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Biosynthesis of Sulfoquinovosyldiacylglycerol in Higher Plants[1]

Use of Adenosine-5′-Phosphosulfate and Adenosine-3′-Phosphate 5′-Phosphosulfate as Precursors

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ABSTRACT

Adenosine-5′-phosphosulfate (APS) and adenosine-3′-phosphate 5′-phosphosulfate (PAPS) have been used as precursors of sulfoquinovosyldiacylglycerol (SQDG) in intact chloroplasts incubated in the dark. Competition studies demonstrated APS was preferred over PAPS and SO_4^{2-}. Rates of SQDG synthesis up to 3 nanomoles per milligram of chlorophyll per hour were observed when [^{35}S]APS and appropriate cofactors were supplied to chloroplasts incubated in the dark. The pH optimum for utilization of APS was 7.0. The incorporation was linear for at least 30 minutes. ATP and UTP stimulated the incorporation of sulfur from APS into SQDG, but the most stimulatory additions were DHAP and glycerol-3-P. The concentration curve for APS showed a maximum at 20 micromolar in the absence of DHAP and 30 micromolar in the presence of DHAP. The optimum concentration of DHAP for conversion of APS into SQDG was 2 millimolar. Rates of synthesis up to 4 nanomoles per milligram of chlorophyll per hour were observed when [^{35}S]PAPS was the sulfur donor and appropriate cofactors were supplied to chloroplasts. Optimal rates for conversion of sulfur from PAPS into SQDG occurred with concentrations of DHAP between 5 and 10 millimolar. DHAP was by far the most effective cofactor, although ATP and UTP also stimulated the utilization of PAPS for SQDG biosynthesis. In general, triose phosphates, including glycerol-3-P were not effective cofactors for SQDG biosynthesis.

The biosynthetic pathway involved in sulfate incorporation into the headgroup of SQDG[2] remains undetermined, over 30 years after the discovery of SQDG by Benson (2, 18). Higher plant chloroplasts are completely autonomous for the synthesis of SQDG, both the diacylglycerol moiety (13, 17) and the headgroup (7, 17), as well as for sulfate metabolism (24, 28). Sulfate must first be activated prior to its metabolism to reduced sulfur-containing compounds such as cysteine and methionine (23, 28). Substitution of ATP or an ATP-generating system for the light requirement for SQDG synthesis from $^{35}SO_4^{2-}$ in spinach chloroplasts (15) implies the activation of sulfate to APS is also an essential step in SQDG biosynthesis. The possible involvement of APS in SQDG synthesis has been suggested by many (1, 2, 8, 9, 18, 20). However, the direct involvement of APS as an intermediary in SQDG biosynthesis, either by competition studies or by the incorporation of [^{35}S]APS into SQDG, has not been shown to date as it has for cysteine biosynthesis (23).

There are several advantages in demonstrating [^{35}S]APS incorporation into SQDG. Sulfate incorporation into SQDG requires light or ATP and magnesium ions and is stimulated by the addition of UTP, DHAP, and Pi (15, 17). These compounds may be affecting sulfate uptake, the synthesis of APS or the further incorporation of APS into SQDG. In identifying APS as the next metabolic step in SQDG biosynthesis, participation of one enzyme on the pathway, the ATP sulfurylase, is established. The study of SQDG synthesis is also simplified in bypassing the light or ATP requiring step and true cofactors required for the remaining steps should become more evident (18). Such studies require a more sophisticated precursor such as APS.

APS can form bound sulfite or PAPS (28). PAPS is the sulfur donor for reactions involving sulfate-ester formation in other systems (28). It would be of interest to test PAPS in this system to determine whether it can be used as a precursor for SQDG. Hoppe and Schwenn (11) utilized [^{35}S]PAPS, $^{35}SO_3$ and $^{35}SO_4$ as precursors of SQDG in a homogenate of *Chlamydomonas*. In that system, PAPS proved the better precursor. APS was not tested. Sulfite is not a satisfactory precursor for such studies since (a) it is readily oxidized to sulfate, (b) sulfite and 5′-AMP can form APS by the reverse reaction of the APS-reductase (21, 22), and (c) the sulfite reductase can interconvert free sulfite and sulfide (28). Sulfite is also undesirable as a precursor because it inhibits photosynthesis (27), oxidizes fatty acid double bonds (19), and reacts with sulfhydryl groups (12). The nonsaturable kinetics of sulfite incorporation into SQDG in *Chlamydomonas* (11) emphasize the difficulties of using sulfite.

A competition study between APS, PAPS, and sulfate reported in a preliminary communication (16) indicated APS was the preferred precursor of SQDG in intact spinach chlo-

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[2] Abbreviations: SQDG, sulfoquinovosyldiacylglycerol; APS, adenosine-5′-phosphosulfate; BTP, bistrispropane; DHAP, dihydroxyacetone phosphate; DG, diacylglycerol; OAA, oxaloacetate; PAPS, adenosine-3′-phosphate 5′-phosphosulfate; 3-PGA, 3 phosphoglycerate.

roplasts. However, the reported rates of SQDG synthesis from APS or PAPS were lower than those when sulfate was the precursor. This prompted a more thorough study, to determine the suitability of APS as a precursor of SQDG, to confirm its involvement as an intermediary at rates comparable to those of sulfate and to verify the involvement of several compounds, previously shown to influence sulfate incorporation into SQDG, in the later biosynthetic steps of the headgroup.

In the current study of SQDG biosynthesis, intact spinach chloroplasts are incubated with [^{35}S]APS or [^{35}S]PAPS in the dark to eliminate the light dependent reductive steps of sulfate metabolism and several competing reactions which require APS. Rates of SQDG synthesis are comparable or in some cases superior to those obtained in a similar study (15) which utilized $^{35}SO_4^{2-}$ as the precursor of SQDG, in the presence of ATP, in dark incubated spinach chloroplasts. These studies verify that UTP stimulates SQDG synthesis and indicate the involvement of DHAP in the synthesis of SQDG.

