In Vitro Biosynthesis of the Plant Sulpholipid: On the Origin of the Sulphonate Group*

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Biosynthesis of the plant sulpholipid (diacylsulphoquinovosyl glycerol) has been demonstrated in a cell-free assay system from the green alga *Chlamydomonas reinhardii* CW 15. The sulpholipid was labelled with [^{35}S]sulphate when ATP, Mg^{2+} and a reductant were provided. Unlabelled cysteic acid failed to suppress the labelling of the sulphonate group in the lipid. Labelling with [^{35}S]PAPS or [^{35}S]sulphite has been studied in a fractionated cell extract. Sulphite as a precursor only required a particulate fraction of the cell while with PAPS the assay had to be complemented with a soluble protein and a thiol compound. [^{35}S]PAPS was incorporated into the diacylsulphoquinovosyl glycerol with a higher specificity than sulphite.

Introduction

The plant sulpholipid represents a minor constituent of the chloroplasts lipids, usually not exceeding 5% of the total lipid of a mature chloroplast. Its function in the plastid is not well understood and, in particular, very little is known about the biosynthesis of this compound. The present literature as thoroughly reviewed by Harwood [1] reveals two different hypotheses for its biosynthesis: a) the sulphoglycolytic pathway [2] and b) the cysteic acid pathway [3]. Both proposals agree in that the carbon sceleton of the sulphoquinovose is synthesized *de novo* either, as in (a) from phosphoenolpyruvate (*via* 2-phospho-3-sulpho-lactate) or, as in (b) from acetylserine (*via* cysteic acid and sulphopyruvate) by a condensation of the intermediate 3-sulpho-lactaldehyde with dihydroxyacetonephosphate. The sulpho group in the sulphoglycolytic pathway is suggested to derive from PAPS whereas in the cysteic acid pathway it is assumed to derive from sulphite.

So far, most of the evidence for the sulpholipid biosynthesis is obtained by experiments with intact algae or plants. Concerning the enzymatic mechanism, intact cells or tissue, however, have severe limitations in isotope feeding of precursor experiments. Therefore, in the present paper a cell-free assay system for the biosynthesis of the sulpholipid is introduced employing extracts from the green alga *Chlamydomonas reinhardii* CW 15. Conditions for the incorporation of [^{35}S]SO_4, [^{35}S]PAPS or [^{35}S]SO_3 as possible sulpho group donors have been studied. The intracellular distribution of the corresponding biosynthetic activities between the soluble cell protein and the membraneous fraction of the algae have been investigated.

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Abbreviations: Chl, chlorophyll; $CysSO_3H$, cysteic acid; DTE, dithioerythritol; DTNB, dithio-bis-nitrobenzoic acid; DTT, dithiothreitol; α-KG, α-keto glutaric acid; PAPS, adenosine 3′-phosphate 5′-sulphatophosphate.

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Materials and Methods

Growth of C. reinhardii CW 15

The algae were cultured photoautotrophically (3500 lux white light) with CO_2-enriched air (5% CO_2) in a medium as described in ref. [4]. The cells were grown to a concentration of 10–30 µg algal chlorophyll per ml nutrient solution (early phase of logarithmic growth) before harvest. Sulphate was used as sole source of sulphur at 0.4 mM. For identification purposes authentic [^{25}S]sulpholipid was isolated from the intact algae grown under identical conditions except for the use of [^{35}S]Na_2SO_4 (Amersham Buchler, Braunschweig) with a specific activity of 38.3 $sec^{-1} \times$ nmol.

