlation between G3P uptake and lipid synthesizing capacity of intact chloroplasts. The G3P level in the stroma space was manipulated with high exogenous concentrations of phosphate ($P_i$).

Reprint requests to Dr. A. Sauer and K.-P. Heise.

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## Materials and Methods

Spinach was grown in water cultures [5] and intact chloroplasts ($O_2$-evolution: 80–140 µmol · $mg^{-1}$ Chl · $h^{-1}$) were rapidly prepared in ice-cold media as in [2]. For determinations of the stromal G3P level, the ice-cold chloroplast suspension immediately after isolation was centrifuged through a layer of silicone oil into 20 µl 1 M $HClO_4$ using the method of Heldt *et al.* [2]. The G3P

concentration in the neutralized chloroplast extracts (bottom layer) was measured enzymatically via glycerophosphate dehydrogenase. For incubation experiments with [$^{14}$C]oleoyl-CoA the chloroplast preparations were further purified on preformed Percoll gradients according to Heinz *et al.* [6] in order to prevent microsomal contamination. The chloroplasts were incubated under saturating white light in a medium containing 0.33 M sorbitol, 50 mM Tricine/NaOH (pH 7.9), 10 mM $NaHCO_3$, 0.3 mM $K_2HPO_4$, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.15 mM Na-acetate. Further additions are noted in the legends of the figures and tables. Metabolite uptake was initiated by adding 10 µl of $^{14}$C- or $^{3}$H-labelled substrate to the chloroplast suspension and terminated by rapid centrifugation of the chloroplasts through silicone oil into 20 µl 1 M $HClO_4$ [2]. Extraction and detection of labelled lipids in the chloroplast sediment has been described earlier [7].

## Results and Discussion

Fig. 1 shows the concentration dependence of [$^{14}$C]G3P uptake by isolated intact chloroplasts together with the amount of G3P which is concurrently acylated to glycerolipids under fatty acid synthesizing conditions. The simultaneous acetate transport into the chloroplast apparently proceeds by free diffusion [8, 9]. The uptake of G3P follows saturation kinetics (Fig. 1 A). But the constants derivable from these kinetics [9] are not comparable with that found for G3P transport by Heldt *et al.* [2], because these authors measured the transport of phosphorylated metabolites in media free of inorganic phosphate and acetate at low temperatures (4 °C) in the dark. Up to 10% of the accumulating [$^{14}$C]G3P is acylated by newly synthesized fatty acids to form glycerolipids (Fig. 1 A, $-P_i$; Fig. 2 B).

In contrast, acyl-CoA synthesizing conditions (*i.e.* the presence of CoA and ATP, Fig. 1 B) in the medium, lead to a linear dependence of the G3P uptake on the G3P concentrations investigated and to a significant increase (up to 40% of G3P uptake) of the simultaneous glycerolipid synthesis (Fig. 1 B, $-P_i$). The latter observation may be due to a stimulation of fatty acid synthesis by the presence of CoA alone (unpublished results) or by the concerted action of CoA and ATP, causing the rapid conversion of newly synthesized long chain fatty acids to the corresponding acyl-CoA-thioesters [4] in the outer envelope membrane [10]. As recently shown [11], at a acyl-CoA/chlorophyll ratio (w/w) of more than 0.2, the acyl-CoA behaves like a detergent and thus permits acyl-CoA-thioesters to penetrate the