MATERIALS AND METHODS

$^{35}SO_4^{2-}$ and [^{35}S]PAPS were purchased from New England Nuclear. [^{35}S]APS was synthesized by incubating carrier free $^{35}SO_4^{2-}$ with 1 mM ATP, 1 mM $MgCl_2$, 25 units ATP sulfurylase (Sigma), and 35 units pyrophosphatase (Sigma) in 100 mM Tricine buffer (pH 8.0) for 3 h at 25°C. Purification of [^{35}S]APS from $^{35}SO_4^{2-}$ by TLC on Brinkmann silica gel N plates in the solvent *n*-propanol:ammonium hydroxide:water (6:3:1, v/v). The [^{35}S]APS (R_F of 0.3), was identified as APS by cochromatography with nonlabeled APS. APS was well separated from $^{35}SO_4^{2-}$ (R_F of 0.15). The radiolabeled APS was eluted from the silica gel with ammoniacal aqueous ethanol (1:50:50, v/v) and the solution lyophylized or flash evaporated dry. The [^{35}S]APS was then redissolved in a minimal volume of 5 mM BTP (pH 6.5) to give approximately 1 μCi per μL. [^{35}S]APS purity was verified by TLC after purification. Aliquots were stored at −80°C and thawed no more than twice to minimize breakdown of the APS. Yield of [^{35}S]APS from $^{35}SO_4^{2-}$ varied between 10 and 33%. Adsorption to charcoal (4, 25) was not employed for purification since contamination with ATP necessitated the further purification step of TLC resulting in a lower yield of APS.

Spinach (*Spinacia oleracea*) plants were conditioned in the dark, leaves harvested in the dark, and chloroplasts isolated in darkness as previously described (17). During all manipulations, the light intensity was below the detection limit (less than 0.25 μE/m^2/s) of the photometer used (Li Cor, model Li-185-B).

Chloroplasts were incubated in 1.0 mL of a basic reaction medium containing 0.3 M sorbitol, 2 mM $MgCl_2$, 33 mM BTP at pH 7.9, and 30 μM [^{35}S]APS, [^{35}S]PAPS, or $^{35}SO_4^{2-}$, at 1 μCi each, unless otherwise noted in the legends. Incubations were done in duplicate for 30 min at 25°C in complete darkness. All experiments were performed at least twice. Reactions were stopped, lipids extracted and prepared for analysis as described previously (17). After evaporation under N_2, the lipids were redissolved in chloroform and aliquots removed for assay of radioactivity by scintillation counting. The remaining lipid was separated by TLC on silica gel plates in the solvent chloroform:methanol:ammonium hydroxide:water (65:35:2:2, v/v) to quantitate the amount of SQDG (20). The distribution of radioactivity on the TLC plate was determined with a Bertold linear analyzer. In this system SQDG has an R_F of 0.4; APS, PAPS, and SO_4^{2-} remain at the origin. Other chloroform-soluble sulfur-containing lipophilic compounds (OSL) previously reported (13, 14, 17) migrate with the solvent front.

RESULTS

Competition Studies

The incorporation of sulfate, APS, and PAPS into SQDG in the dark is shown in Table I. Comparing the three radiolabeled compounds as precursors of SQDG, [^{35}S]APS and [^{35}S]PAPS provided greater incorporation into SQDG than $^{35}SO_4^{2-}$. The incorporation of $^{35}SO_4^{2-}$ in the dark without ATP reported here agrees with that previously reported (0.1–0.3 nmol/mg Chl/h) (15) in a study showing the synthesis of SQDG in dark incubated chloroplasts required the presence of ATP. To ensure hydrolysis of [^{35}S]APS or [^{35}S]PAPS was not occurring, these rates of incorporation were compared in the same experiment to those of $^{35}SO_4^{2-}$ plus ATP. In the presence of 2 mM ATP, 30 μM $^{35}SO_4^{2-}$ was still a poorer substrate than 30 μM [^{35}S]APS or [^{35}S]PAPS with rates of incorporation up to 0.25 nmol/mg Chl/h, four- to seven-fold less than those of [^{35}S]APS or [^{35}S]PAPS (data not shown). These rates of 30 μM $^{35}SO_4^{2-}$ incorporation into SQDG in the presence of ATP were comparable to those at similar sulfate concentrations of light-incubated chloroplasts (17). However, the incorporation of sulfur from only 30 μM [^{35}S]APS or [^{35}S]PAPS into SQDG in the presence of DHAP was comparable to that previously reported for 100 μM $^{35}SO_4^{2-}$ in the presence of 2 mM ATP in the dark (1.9 nmol/mg Chl/h) under similar incubation conditions (15, 16).

Competition of each radiolabeled precursors with nonlabeled sulfate, APS, or PAPS indicated APS was preferred over PAPS and sulfate. Addition of unlabeled sulfate did not greatly affect incorporation of [^{35}S]PAPS into SQDG, whereas the addition of unlabeled APS markedly lowered the incorporation. When [^{35}S]APS was supplied to chloroplasts, its incorporation into SQDG decreased only slightly upon the

Table I. *Incorporation of Sulfate, APS, or PAPS into SQDG*

Chloroplasts equivalent to 280 μg Chlorophyll, 2 mM DHAP, and 30 μM of the labeled precursor were present in each test tube. Each unlabeled competitor compound was tested at a concentration of 30 μM.

Labeled Precursor	Competitor	nmol/mg Chl/h
$^{35}SO_4^{2-}$	None	0.14
	PAPS	0.13
	APS	0.10
[^{35}S]PAPS	None	1.68
	Sulfate	1.75
	APS	0.23
[^{35}S]APS	None	1.17
	Sulfate	0.99
	PAPS	0.99

addition of either unlabeled sulfate or PAPS. When $^{35}SO_4^{2-}$ was supplied to chloroplasts, only low incorporation was observed because the incubation was in the dark and there was no source of ATP for the synthesis of APS besides the conversion of DHAP to 3-phosphoglycerate. Under these conditions, no competition by PAPS and only little by APS could be detected.