Isolation and identification of the plant sulpholipid

The lipids from the intact *C. reinhardii* were extracted following the method in ref. [5]. Enzymatic

assays were terminated prior to the extraction by immersing the samples into boiling water for 5 min. Denatured protein and membrane material were pelleted by centrifugation ($3000 \times g$ for 10 min) and washed once with first 1 M KCl and then with destilled water. The lipid extract was fractionated by hydrophobic ionexchange chromatography [6] on DEAE cellulose columns to separate the bulk of galactolipids from the negatively charged lipids. The fraction containing the sulpholipid has been rechromatographed on thin layer plates (Kieselgel 60, Merck, Darmstadt) with chloroform/methanol/acetic acid/H_2O (vol.: 85:25:15:3) as mobile phase [7]. Two ^{35}S-labelled lipids were visualized by an integrating TLC scanner (Labor Berthold, Wildbad). The most highly labelled component (92%) showed the R_f-value of 0.46. This substance has been identified as SL (R_f 0.29) in the solvent system given in ref. [5]. The compound has also been chromatographed in two other solvents as described in ref. [8] and in ref. [9] showing no difference to the published R_f-values. The minor labelled compound (8%) migrating slower than the sulpholipid (R_f 0.29) was suspected to represent lyso-sulpholipid. Acidic hydrolysis of the sulpholipid did not yield sulphate but a water soluble compound which under the same conditions has previously been identified as 6-sulphoquinovose in ref. [10].

For the quantitative determination of [^{35}S]sulpholipid the complete assay was liberated from the galactolipids by passage through a DEAE column and rechromatographed with authentic sulpholipid on thin layer plates as described above. The radioactive spots detected by scanning were collected from the dried plates and counted in commercially available scintillant for aqueous samples in a Packard model 3385 scintillation counter.

Enzymatic assays

a. Extraction of crude cell homogenates, soluble protein and membrane fraction

The intact algae were washed once in isotonic buffer, containing Tris-Cl: 50 mM (pH 8.0), sorbitol 0.4 M, EDTA 2.5 mM, and sedimented by low speed centrifugation ($300 \times g$ for 5 min). They were ruptured osmotically by resuspending the pellet in hypotonic Tris-Cl buffer from which the sorbitol has been omitted. The content of chlorophyll was adjusted to 1 mg/ml and the algae were sonicated briefly with a Branson sonifier (30 sec applied at intervalls of 5 sec, approximately 70 Watt). After removal of intact cells by a second low speed centrifugation the "crude cell homogenate" was obtained. For the soluble protein and membrane fraction a crude cell homogenate (2 mg chlorophyll/ml) treated as above was centrifuged at $45000 \times g$ for 30 min. The pelleted membranes were washed in the original volume of Tris-Cl buffer and sedimented by a second centrifugation ($45000 \times g$ for 15 min). The resultant two supernatants were recombined and designated as "soluble protein". The washed membranes have been resuspended in twice the original amount of buffer and used as "membrane fraction".

b. Reconstituted assay system for sulpholipid labelling

The reconstituted reaction system consisted out of a limited amount of "membrane fraction" to which increasing amounts of "soluble protein" have been added (Fig. 4). The filtrated protein was obtained from a Sephadex G 50 column (∅ 1.5 × 33 cm) developped with 50 mM Tris-Cl buffer (pH 8.0). The low molecular weight fraction of the soluble protein was separated on Biogel P2 (exclusion limit 1200 daltons, ∅ 1.5 × 27 cm) in Tris as above. The reconstitutional effect on the sulpholipid labelling was obtained with 0.4 ml of the low weight fraction (elution volume 30–35 ml, comigrating with GSH) in an assay containing Tris-Cl 100 mM (pH 8.0), $MgCl_2$ 10 mM, [^{35}S]PAPS 30 µM (spec. activity: 183 $sec^{-1} \times nmol^{-1}$), Glc-6-P 5 mM, NADP 0.25 mM, Glc-6-P DH 0.2 I.U. (3.3 nkat), membrane-fraction (40 µg Chl) and 1.02 mg of the soluble protein in a total volume of 1.0 ml. The samples were incubated for 15 min at 25 °C and the labelled sulpholipid was assayed as described.