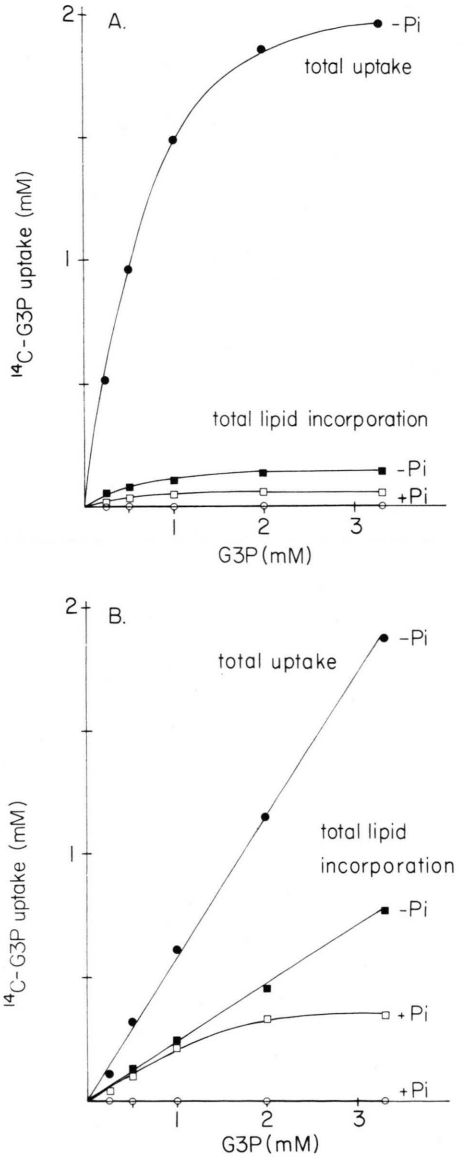


Fig. 1. Concentration dependent uptake (●, $-P_i$; ○, $+P_i$) and simultaneous acylation of [$^{14}$C]G3P (■, $-P_i$; □, $+P_i$) by intact spinach chloroplasts under fatty acid synthesizing conditions in the presence of 0.15 mM acetate (saturating white light, $T = 25$ °C) in the absence ($-P_i$) or presence ($+P_i$) of high concentrations of inorganic phosphate (15 mM) in the reaction mixture (A). The same measurements are repeated under acyl-CoA synthesizing conditions (+ 0.5 mM CoA + 2 mM ATP) (B).

envelope membrane, which normally represents a barrier for these substrates. High $P_i$ concentrations in the medium abolish G3P uptake (Fig. 1 A and 1 B). In earlier studies [2], the phosphate translocator, which mainly catalyzes a counterexchange of 3-phosphoglycerate, triosephosphate and $P_i$, was also found to transport G3P, although the $K_m$ for G3P was more than three times higher than that for the other substrates mentioned. An excessive $P_i$ transport into the stroma will therefore inhibit G3P uptake and simultaneously release phosphorylated stroma metabolites (inclusive G3P) by counterexchange [12]. While a depletion of stromal metabolites leads to an inhibition of $CO_2$ fixation [12] by high $P_i$ concentrations, acetate uptake is not affected and acetate incorporation into the lipid fraction is reduced only by 30–40% (unpublished results). Furthermore, simultaneously occurring pH changes in the stroma space which would result in significant changes in the acetate incorporation by chloroplast lipids [13], can be neglected. Since $P_i$ competitively inhibits G3P transport by the phosphate translocator across the chloroplast envelope, addition of $P_i$ to the medium is a means for manipulating the stromal G3P level and for studying its relation to glycerolipid synthesis by intact chloroplasts. But, corresponding to the relative high $K_m$ for the G3P transport, counterexchange by high $P_i$ concentrations in the medium leads only to a partial displacement of the G3P level, which accumulates in the stroma space during [$^{14}$C]G3P uptake by intact spinach chloroplasts (Fig. 2 $A_1$ and $A_2$). As shown, the remaining stromal G3P pool is about 2 nmol · mg$^{-1}$ Chl. Assuming a stromal volume of about 20 µl · mg$^{-1}$ Chl a residual G3P concentration of about 0.1 mM can be estimated. The latter concentration is nearly equal to the lowest values of the G3P levels, which have been measured in intact chloroplasts, immediately isolated from spinach leaves during a light-dark cycle, and which oscillate between 2.5 and 4 nmol · mg$^{-1}$ Chl. The reduced stromal G3P content (0.1 mM), caused by 15 mM $P_i$ in the medium, is about 3 times higher than the $K_m$ of the primary G3P acylation for G3P ($K_m = 31$ µM) in the chloroplast stroma, which has been recently determined for the stromal glycerol-3-P acyltransferase of spinach chloroplasts with oleoyl-ACP as physiological fatty acid donor [14]. The latter observation may explain, why even in spite of a drastic decrease of the stromal G3P