[^{35}S]APS, [^{35}S]PAPS, and $^{35}SO_4^{2-}$ were also incorporated into other sulfur-containing lipophilic compounds (OSL) partitioning into the chloroform layer as noted in previous studies with sulfate (15, 17). Incorporation of [^{35}S]APS into these compounds occurs at rates of 6.6 nmol/mg Chl/h, while that of [^{35}S]PAPS is 2.8 nmol/mg Chl/h and that of $^{35}SO_4^{2-}$ is only 0.4 nmol/mg Chl/h at these concentrations. Addition of PAPS and sulfate did not diminish label incorporated into these other sulfur-containing compounds from $^{35}SO_4^{2-}$ or APS. However, when [^{35}S]PAPS was the precursor the presence of sulfate increased the rate to 3.8 nmol/mg Chl/h and the presence of APS decreased the rate of 2.0 nmol/mg Chl/h from that of the control (2.8 mg Chl/h). Separate studies of these compounds is currently underway.

It is possible that DHAP might provide sufficient reductant and ATP to dark incubated chloroplasts to permit reactions which could compete for APS. Since this would bias the results of the competition studies, testing of APS and PAPS as potential precursors was also done in the absence of DHAP. The results which appear in a separate preliminary communication (16) support the results presented here. In the absence of DHAP, [^{35}S]APS incorporation into SQDG (115 pmol/mg Chl/h) was superior to that of [^{35}S]PAPS (9 pmol/mg Chl/h) which was comparable to that of $^{35}SO_4^{2-}$ (10 pmol/mg Chl/h) under the same conditions used here. In that competition study (16), [^{35}S]APS incorporation into SQDG showed little change in the presence of sulfate (97 pmol/mg Chl/h) or PAPS (107 pmol/mg Chl/h) compared to the control (115 pmol/mg Chl/h). However, [^{35}S]PAPS incorporation into SQDG changed substantially in the presence of APS (2 pmol/mg Chl/h) compared to that in the presence of sulfate (7 pmol/mg Chl/h) or the control (9 pmol/mg Chl/h).

From both these competition studies, it was evident that APS was a better precursor for SQDG biosynthesis than sulfate or PAPS. Evident as well from these competition studies was that APS was distinct and differed from both sulfate and PAPS as a precursor, especially in the effect of DHAP on the incorporation of the different precursors. Thus, it was necessary to characterize further the incorporation of APS into SQDG. The characteristics of APS incorporation into SQDG could then be compared to those of sulfate (15). APS incorporation into SQDG was also compared, in a more limited manner, to that of PAPS.

Characterization of the System

The incorporation of [^{35}S]APS into SQDG was linear for 30 min. Thirty min was chosen as a standard incubation time. Incorporation of [^{35}S]PAPS into SQDG was linear for up to 60 min, longer than for APS, but similar to that previously reported for sulfate (15). Incorporation of [^{35}S]APS into SQDG increased as a function of chloroplast concentration, but only became linear between 100 and 150 μg Chl (Fig. 1). Incorporation of [^{35}S]APS into SQDG was optimal when the pH of the reaction mixture was 7.0 (Fig. 2), identical to that reported for sulfate incorporation into SQDG (15, 17). Incorporation of [^{35}S]APS into SQDG was greater when the buffer provided was BTP/HCl rather than Mes/KOH, also in agreement with that previously reported for $^{35}SO_4^{2-}$ (17).

Incorporation of [^{35}S]APS into SQDG as a function of APS concentration was measured with and without DHAP present (Fig. 3). In the absence of DHAP, maximal incorporation was at 20 μM. A decreased rate of incorporation or inhibition was observed above 30 μM APS, but by 40 μM a plateau was reached wherein no further decrease of incorporation was evident. The concentration curve was repeated in the presence of DHAP since DHAP was previously shown to stimulate the incorporation of APS into SQDG. In the presence of 2 mM DHAP, the shape of the substrate curve changed considerably. The original peak of activity at 20 μM APS in the absence of DHAP shifted to between 30 and 40 μM APS beyond which a slight inhibition followed and was resumed by unsaturable kinetics.

Cofactors

The effect of various concentrations of DHAP on the incorporation of [^{35}S]APS or [^{35}S]PAPS into SQDG was tested (Fig. 4). Optimal incorporation of APS into SQDG was evident with 2 mM DHAP. However, maximal incorporation of PAPS into SQDG required the presence of at least 5 mM DHAP. With DHAP present, incorporation of PAPS into SQDG surpassed that of APS as evident in the competition

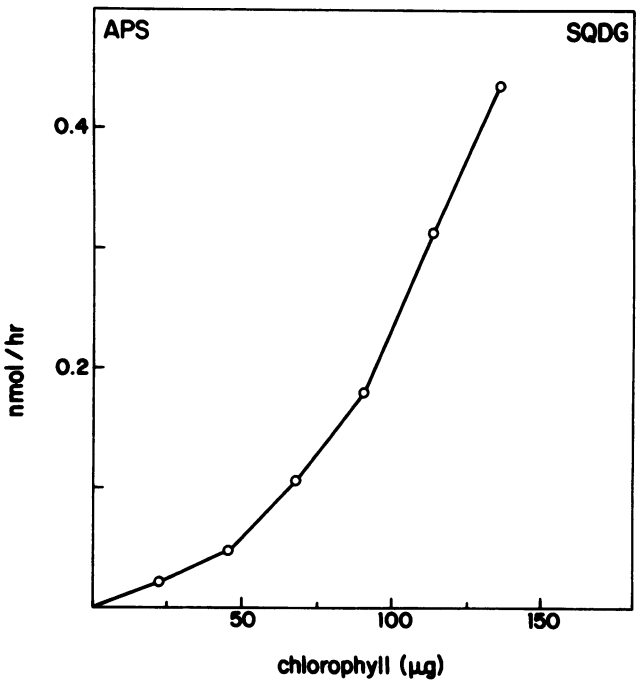

Figure 1. SQDG synthesis from APS as a function of Chl concentration. Reaction mixtures and conditions were as described in "Materials and Methods." APS concentration was 25 μM. Chl concentration was varied by adding various volumes of chloroplast suspension.