c. α-Keto glutarate transaminase activity

The assay contained in a total volume of 1.0 ml Tris-Cl 50 mM, (pH 8.0), α-KG 4 mM, Cysteic acid 4 mM, NADH 0.1 mM, malate dehydrogenase 3.0 I.U. (50 nkat), and cell extract filtrated on Sephadex G 50 with 580 µg protein. The reaction was followed as the cysteic acid dependent oxidation of NADH at 25 °C. Rates obtained were in the range of 0.3 to 0.4 µmol NADH oxidized × mg $Chl^{-1} \times min^{-1}$ (5 to 6.7 nkat).

d. Substrates for sulpholipid labelling

[^{35}S]sulphate and -sulphite were purchased from Amersham Buchler (Braunschweig) as the sodium salts. [^{35}S]PAPS was prepared enzymatically with the sulphate activating system from bakers yeast [11]. 50 mg of the partially purified protein were used in a volume of 10 ml containing Tris-Cl 100 mM (pH 8.5), $MgCl_2$ 10 mM, ATP 20 mM, and [^{35}S]sulphate 1.0 mM. The sample has been incubated at 25 °C for 10 h. The deproteinized assay mixture has been applied to a DEAE-cellulose column and developped with a linear gradient (300 ml volume) of 0–0.4 M KCl in 25 mM Tris-Cl (pH 8.0). The labelled nucleotide was eluted after 240 ml, concentrated by evaporation and desalted on Biogel P 2. Its specific activity was calculated as described previously [12].

e. Chlorophyll-, protein- and thiol group determination

Chlorophyll was extracted with aqueous acetone and determined as given in ref. [13]. The soluble proteins were measured after staining with Coomassie [14] while DTNB was used as reporter group for reduced thiols [15].

Results

a. Incorporation of [^{35}S]sulphate

Crude extracts from *C. reinhardii* incorporated [^{35}S]sulphate into the sulpholipid. The cell homogenate, required ATP and Mg^{2+} (Table I) indicating the need to activate the sulphate prior to its incorporation. The overall reaction from sulphate to the SL showed an apparent K_m of 0.1 mM for sulphate (Fig. 1a) which is in the same range as found for the plant ATP-sulphurylase [16]. Unlabelled cysteic acid as a possible precursor [2, 5] did not affect the incorporation of sulphate significantly, despite the relatively high activity of α-KG transaminase found in these extracts (Materials and Methods). The cysteic acid pathway may therefore not be operative in *C. reinhardii*. The lack of linearity between the rate of sulpholipid biosynthesis and the amount of cell extract as the enzymatic system (Fig. 1b) presumably referred to a dilution of cooperative enzymes and (or) cofactors. In this respect, it is noteworthy that $NADPH_2$ generated by glc-6-P stimulated the incorporation of sulphate by 27% and the ubiquitous thiol gluthatione even by 275% (Table I). This positive effect of reductants may either have visualized a protection of enzymes sensitive to oxidation or the involvement of a reductive step in the process of SL biosynthesis.

Table I. Incorporation of [^{35}S]sulphate into sulpholipid.

Additions	SL [nmol × mg Chl^{-1} × h^{-1}]
sulphate	0.08 –
sulphate, ATP, $MgCl_2$	2.32 (± 0%)
sulphate, ATP, $MgCl_2$, Glc-6-P	2.88 (+ 24%)
sulphate, ATP, $MgCl_2$, Glc-6-P, NADP, Glc-6-P DH	2.72 (+ 17%)
Sulphate, ATP, $MgCl_2$, GSH	8.72 (+ 275%)
sulphate, ATP, $MgCl_2$, α-KG	2.24 (– 3.5%)
sulphate, ATP, $MgCl_2$, α-KG + $CysSO_3H$	2.24 (– 3.5%)

Crude cell homogenate from *C. reinhardii* was incubated in 100 mM Tris/Cl (pH 8.0), [^{35}S]SO_4 0.5 mM (spec. activ.: 666 sec^{-1}/nmol), 5.5 mg protein (containing 0.2 mg Chl). Additions: ATP 5 mM, $MgCl_2$ 10 mM, Glc-6-P 5 mM, $NADP^+$ 0.5 mM, Glc-6-P dehydrogenase 0.2 U, reduced glutathione 0.5 mM, α-keto glutarate 5 mM, cysteic acid 2 mM, for assay of α-KG transaminase see "Materials and Methods". After 30 min of incubation at 25 °C the sulpholipid as been extracted according to ref. [5].