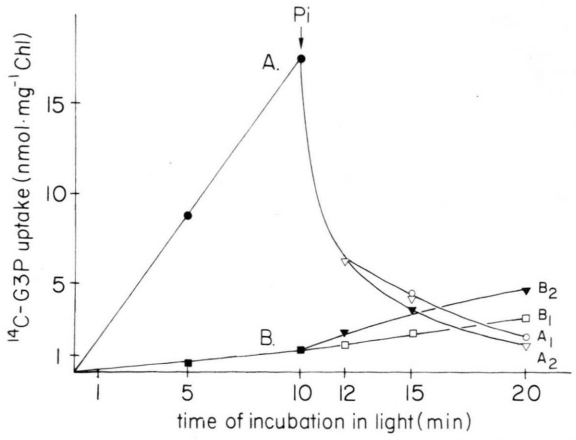


Fig. 2. Changes in the G3P level (A) and in the total glycerolipid synthesis (B) from [$^{14}$C]G3P (0.5 mM; specific activity: 10–20 mCi · mmol$^{-1}$) of intact spinach chloroplasts after addition of 15 mM $P_i$ in the absence ($A_1$, $B_1$) and presence ($A_2$, $B_2$) of acyl-CoA synthesizing conditions (+ 0.5 mM CoA + 2 mM ATP).

uptake by high $P_i$ concentrations (Fig. 1 A, 1 B and 2), glycerolipid synthesis is only partially reduced.

In order to characterize further the dependence of the lipid synthesizing capacity of intact chloroplasts on the G3P-level in the stroma space, the incorporation of labelled fatty acids from [$^3$H]acetate into the different lipid fractions in the absence and presence of high $P_i$-concentrations has been investigated (Table I, Fig. 3). As shown in Table 1, intact chloroplasts show in G3P free media a reduced capacity to incorporate freshly synthesized fatty acids (from [$^3$H]acetate) into glycerolipids (about 30%). This diminished ability for G3P acylation may be due to stromal G3P dilution by counterexchange during isolation procedures. Initiation of G3P transport into the stroma, in contrast, mediated by addition of G3P (0.5 mM) to the medium, leads to a significant enhancement of G3P acylation, which is demonstrated by an accumulation of labelled fatty acids in diglycerides (44%) and phospholipids (24%) at the expense of free fatty acids. The stimulation of G3P acylation is further accompanied by a significant increase in the portion of free and lipid bound $C_{16}$-fatty acids (Table I, Fig. 3). Similar data have already been reported by other authors (4).

The observed changes in glycerolipid synthesizing capacity of intact chloroplasts, caused by increasing G3P levels in the stroma, are partially