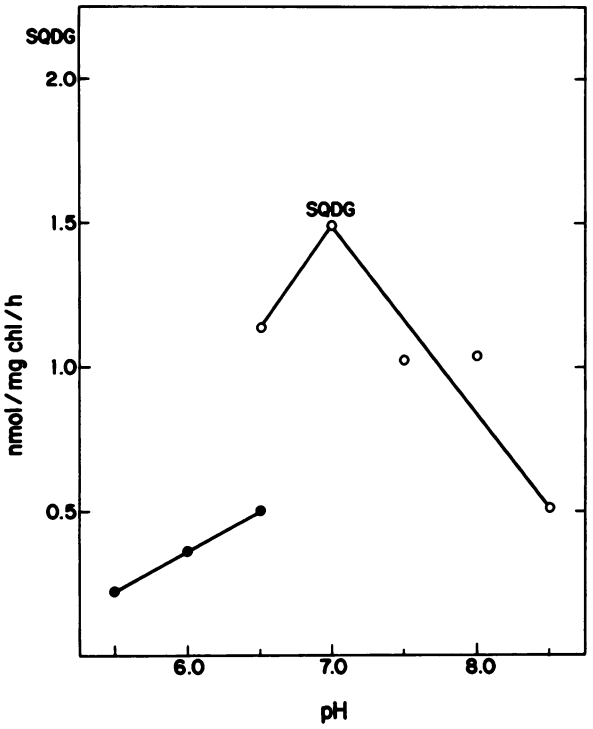

Figure 2. Effect of pH on the synthesis of SQDG from APS. Reaction mixtures were as described in "Materials and Methods." Each reaction mixture contained chloroplasts equivalent to 147 μg Chl. Mes/KOH was used for pH values 5.5 to 6.5 (●) and BTP/HCl for pH values 6.5 to 8.5 (○).

studies of the two precursors in the presence (Table I) and absence (16) of DHAP. DHAP may stimulate by providing ATP, NADPH or carbon skeleton for synthesis of SQDG. DHAP may also serve a regulatory role (6) and may deplete Pi (6, 30). Further experiments were directed toward understanding how DHAP acts and identifying other cofactors which could affect the incorporation into SQDG.

Various triose phosphate compounds were tested to determine if DHAP was providing a carbon skeleton for synthesis of the headgroup or glycerol backbone of SQDG (Table II). Of the compounds tested, only glycerol-3-P and DHAP increased incorporation of [^{35}S]APS into SQDG (part A). DHAP was the only compound tested which greatly enhanced the incorporation of [^{35}S]PAPS into SQDG (part B). Addition of 3-PGA inhibited APS incorporation into SQDG although there was relatively no effect on the incorporation of PAPS into SQDG. Synthesis of SQDG from either APS or PAPS was totally inhibited upon addition of 2 mM glyceraldehyde-3-phosphate (data not shown).

Whether DHAP provides ATP and/or reductant for SQDG synthesis was tested by comparing the addition of DHAP to that of ATP or reductant or both. Addition of ATP, UTP, or malate increased the incorporation of [^{35}S]APS but not nearly as effectively as did DHAP or DHAP plus glycerol-3-P (Table III). DHAP and glycerol-3-P were both shown to increase independently the incorporation of [^{35}S]APS into SQDG (Table II). In the presence of DHAP and glycerol-3-P at 2 mM concentration each, incorporation into SQDG was more than twice that when both were present at only 1 mM. When DHAP was present simultaneously with either ATP, UTP, or malate, a lower rate of synthesis was observed in comparison to that of DHAP alone. UTP was more effective than ATP in increasing SQDG synthesis. ATP and UTP when present simultaneously were no more effective than each added alone. Malate, which generates NADPH internally during its conversion to oxaloacetate, was no more effective than ATP. ATP and malate added simultaneously were less effective in increasing the incorporation than each compound added separately. NADPH added to the chloroplasts decreased incorporation of APS into SQDG as did DTT.

ATP and NADPH were also added via the triose phosphate shuttle mechanism (30) wherein DHAP, OAA and Pi, added simultaneously, supply both ATP and NADPH internally to the chloroplasts by the metabolism of DHAP to 3-PGA. When the complete shuttle mechanism was tested with [^{35}S]APS as the precursor, the combined presence of DHAP, OAA, and Pi slightly inhibited synthesis of SQDG (Table IV). OAA alone or in conjunction with Pi or DHAP had little effect or decreased slightly the incorporation into SQDG. However, addition of Pi, with or without DHAP, stimulated SQDG synthesis (Tables II and IV).