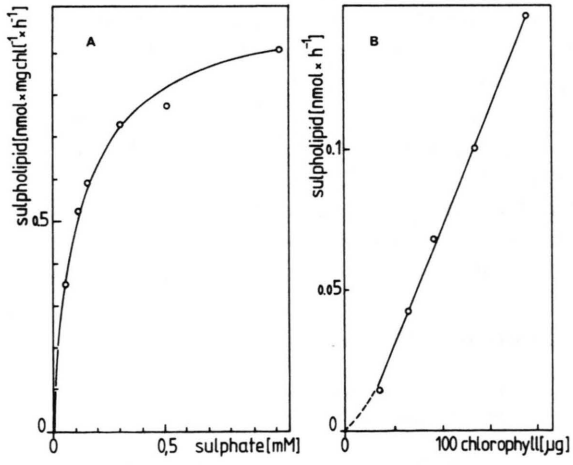

Fig. 1. Labelling of sulpholipid in *C. reinhardii* extracts with [^{35}S]SO_4 as source of sulphonyl group: (a) saturation with sulphate in the presence of ATP: 5 mM, and Mg^{2+}: 10 mM, crude cell homogenate: 3.75 mg protein (containing 125 µg Chl). (b) Effect of dilution on the rate of sulpholipid labelling at low amounts of cell homogenate as enzymatic system. Assay conditions as in (a), cell homogenate 33.7 µg protein × µg $chlorophyll^{-1}$, for experimental details and determination of the sulpholipid see Materials and Methods and legend to Table I.

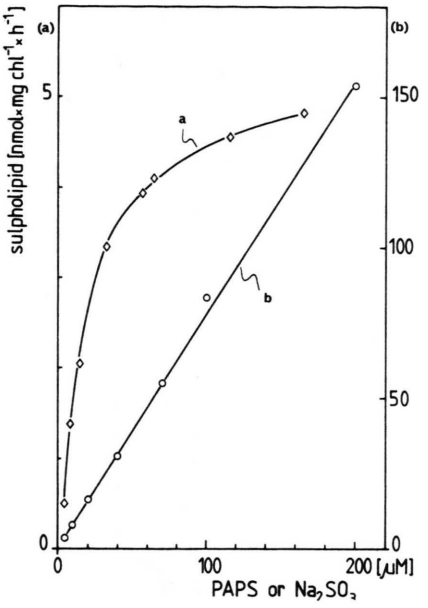

Fig. 2. Labelling of the sulpholipid with [^{35}S]PAPS or [^{35}S]SO_3 as sulphonyl group donors: (a) incorporation of the ^{35}S-label from PAPS in the presence of a reducing thiol (DTT 0.3 mM), cell homogenate: 4.35 mg protein (containing 125 μg Chl), [^{35}S]PAPS spec. activity 583 sec^{-1} × nmol^{-1}, Mg^{2+} 15 mM, and (b) from sulphite, thiol and Mg^{2+} omitted, cell homogenate 2.9 mg protein (containing 60 μg Chl), [^{35}S]Na_2SO_3 spec. activity 60 sec^{-1} × nmol^{-1}, experimental set up as described in Materials and Methods and legend to Table I.