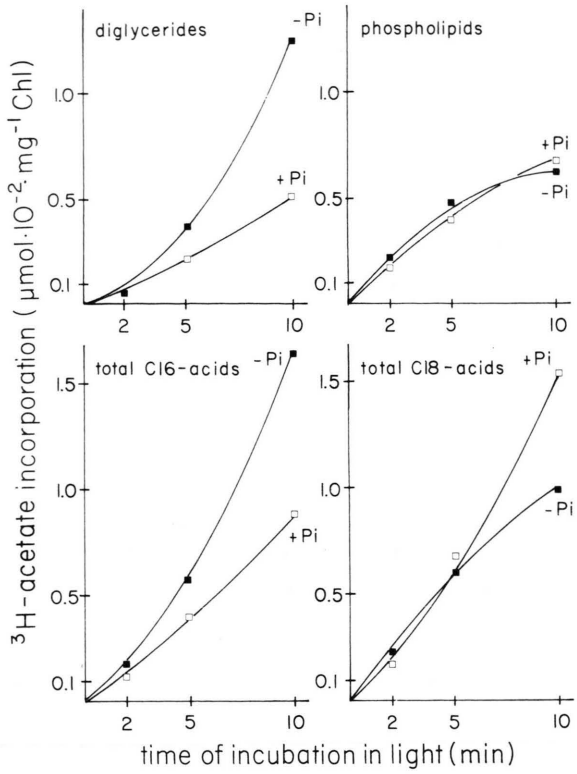


Fig. 3. Lipid synthesis from [$^3$H]acetate (presence of 0.5 mM G3P) into the mainly labelled lipid fractions of intact spinach chloroplasts in the absence (− $P_i$) and presence (+ $P_i$) of 15 mM $P_i$ in the reaction mixture. Final acetate concentration was 0.15 mM (specific activity: 520 mCi · mmol$^{-1}$).

neutralized by high $P_i$ concentrations (15 mM) in the medium. This observation is essentially manifested by a reduced fatty acid incorporation (especially palmitic acid) into the diglyceride fraction combined with an accumulation of free fatty acids (preferentially oleic acid) (Table I, Fig. 3). Compared to the $P_i$ induced inhibition of diglyceride synthesis, the fatty acid incorporation into phosphatides, which are predominantly represented by phosphatidic- and lysophosphatidic acids, is much less affected (Table I) and shows an increasing tendency to accumulate $C_{18}$-fatty acids (especially oleic acid). With reference to the above calculations, this observations suggest, that the reduced stromal G3P level will suffice to drive the primary G3P acylation in the stroma with oleoyl-ACP as preferred physiological fatty acid donor, but will limit an introduction of $C_{16}$-fatty acids by the envelope bound secondary acylation step, which specifically transfers palmitic acid onto the C-2-position of the glycerol backbone of chloroplast lipids [14]. Analogous to increasing stromal G3P levels, the stimulation of acyl-CoA-thioesterformation in the outer envelope membrane by the presence of CoA and ATP in the medium [10] leads not only to an enhancement of lipid biosynthesis (Fig. 1 B) of intact chloroplasts from acetate, but also to an accumulation of $C_{16}$-fatty acids in these lipids (Table I). Therefore we suggest, that increasing amounts of substrates (like G3P or CoA

Table I. Lipid synthesis from [$^3$H]acetate (final concentration: 0.15 mM-specific activity: 520 mCi · mmol$^{-1}$) by isolated intact spinach chloroplasts. The chloroplasts were incubated at pH 8.0 under saturating white light (25 °C). Lipid synthesizing conditions in the reaction mixture were manipulated by the addition of G3P (0.5 mM), CoA (0.5 mM) + ATP (2 mM) and $P_i$ (15 mM) in accordance with Figs. 2 and 3. In brackets the distribution of label between oleic- and palmitic acid (expressed as $^{14}C_{18:1}/^{14}C_{16:0}$-ratio) is given.

| "Cold" additions to basal medium | Total incubation time [min] | Percentage of total lipid radioactivity in the fatty acid fraction of [Proportion of oleic-compared to palmitic acid label in] | | | |
|---|---|---|---|---|---|
| | | Phospholipids[a] | 1-Mono-glycerides | Diglycerides | Free fatty acids |
| – | 10 | 9 [1.3] | 4 [5.4] | 15 [1.2] | 70 [9.3] |
| CoA, ATP | 10 | 16 [1.8] | 9 [7.5] | 30 [0.7] | 44 [4.2] |
| G3P | 10 | 24 [1.1] | 5 [2.6] | 44 [0.8] | 24 [2.4] |
| G3P + $P_i$ | 10 | 19 [2.2] | 7 [3.4] | 22 [1.0] | 48 [3.6] |
| G3P + $P_i$ after 10 min | 20 | 16 [1.3] | 3 [3.4] | 43 [0.7] | 34 [4.5] |
| G3P + $P_i$, CoA, ATP after 10 min | 20 | 23 [1.5] | 1 [2.3] | 49 [0.5] | 24 [3.4] |
| G3P, CoA, ATP | 10 | 27 [1.6] | 6 [4.0] | 45 [0.6] | 20 [1.9] |
| G3P, CoA, ATP + $P_i$ | 10 | 22 [0.8] | 3 [2.7] | 33 [0.8] | 40 [1.3] |

[a] The phospholipids are predominantly represented by phosphatidic- and lysophosphatidic acids.