DISCUSSION

As precursors of SQDG, APS, and PAPS offer many advantages over sulfate since the light or ATP requirement of

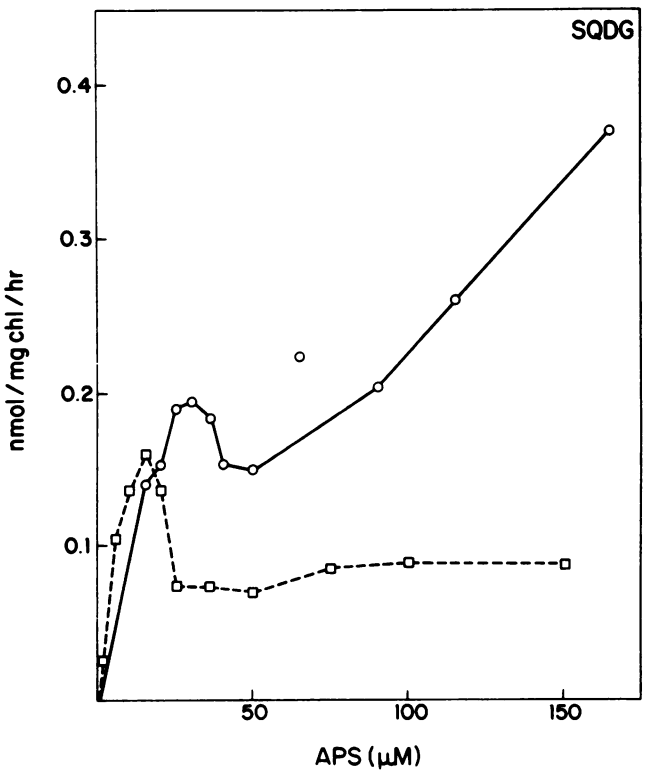

Figure 3. Effect of APS concentration on SQDG biosynthesis. Chloroplasts equivalent to 167 μg Chl were added at each concentration tested. Incorporation of APS into SQDG was measured in the presence (○——○) and in the absence (□——□) of 2 mM dihydroxyacetone phosphate.

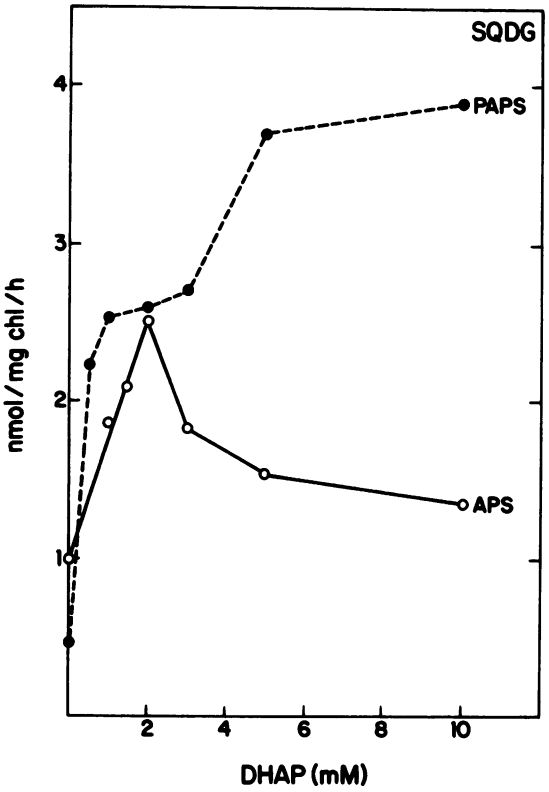

Figure 4. Effect of dihydroxyacetone phosphate concentration on SQDG biosynthesis. Chloroplasts equivalent to 200 μg Chl were added to standard reaction mixtures at each concentration of DHAP tested. Incorporation of APS (○) and PAPS (●) into SQDG was measured as described in "Materials and Methods."

Table II. *Incorporation of APS or PAPS into SQDG in the presence of triose phosphate compounds*

Chloroplasts equivalent to 76 μg Chl and 15 μM [^{35}S]APS were present in each test tube in part A. Chloroplasts equivalent to 109 μg Chl and 20 μM [^{35}S]PAPS were present in each test tube in part B. Each compound was tested at a concentration of 2 mM.

Additions	[^{35}S]APS Part A	[^{35}S]PAPS Part B
	nmol/mg Chl/h	
None	0.210	0.020
+ DHAP	0.330	0.140
+ 3-PGA	0.110	0.025
+ G-3-P	0.320	0.029
+ PEP	0.220	0.029

sulfate incorporation into SQDG is bypassed. WIthout the addition of ATP to the media, very low incorporation of $^{35}SO_4^{2-}$ into SQDG was observed, in agreement with previous reports (15, 17), because in the dark there was no source of ATP for the synthesis of APS besides the conversion of DHAP to 3-phosphoglycerate. As no ATP is necessary with APS, rates of [^{35}S]APS and [^{35}S]PAPS incorporation into SQDG (1.17 nmol/mg Chl/h) are 10-fold higher than those of $^{35}SO_4^{2-}$ incorporation into SQDG without ATP (0.14 nmol/mg Chl/h) at the same precursor concentration. Upon addition of 2

Table III. *Incorporation of APS into SQDG in the Presence of Various Compounds*

Chloroplasts equivalent to 280 μg Chl, 0.42 μCi, and 30 μM [^{35}S] APS were present in each test tube. Each compound was tested at a concentration of 2 mM except where indicated.

Additions	nmol/mg Chl/h
None	0.28
ATP	0.47
UTP	0.58
ATP + UTP (1 mM each)	0.53
DHAP	1.17
DHAP + Glycerol-3-P	2.96
DHAP + Glycerol-3-P (1 mM each)	0.83
Malate	0.48
ATP + Malate	0.38
DHAP + Malate	0.79

Table IV. *Incorporation of APS into SQDG in the Presence of the DHAP Shuttle Compounds*

Chloroplasts equivalent to 76 μg Chl, 0.80 μCi, and 15 μM [^{35}S] APS were present in each test tube. Each compound was tested at a concentration of 2 mM except for Pi which was tested at a concentration of 4 mM.