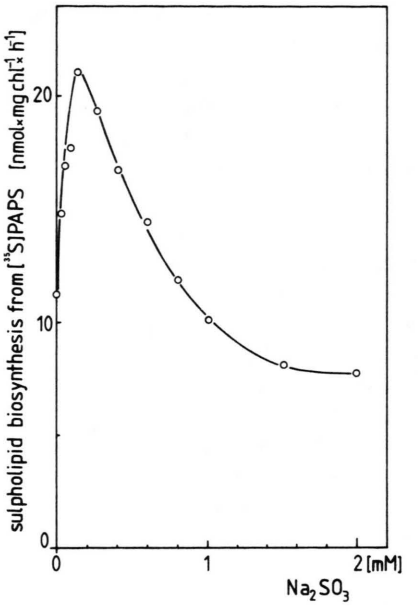

Fig. 3. Twofold effect of unlabelled sulphite on the sulpholipid labelling with [^{35}S]PAPS as sulphonyl group donor: stimulation of the incorporation at low concentration of sulphite and progressing inhibition at concentrations exceeding 100 μM. The assay contained [^{35}S]PAPS 30 μM (spec. activity: 580 sec^{-1} × nmol^{-1}), sulphite varied as indicated, cell homogenate: 0.93 mg protein (containing 20 μg Chl), DTT 0.3 mM, Mg^{2+} 10 mM, other experimental details as described in Materials and Methods and in the legend to Table III.

b. Incorporation of [^{35}S]PAPS or [^{35}S]SO_3

The activity for sulpholipid labelling from either PAPS or sulphite has been compared using the same cell extract as for the incorporation of sulphate. PAPS was found a more specific donor of the sulphonate group than sulphite (Fig. 2). The apparent K_m for PAPS was 25 μM whereas no saturation was obtained for sulphite (Fig. 2, curve b). As compared to PAPS, labelling of SL from sulphite was increased by a factor of 30, suggesting that sulphite would be incorporated more effectively. However, the use of unlabelled sulphite as competitive substrate in the presence of equimolar concentrations of [^{35}S]PAPS (Fig. 3) increased rather than suppressed the labelling of the sulpholipid. The highest rate of incorporation was observed in the presence of 150 – 160 μM sulphite (ratio of unlabelled to labelled substrates 5 : 1). Higher concentrations of sulphite lead to a progressive decline of the labelling and only at 800 μM of sulphite gave rise to a weak inhibition of the incorporation (20%).

Since, in direct comparison to PAPS, sulphite was not used by the cell extract as a substrate it may have served as a reductant due to its tendency to undergo slow oxidation. In fact, other reductants like thiols were found to stimulate the labelling of the sulpholipid from [^{35}S]PAPS in a fashion which resembled that of sulphite. Glutathione gave the highest rates of sulpholipid labelling (Table II) saturating at concentrations exceeding 1 mM . With the

Table II. Requirement for thiols.

Thiol	SL [nmol × mg Chl^{-1} × h^{-1}]
–	2.4
L-cysteine	2.0
glutathione	32.4
dithiothreitol	29.6
dithioerythritol	28.8

Crude cell homogenate from *C. reinhardii* was incubated as described in the legend to Table I. Thiols were used at 0.3 mM, [^{35}S]PAPS as substrate 30 μM (spec. activ.: 483 sec^{-1} × nmol^{-1}).

dithiols DTE or DTT 0.2 or 0.5 mM were required for maximal labelling (details are not shown) – higher concentrations were increasingly inhibitory. L-cysteine or β-mercaptoethanol were ineffective to promote the labelling of sulpholipid from PAPS.

Further fractionation of the cell homogenate into soluble protein and not soluble membrane particles revealed that PAPS was predominantly used by the soluble cell fraction (Table III) which retained 77% of the activity observed in the cell homogenates. With sulphite as donor, the soluble protein only showed residual activity (17%, attributable to the contaminating membrane fragments) but the resuspended membrane fraction was highly active (179%). When the membranes were reconstituted with soluble protein originated from the same preparation (Fig. 4) the activity for sulpholipid biosynthesis with PAPS as sulphonate donor was regained. The membranes required 18.8 mg of soluble protein per mg of chlorophyll for a complete saturation of the reconstitutional effect. Since this property was destroyed by heat it has been assumed that the sulpholipid biosynthesis from PAPS is dependent on a specific enzyme activity of the soluble part of the cell. Filtration of the soluble protein through Sephadex G 50 caused a loss of 30% of its reconstitutional activity. When the soluble protein was fractionated on Biogel P 2 (exclusion limit 1.2 kdaltons) its reconstitutional activity was completely lost. In this case, a partial restoration of the sulpholipid labelling was obtained when the assay was supplemented with a low weight fraction (MW 300 to 400 daltons, Material and Methods) which was retarded by the gel. This low weight fraction contained thiol group(s) as evidenced by the colorimetric detection with DTNB.