Table II. Incorporation of oleic acid from [$^{14}$C]oleoyl-CoA into lipid fractions of intact spinach chloroplasts, purified on Percoll gradients, under glycerolipid synthesizing conditions (presence of 0.5 mM G3P). The samples were incubated for 20 min at pH 7.6 under saturating white light (25 °C). Final concentration of [$^{14}$C]oleoyl-CoA was 7.8 μM (specific activity: 40 mCi · mmol$^{-1}$). Furthermore following modifications of lipid synthesizing conditions in the reaction mixture during chloroplast incubations were adjusted: Stimulation of chloroplast fatty acid synthesis by addition of acetate (0.15 mM). Initiation of galactolipid synthesis by addition of UDP-galactose (0.1 mM) after 20 min (for further 10 min). Introduction of G3P deficiency in the stroma space by the presence of 15 mM $P_i$ in the medium. Total uptake of [$^{14}$C]oleoyl-CoA by intact chloroplasts was about 10–15%. Most of the label was detected as free oleic acid (7–10%) while only 0.5–1% was found in the stroma fraction.

| "Cold" additions to basal medium | Total incubation time | Percentage of radioactivity from [$^{14}$C]oleoyl-CoA incorporated into the fatty acid fractions of | | | | | |
|---|---|---|---|---|---|---|---|
| | min | Phosphatidylcholine | lyso-Phosphatidic acid | Phosphatidic acid | Monoglycerides | Diglycerides | Galactolipids |
| G3P | 20 | 0.5 | 1.0 | 0.8 | 0.3 | 0.6 | 0.2 |
| G3P, $P_i$ | 20 | 0.5 | 1.3 | 0.8 | 0.3 | 0.5 | 0.1 |
| G3P, acetate | 20 | 0.5 | 0.9 | 0.9 | 0.4 | 1.3 | 0.2 |
| G3P, acetate, $P_i$ | 20 | 0.4 | 0.6 | 0.8 | 0.4 | 1.3 | 0.1 |
| G3P, acetate + UDP-gal. (after 20 min) | 30 | 0.7 | 0.8 | 0.4 | 0.1 | 2.1 | 1.3 |
| G3P, acetate, $P_i$ + UDP-gal. (after 20 min) | 30 | 0.2 | 0.4 | 0.3 | 0.1 | 1.9 | 2.0 |

and ATP), which drive fatty acid consuming reactions either in the stroma (*e.g.* glycerol 3-P acyltransferase) or in the envelope membrane (*e.g.* acyl-CoA synthetase) of chloroplasts, may induce a direct channeling of palmitic acid transfer from palmitoyl-ACP, which is known to be a poor substrate for the acyl-ACP hydrolase [15] as well as for the primary acylation reaction of G3P [14], onto the C-2-position of 1-oleoyl glycerol 3-P. An explanation for this specific $C_{16}$ channeling reaction may be a limitation of the elongation reaction from palmitoyl- to stearoyl-ACP [15] by high levels of above mentioned substrates.