Additions	nmol/mg Chl/h
None	0.21
DHAP	0.33
OAA	0.20
Pi	0.25
DHAP + Pi	0.30
OAA + Pi	0.11
DHAP + OAA	0.19
DHAP + OAA + Pi	0.16

mM ATP, $^{35}SO_4^{2-}$ incorporation into SQDG at 30 μM still proved a much poorer substrate than 30 μM [^{35}S]APS or [^{35}S] PAPS with rates of incorporation 4-fold to 7-fold less. At 30 μM sulfate, rates of $^{35}SO_4^{2-}$ incorporation into SQDG with ATP were comparable to those at similar sulfate concentrations of light-incubated chloroplasts (17) but lower than those previously reported for 100 μM sulfate (15), since conditions were no longer optimal for sulfate incorporation into SQDG. APS and PAPS can be more effective than sulfate in studies of SQDG biosynthesis. In the presence of DHAP, 30 μM [^{35}S] APS or [^{35}S]PAPS incorporation into SQDG was comparable to the previously reported incorporation of 100 μM $^{35}SO_4^{2-}$ in the presence of ATP or a ATP-generating system, composed of DHAP, oxaloacetate and inorganic phosphate (1–3 nmol/mg Chl/h) in the dark (15, 16). This shows that the effective concentrations of these precursors are less than that of sulfate.

The involvement of APS as an intermediate between sulfate and SQDG has been confirmed. The competition study indicated sulfate and PAPS are converted to APS prior to the formation of the sulfoquinovose headgroup since cold APS decreased incorporation of $^{35}SO_4^{2-}$ or [^{35}S]PAPS into SQDG. No appreciable decrease in [^{35}S]APS incorporation into SQDG was evident when cold sulfate or cold PAPS were present. APS was confirmed as an intermediate in cysteine synthesis by similar competition studies (23). These results

confirm those of a similar competition study done in the absence of DHAP reported in a preliminary communication (16). However, here rates of [^{35}S]APS incorporation into SQDG in the presence of DHAP (1–2.5 nmol/mg Chl/h) are comparable to those previously reported for sulfate (13, 15, 16).

Hydrolysis of APS and PAPS back to sulfate prior to incorporation into SQDG is not occurring. In theory [^{35}S]APS can break down to AMP and $^{35}SO_4^{2-}$. However, if hydrolysis of [^{35}S]APS were occurring, rates of synthesis would be less than, not even equal to, the rates of $^{35}SO_4^{2-}$ incorporation into SQDG since AMP was previously shown to inhibit $^{35}SO_4^{2-}$ incorporation into SQDG (15). This is not the case. Furthermore, if hydrolysis were occurring, the competition of unlabeled sulfate with radiolabeled APS would have diluted out the incorporation into SQDG. From the competition studies both in the presence and absence of DHAP the addition of APS decreased sulfate or PAPS incorporation into SQDG but sulfate did not substantially decrease APS incorporation into SQDG. This implies that these precursors are accessible to reactions leading to the synthesis of SQDG but does not indicate necessarily where those reactions are localized.

APS and PAPS can also be used effectively as precursors of other sulfur-containing lipophilic compounds (OSL). Joyard *et al.* (14) have identified elemental sulfur as a major constituent of this fraction. Incorporation of APS into the OSL is much greater than that of PAPS or sulfate. This further indicates that APS is not broken down to sulfate prior to its incorporation. Otherwise, the incorporation of APS into the OSL would be less than that of SQDG, since synthesis of these compounds is evident only when the added sulfate concentration exceeds 50 μM (17). Addition of unlabeled sulfate or PAPS also did not decrease the incorporation of APS into the OSL. In the case of sulfate, the incorporation increased due to the stimulatory effect of sulfate on synthesis of the OSL (17). The synthesis of OSL peaks at 2 mM DHAP for APS, 1 mM DHAP for PAPS, and is inhibited by Pi, DTT, and the DHAP shuttle mechanism (15, 17), conditions also favoring DHAP conversion to glycerol-3-P and ultimately DG (6). These results set the precedent for utilizing APS and PAPS for further studies of the biosynthesis of these compounds in addition to SQDG.

Characteristics of [^{35}S]APS incorporation into SQDG differed slightly from those of $^{35}SO_4^{2-}$ previously reported (15, 17). Although the pH optima of both (pH 7.0) was the same, the incorporation of APS was linear for only 30 min whereas that of $^{35}SO_4^{2-}$ (17) and of [^{35}S]PAPS were linear beyond 60 min. This was not surprising since the conversion of APS back to sulfate by the ATP sulfurylase is favored energetically (25, 28) whereas the conversion of sulfate to APS, by the ATP sulfurylase, and of PAPS to APS, by the reverse reaction of the APS kinase controls the amount of APS available at any given time (28) when either sulfate or PAPS is the precursor. The effect of increasing concentrations of Chl was also different for both. When sulfate was the precursor, no lag below 50 μg was evident (15) and incorporation diminished above 200 μg Chl whereas that for APS was linear to at least 250 μg Chl. The response of APS incorporation into SQDG to increasing concentrations of Chl more closely resembled that of APS incorporation into cysteine (23) even though intact chloroplasts were used in the current study. Under the conditions of incubation used in these experiments, APS could not be converted to cysteine as sufficient reductant was not available (23, 24, 28).

Incorporation of $^{35}SO_4^{2-}$ into SQDG at various concentrations of sulfate exhibited characteristic Michaelis-Menten properties (17). However, with APS as the precursor, SQDG synthesis with increasing APS concentrations behaved similarly to the substrate curve of the cytosolic fructose 1,6-bisphosphatase which is considerably enhanced in the presence of DHAP and does not show Michaelis-Menten characteristics (10). The optimal concentration of sulfate required was above 50 μM while that for APS was below 50 μM; either 30 μM when DHAP was present or 20 μM when DHAP was absent. Above 50 μM, APS incorporation into SQDG increases with nonsaturable kinetics over the concentrations tested. In the absence of DHAP, the APS substrate curve is modulated not only by the APS concentration but also by the degradation of APS via the ATP sulfurylase. In the presence of DHAP, APS breakdown products are minimized since some APS is reformed from ATP generated through DHAP conversion to PGA. These results, coupled with those summarized above, indicate that APS differs from sulfate as a precursor and is not broken down to sulfate prior to its incorporation into SQDG.