Table III. Intracellular distribution of enzymatic activity for sulpholipid labelling.

Origin of sulphonate	PAPS		Sulphite	
Cell fraction	Rate [a]		Rate	
soluble proteins reconst. with membranes	6.4	100%	5.2	100%
soluble proteins S 45000 × *g*	4.9	77	0.9 [b]	17
membranes P 45000 × *g*	0.05	1	9.3	179
soluble proteins heat inactivated	0	–	0.1	2
membranes heat inactivated	n.d.	–	0.9	19

[a] Rates expressed as [nmol SL × mg Chl^{-1} × h^{-1}].
[b] Contains chlorophyll containing fragments 0.01 mg Chl × ml^{-1}.

The assay for sulpholipid labelling employed as sulphonyl group donor [^{35}S]PAPS 30 µM (spec. activ.: 483 sec^{-1} × $nmol^{-1}$ or [^{35}S]SO_3 20 µM (spec. activ.: 233 sec^{-1} × $nmol^{-1}$), soluble protein 7.3 mg × ml^{-1}, membranes 3.3 mg protein × ml^{-1} containing 0.2 mg chlorophyll × ml^{-1} (ratio of reconstitution 1:1 volume), Tris-Cl 100 mM, $MgCl_2$ 10 mM and DTT 0.3 mM. Samples of 1 ml have been incubated for 15 min at 25 °C and assayed for sulpholipid as described in Materials and Methods.

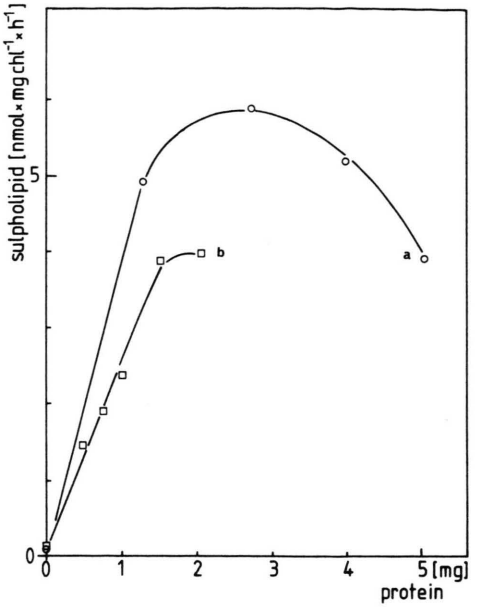

Fig. 4. Reconstitution of the sulpholipid labelling with [^{35}S]PAPS by a protein fraction from the cell extract. Washed membrane particles have been supplemented with untreated soluble protein (a) or with the corresponding amount of protein submitted separately to gel filtration (b) in order to remove low weight compounds. The assay contained per 1.0 ml: membrane particles 80 µg chlorophyll, soluble protein as indicated, [^{35}S]PAPS 30 µM (spec. activity 583 sec^{-1} × $nmol^{-1}$), DTT 0.3 mM and Mg^{2+} 10 mM, other conditions as described in Materials and Methods and in the legend to Table III.

Discussion

Incorporation of appropriate [^{35}S]sulphonates into the sulpholipid was observed in a cell-free assay system from *Chlamydomonas reinhardii* CW 15. The algae have been disrupted by osmotic shock and subsequent sonication as to remove diffusion- or transportation harriers (*i.e.* cell wall or chloroplast envelope). Compared to tracer feeding with intact al-

gae or part of higher plants the conditions have been considered as *in vitro*. When left unfractionated the crude extract still contained all the enzymes and the sulphonyl group acceptor necessary for sulpholipid labelling. Fractionation of the crude cell homogenate into soluble protein and into particulate material indicated that the sulphonyl group acceptor presumably was located in the chlorophyll containing membranes.