In order to elucidate the function of long-chain acyl-CoA thioesters in chloroplast lipid biosynthesis, we investigated lipid labelling from exogenous [$^{14}$C]oleoyl-CoA in intact spinach chloroplasts under glycerolipid synthesizing conditions (presence of G3P, Table II). The chloroplast preparations are purified on Percoll gradients to avoid microsomal contamination and oleoyl-CoA is offered in low concentrations to prevent its detergent character [11]. Even in spite of the fact, that only long-chain acyl-ACP thioesters are taken to be the physiological fatty acid donors for G3P acylation in chloroplasts and that the chloroplast envelope represents a barrier for physiological concentrations of long-chain acyl-CoA-thioesters [14], measurable incorporation of oleic acid from [$^{14}$C]oleoyl-CoA into chloroplast glycerolipids (3–4%) has been observed (Table II). Most of the label, however, has been detected as free oleic acid (7–10%) indicating thioesterase activity in the chloroplast envelope [11]. The observed incorporation of oleic acid into glycerolipids is not affected by $P_i$ induced lowering of the stromal G3P level. Simultaneous stimulation of fatty acid synthesizing conditions in the medium by addition of cold acetate leads to a duplication of oleic acid incorporation from [$^{14}$C]oleoyl-CoA into diglycerides, which may be due to the enhanced secondary acylation of G3P with freshly synthesized palmitic acid. After addition of UDP-galactose the resulting diglycerides are further galactosylated to form galactolipids (Table II). In contradiction to earlier findings [11], these results indicate, that a fatty acid transfer from exogenously added long-chain acyl-CoA thioesters onto typical plastidary lipid fractions of intact Percoll chloroplasts cannot be totally excluded.

[1] M. Bertrams and E. Heinz, Plant Physiol. **68,** 653–657 (1981).
[2] R. Fliege, U. I. Flügge, K. Werdan, and H. W. Heldt, Biochim. Biophys. Acta **502,** 232–247 (1978).
[3] J. Joyard and R. Douce, Biochim. Biophys. Acta **486,** 273–285 (1977).
[4] P. G. Roughan and C. R. Slack, Ann. Rev. Plant Physiol. **33,** 97–132 (1982).
[5] R. McC. Lilley and D. A. Walker, Biochim. Biophys. Acta **368,** 269–278 (1974).
[6] R. Haas, H. P. Siebertz, K. Wrage, and E. Heinz, Planta **148,** 238–244 (1980).
[7] A. Sauer and K.-P. Heise, Z. Naturforsch. **37c,** 218–225 (1982).
[8] B. S. Jacobson and P. K. Stumpf, Arch. Biochem. Biophys. **153,** 656–663 (1972).
[9] A. Sauer and K.-P. Heise, Biochemistry and Metabolism of Plant Lipids (P. J. C. Kuiper and J. F. G. M. Wintermans, eds.), pp. 187–190, Elsevier/North-Holland Biomedical Press, Amsterdam 1982.
[10] A. J. Dorne, M. A. Block, J. Joyard, and R. Douce, Biochemistry and Metabolism of Plant Lipids (P. J. C. Kuiper and J. F. G. M. Wintermans, eds.), pp. 153–164, Elsevier/North-Holland Biomedical Press, Amsterdam 1982.
[11] M. Bertrams, K. Wrage, and E. Heinz, Z. Naturforsch. **36c,** 62–70 (1981).
[12] U. I. Flügge, M. Freisl, and H. W. Heldt, Plant Physiol. **65,** 574–577 (1980).
[13] A. Sauer and K.-P. Heise, submitted for publication in Plant Physiol.
[14] M. Frentzen, E. Heinz, T. McKeon, and P. K. Stumpf, Biochemistry and Metabolism of Plant Lipids (P. J. C. Kuiper and J. F. G. M. Wintermans, eds.), pp. 141–152, Elsevier/North-Holland Biomedical Press, Amsterdam 1982.
[15] P. K. Stumpf, T. Shikamata, K. Eastwell, D. J. Murphy, B. Liedvogel, J. B. Ohlrogge, and D. N. Kuhn, Biochemistry and Metabolism of Plant Lipids (P. J. C. Kuiper and J. F. G. M. Wintermans, eds.), pp. 3–11, Elsevier/North-Holland Biomedical Press, Amsterdam 1982.