In the current studies, there was no need for additional reductant such as DTT. The decreased incorporation of [^{35}S]APS into SQDG upon addition of DTT to dark incubated chloroplasts is attributed to reactions which compete for APS. Upon addition of DTT in the dark, APS can be further metabolized to sulfide, cysteine, and other reduced sulfur forms at rates up to 2 nmol/mg Chl/h (23, 24, 28). The previously reported increase in $^{35}SO_4^{2-}$ incorporation into SQDG in the presence of DTT (15) is attributed to either sulfate transport, conversion of suflate to APS, or stabilization of enzymes.

DHAP may provide a source of carbon for SQDG synthesis. Other triose phosphate compounds tested (Table II) were ineffective in substituting for DHAP. Glycerol-3-P greatly enhanced APS incorporation into SQDG with levels of incorporation equivalent to those of DHAP. Gee *et al.* (6) have shown conversion of DHAP to glycerol-3-P within the chloroplast. That reaction is stimulated by DTT, ATP, and Pi but inhibited by glycerol-3-P, free fatty acids, and phosphatidylcholine. The data suggest DG, formed from glycerol-3-P conversion first to phosphatidate, may be one limiting factor in SQDG synthesis, especially APS incorporation into SQDG. Because PAPS and sulfate are one step further removed in SQDG synthesis, other factors more limiting may minimize, or eliminate, possible stimulation by glycerol-P there.

The data suggest a multiple role of DHAP. If DHAP were serving solely as a source of glycerol-3-P, the addition of both together at 2 mM would not increase the level of incorporation more than the presence of DHAP alone at 2 mM. The optimal DHAP concentration for these reactions peaks sharply at 2 mM (Fig. 4). However, levels of APS incorporation were far greater with both DHAP and glycerol-3-P present at 2 mM

than with DHAP alone at 2 mM or DHAP and glycerol-3-P both at 1 mM (Table III). Also, glycerol-3-P did not influence PAPS incorporation into SQDG, nor that of sulfate, under these conditions.

DHAP does not serve as a source of ATP for these reactions. APS incorporation into SQDG does not increase in the presence of the DHAP shuttle mechanism, utilized in previous studies of sulfate incorporation into SQDG in the dark (15) to provide an internal source of ATP (Table IV). In fact, the incorporation of sulfur from APS into SQDG in the presence of the entire shuttle mechanism is lower than the incorporation when the shuttle mechanism is not present. The incorporation of sulfur from APS is 10-fold lower than that of sulfate into SQDG in the presence of the DHAP shuttle mechanism in the dark (15) indicating the presence of the DHAP shuttle mechanism is not required to generate ATP and reductant. This further demonstrates that APS and sulfate are distinctly precursors in these reactions.

The stimulation afforded by DHAP also differs when PAPS is the substrate. This reflects both the multiple role of DHAP in these reactions and differences in the incorporation of APS *versus* PAPS into SQDG. The optimal concentration of DHAP required for APS incorporation (2 mM) differed from that of PAPS which saturated above 5 mM DHAP. Dark incubated chloroplasts can convert APS to sulfate, PAPS, or a limited amount of bound or free sulfite since sufficient reductant is lacking for metabolism of APS to cysteine (23, 24). The equilibrium between PAPS and APS is influenced by Pi levels, ATP levels, and APS levels (22, 28). Higher concentrations of DHAP may begin to generate enough ATP and reductant to allow further metabolism of APS to reduced sulfur compounds such as cysteine thus shifting the equilibrium more in favor of PAPS conversion to APS. Differences in metabolic pools of Pi, ATP, APS, and other compounds at the time of chloroplast isolation may explain the rates of APS and PAPS incorporation into SQDG observed in the various experiments. As mentioned previously, the competition experiments indicate APS is the preferred precursor of SQDG, regardless of the presence or absence of DHAP.

Other cofactors were also found to stimulate SQDG synthesis from APS. UTP, which stimulated $^{35}SO_4^{2-}$ incorporation into SQDG (15, 17) also stimulated [^{35}S]APS incorporation into SQDG. The report by Shibuya *et al.* (26) of a nucleotide sugar compound in *Chlorella* first suggested the possible role of UTP in the formation of a nucleotide sugar, such as UDP-SQ, in reactions analogous to UDP-galactose formation, a precursor in galactolipid synthesis. Our results further support this proposal.

Addition of Pi increased APS incorporation into SQDG, as previously reported for $^{35}SO_4^{2-}$ (15). The role of Pi in SQDG synthesis from APS remains uncertain. Addition of Pi activates light modulated enzymes (3), including DHAP conversion to glycerol-3-P (6), and influences the transport of triose phosphate compounds, including 3-PGA and DHAP (30). Addition of Pi also affects the phosphorylation state of the chloroplast (5, 29), and inhibits the ATP sulfurylase (21). The stimulation of SQDG biosynthesis by Pi may result indirectly from one or more of these factors.

In conclusion, APS was shown to be an intermediate between sulfate and SQDG in competition studies and proved a good precursor of SQDG. APS incorporation into SQDG in the dark occurs at rates comparable to those of sulfate in the light. DHAP stimulates the incorporation by either providing a carbon source for the headgroup or the DG moiety via synthesis of glycerol-3-P. DHAP may also modulate enzymatic activity necessary for SQDG synthesis. UTP and Pi also stimulate these reactions although no absolute requirement for either was indicated.