When supplied with [^{35}S]sulphate and ATP the sulpholipid labelling in the crude cell homogenate was not reduced by unlabelled cysteic acid (or 3-sulpholactate to which it was rapidly metabolized). Cysteic acid therefore may not act as precursor as proposed by Davies *et at.* [1]. Presumably, the sulphoglycolytic [2] or cysteic acid pathway are not at all operative in *C. reinhardii* because no intermediates of either pathway have been discovered. Nevertheless, it should not be ruled out completely that in the case of extremely small pools of these intermediates, they may have escaped detection. As a working hypothesis it is assumed that the membrane fraction of *C. reinhardii* already contained a suitable precursor lipid which served as sulphonyl group acceptor *in situ*.

In this respect, it is noteworthy that Zill and Cheniae [18] already discussed the transfer of an activated sulphonyl group onto carbonyl atom of a lipid precursor. The requirement for the membrane fraction significantly parallelled the recent finding of Haas *et al.* [19] who showed that in the higher plant chloroplasts the labelling of the sulpholipid is virtually restricted to the thylakoid membrane system.

After separation of the crude cell extract into soluble protein and particulate membrane material, labelling with [^{35}S]PAPS required an enzymatic activity from the soluble. This enzymatic activity was characterized by a high affinity for PAPS as sulphonyl donor (apparent K_m 25 μM). ATP was no longer necessary as for the sulpholipid labelling from sulphate but the stimulation by thiols of the sulphonyl group incorporation persisted. In a reconstituted assay system it was observed that after gel filtration the PAPS transferring activity became dependent on a low weight compound containing thiol group(s). Exogenous thiol compounds such as reduced glutathione, DTE or DTT which increased the rate of labelling nearly tenfold could replace the endogenous cofactor. This stimulation by thiols may have reflected the necessity of a reductive step in the biosynthesis of the sulphonic acid from the sulphuric acid in PAPS.

With sulphite as sulphonyl group donor and washed membranes as acceptor a much higher rate of sulpholipid labelling was observed than with PAPS as donor (150 nmol × mg Chl^{-1} × h^{-1} vs. 5 nmol × mg Chl^{-1} × h^{-1}). Yet, the labelling of sulpholipid by sulphite appeared less specific since no saturation was obtained up to 0.2 mM. The apparent lack of specificity was parallelled by the ineffectiveness of unlabelled sulphite to suppress the incorporation of [^{35}S]sulphonyl groups provided by PAPS. In this respect, it should not be overlooked that free sulphite may have reacted unspecifically with the cell protein needed for the PAPS dependent sulphonyl transfer so that much higher concentrations are to be employed to reduce the labelling. Such speculations may be misleading because the cell protein may also have affected the accessibility or altered the chemical properties of the hypothetical precursor lipid. It is noteworthy that the reducing thiols were not required for the sulphite labelling of the sulpholipid. This finding may be seen in support of our suggestion that the effect of the thiols in the PAPS dependent assay was due to a reduction of the sulphuric acid in PAPS (oxidation state + 6) to the sulphonic acid in the sulpholipid (oxidation state + 4) because sulphite is already in the suitable oxidation state. It is pertinent to report that sulphite (very likely as the hydrogensulphite radical) readily reacts with 6-deoxy-hex-5-enopyranosides yielding the corresponding hexopyranoside-6-sulphonic acids [20]. If indeed sulphite, generated under physiological conditions from PAPS is the sulphonyl donor without the formation of sulpholactate or similar intermediates, the hypothesis of a sulphonyl transfer to a precursor glycolipid has to be reconsidered [18]. Moreover, the location of this precursor in the lamellar system as discussed here may bear further significance for the very reason that the enzymes of sulphate activation and assimilatory reduction have been found associated with the thylakoids of the higher plant chloroplasts [21, 22].

Acknowledgment

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