LITERATURE CITED

1. **Abraham A, Bachhawat BK** (1963) Effect of light and streptomycin on the incorporation of sulfate into sulfolipids in *Euglena gracilis*. Biochim Biophys Acta **70:** 104–106
2. **Benson AA** (1963) The plant sulfolipid. Adv Lipid Res **1:** 387–394
3. **Buchanan BB** (1980) Role of the light on the regulation of chloroplast enzymes. Annu Rev Plant Physiol **31:** 341–374
4. **Burnell JM, Anderson JW** (1973) Adenosine 5′ sulphatophosphate kinase activity in spinach leaf tissue. Biochem J **134:** 565–579
5. **Carver KA, Hope AB, Walker DA** (1983) Adenine nucleotide status, phosphoglycerate reduction and photosynthetic phosphorylation in a reconstituted chloroplast system. Biochem J **210:** 273–276
6. **Gee RW, Byerrum RU, Gerber DW, Tolbert NE** (1988) Differential inhibition and activation of two leaf dihydroxyacetone phosphate reductases. Plant Physiol **87:** 379–383
7. **Haas R, Sieberts HP, Wrage K, Heinz E** (1980) Localization of sulfolipid labelling within cells and chloroplasts. Planta **148:** 238–244
8. **Harwood JL** (1975) Synthesis of sulfoquinovosyldiacylglycerol by higher plants. Biochem Biochim Biophys Acta **398:** 224–230
9. **Harwood JL** (1980) Sulfolipids. *In* PK Stumpf, EE Conn, eds, The Biochemistry of Plants, Vol. 4, Lipids: Structure and Function. Academic Press, New York, pp 301–320
10. **Herzog B, Stitt M, Heldt HW** (1984) Control of synthetic sucrose synthesis by fructose 2,6 bisphosphate III Priorities of the cytosolic fructose 1,6 bisphosphatase. Plant Physiol **75:** 561–656
11. **Hoppe W, Schwenn JD** (1981) *In vitro* biosynthesis of the plant sulpholipid: On the origin of the sulphonate group. Z Naturforsch **36:** 820–826
12. **Jocelyn PC** (1972) Chemical reactions of thiols. *In* Biochemistry of the SH Group. Academic Press, New York, pp 63–88
13. **Joyard J, Blée E, Douce R** (1986) Sulfolipid synthesis from $^{35}SO_4$ and [1-^{14}C] acetate in isolated spinach chloroplasts. Biochim Biophys Acta **879:** 78–87
14. **Joyard J, Forest E, Blée E, Douce R** (1988) Characterization of elemental sulfur in isolated intact spinach chloroplasts. Plant Physiol **88:** 961–964
15. **Kleppinger-Sparace KF, Mudd JB** (1987) Biosynthesis of sulfoquinovosyldiacylglycerol in higher plants: the incorporation of $^{35}SO_4$ by intact chloroplasts in darkness. Plant Physiol **84:** 682–687
16. **Kleppinger-Sparace KF, Mudd JB** (1987) Biosynthesis of sulfoquinovosyldiacylglycerol in chloroplasts of higher plants. *In* PK Stumpf, JB Mudd, WD Nes, eds, The Metabolism, Structure and Function of Plant Lipids. Plenum Press, New York, pp 309–311
17. **Kleppinger-Sparace KF, Mudd JB, Bishop DG** (1985) Biosynthesis of sulfoquinovosyldiacylglycerol in higher plants: the incorporation of $^{35}SO_4$ by intact chloroplasts. Arch Biochem Biophys **240:** 859–865
18. **Kleppinger-Sparace KF, Mudd JB, Sparace SA** (1990) Plant Sulfolipids. *In* H Rennenberg, C Brunold, L J de Kok, I Stulen, eds, Sulfur Nutrition and Sulfur Assimilation in Higher Plants. SPB Academic Publishing, The Hague (in press)

19. **Lizada MCC, Yang SF** (1981) Sulfite-induced lipid peroxidation. Lipids **16:** 189–194
20. **Mudd JB, Dezacks R, Smith J** (1980) Studies on the biosynthesis of sulfoquinovosyldiacylglycerol in higher plants. *In* P Mazliak, P Benveniste, C Costes, R Douce, eds, Biogenesis and Function of Plant Lipids. Elsevier/North Holland Biomedical Press, New York, pp 57–66
21. **Sawhney SK, Nicholas OJD** (1977) Effects of adenine nucleotides and phosphate on adenosine triphosphate sulfurylase from *Anabaena cylindrica*. Biochem J **164:** 161–167
22. **Schmidt A** (1975) Inhibition of the adenosine-5′-phosphosulfate-sulfotransferase activity from spinach, maize, and *Chlorella* by adenosine-5′-monophosphate. Planta **127:** 93–95
23. **Schürmann P, Brunold C** (1980) Formation of cysteine from adenosine 5′-phosphosulfate (APS) in extracts from spinach chloroplasts. Z Pflanzenphysiol Bd **100S:** 257–268
24. **Schwenn JD, Depka B, Hennies HH** (1976) Assimilatory sulfate reduction in chloroplasts: Evidence for the participation of both stromal and membrane-bound enzymes. Plant Cell Physiol **17:** 165–176
25. **Shaw WH, Anderson JW** (1972) Purification properties and substrate specificity of ATP sulfurylase EC-2.7.7.4 from spinach leaf tissue. Biochem J **127:** 237–247
26. **Shibuya I, Yagi T, Benson AA** (1963) Sulfonic acids in algae. *In* Microalgae & Photosynthetic Bacteria. Japanese Society of Plant Physiologists. University of Tokyo, Tokyo, Japan, pp 627–636
27. **Shimazaki K-I, Nakamachi K, Kondo N, Sugahara K** (1984) Sulfite inhibition of photosystem II in illuminated spinach leaves. Plant Cell Physiol **25:** 337–341
28. **Siegel LM** (1975) Biochemistry of the sulfur cycle. *In* DM Greenberg, ed, Metabolism of Sulfur Compounds. Academic Press, New York, pp 217–286
29. **Stitt M, Lilley RM, Heldt HW** (1982) Adenine nucleotide levels in the cytosol, chloroplasts, and mitochondria of wheat leaf protoplasts. Plant Physiol **70:** 971–977
30. **Werdan K, Heldt HW, Ilovancev MM** (1975) The role of pH in the regulation of carbon fixation in the chloroplast stroma. Studies on CO_2 fixation in the light and dark. Biochem Biophys Acta **396:** 276–292