Some Observations on the Biosynthesis of the Plant Sulpholipid by *Euglena gracilis*

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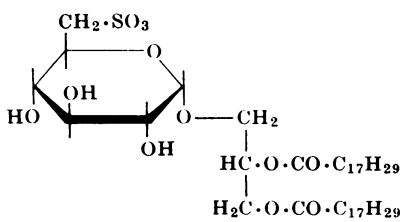
(*Received* 1 *July* 1965)

1. DL-Cysteine decreases the uptake of $^{35}SO_4^{2-}$ by *Euglena gracilis* but does not decrease the relative incorporation of the isotope into sulpholipid; cysteic acid, on the other hand, does not affect the uptake of $^{35}SO_4^{2-}$ but does dilute out its incorporation into the sulpholipid. 2. Both L-[^{35}S]cysteic acid and DL-+*meso*-[3-^{14}C]cysteic acid appear almost exclusively in 6-sulphoquinovose. 3. Molybdate inhibits the incorporation of $^{35}SO_4^{2-}$ into sulpholipid but not its uptake into the cells; this suggests that adenosine 3′-phosphate 5′-sulphatophosphate may be concerned with the biosynthesis of sulpholipid, and it was shown to be formed by chloroplast fragments. 4. An outline scheme for sulpholipid biosynthesis based on these observations is discussed.

Benson, Daniel & Wiser (1959) first showed that ^{35}S in the form of $^{35}SO_4^{2-}$ was rapidly incorporated into the lipids of green algae, photosynthetic bacteria and the leaf tissue of many higher plants. The structure of the sulpholipid involved was eventually shown to be 6-deoxy-6-sulpho-α-D-glucopyranosyl diglyceride [6-sulpho-α-D-quinovosyl diglyceride (I) (Daniel *et al.* 1961)]. The major fatty acid components are palmitic acid and linolenic acid (O'Brien & Shibuya, 1964). The major portion of the sulpholipid is present in the chloroplasts of algae and higher plants (Davies, Mercer & Goodwin, 1965), and the same lipid was present in all tissues (maize, runner beans, *Euglena gracilis* and cambial tissue culture of Paul's scarlet rose) examined by Davies *et al.* (1965).

The existence of 6-sulphoquinovose in the molecule raises a number of important questions of biosynthesis. We have investigated some aspects of origin of the sulphoquinovose molecule from DL-[^{35}S]cysteine, DL-[2-^{14}C]cysteic acid and L-[^{35}S]-cysteic acid as possible precursors of the sulphonic acid residue. In addition, the possibility of the existence of the sulphate-activating system which synthesizes adenosine 3′-phosphate 5′-sulphatophosphate from reduced sulphur has been examined.



(I)

EXPERIMENTAL

Organism. Euglena gracilis Z (1221–5Z) was obtained from The Collection of Algae and Protozoa, Botany School, Cambridge. It was maintained on slopes prepared from agar (1·5%, w/v) and yeast extract (Difco; 0·2%, w/v) in distilled water. It was cultured autotrophically on the medium of Brawerman & Chargaff (1959) and on the heterotrophic medium of Wolken (1961). Conical flasks (250ml.) containing 100ml. of medium were incubated at 18° without shaking. The cells were harvested after 6 days' growth by centrifugation.

Extration of sulpholipid. The method of Davies *et al.* (1965) was used.

Chromatography of sulpholipid. The thin-layer system of Davies *et al.* (1965) was generally used but occasionally the two-dimensional paper system of Benson *et al.* (1959) was used for reference purposes.

Detection and assay of sulpholipid. The administration of ^{35}S to a tissue is by far the most sensitive and unambiguous means of detecting the plant sulpholipid. The radioactive spots on the thin-layer chromatograms were located by radioautography with X-ray film (Kodirex). In this respect thin layers are some 20 times as sensitive as paper chromatograms (Goodwin, 1964). The radioactivity in the sulpholipid spot was quantitatively assayed by eluting it with toluene, adding the extract to a scintillator dissolved in toluene (5g. of 2,5-diphenyloxazole and 0·3g. of 2,2-phenyl-5-phenyloxazole in 1l.) and counting it in a Packard Tri-Carb Scintillation Spectrometer series 314E. All samples were bleached with u.v. light to prevent quenching by any pigment present.

Isolation of 6-sulphoquinovose from the sulpholipid. To a sample of [^{35}S]sulpholipid in 1ml. of aq. 50% ethanol, 0·5ml. of 6N-HCl was added and the mixture heated at 100° for 30min. On cooling 2ml. of water was added and the solution extracted with 5ml. of diethyl ether followed by 5ml. of chloroform. The aqueous layer was evaporated to dryness *in vacuo* and the residue dissolved in 1ml. of water. It was chromatographed on paper with either phenol saturated with water at 23° (Benson *et al.* 1959) or a butanol–propionic acid–water system (Benson *et al.* 1959). The R_F of 6-sulphoquinovose was 0·05 in both solvents. The diethyl ether and chloroform fractions were bulked and the total activity present was measured in the Packard machine. This activity was taken as that present in the diglyceride.

Preparation of chloroplasts from Euglena. Crude chloroplasts were obtained by ultrasonic treatment at 2° (MSE–Mullard Ultrasonic Disintegrator) of intact cells suspended in the medium of Stumpf & James (1963). The time required for ultrasonic treatment depended on the type of cells used: etiolated cells were given two periods of 30sec. duration separated by an interval of 60sec.; cells grown heterotrophically in the dark had three bursts of 30sec., each separated by an interval of 60sec.; autotrophic cells were given one burst of 60sec. followed by two bursts of 30sec., each separated by an interval of 60sec. The intervals were necessary to prevent excessive rises in temperature around the probe. These were then purified in two ways. In the first the disrupted cells were centrifuged at 140*g* for 5min.; the supernatant was then centrifuged for a further 5min. to ensure complete sedimentation of intact cells. The resulting supernatant was then centrifuged at 1000*g* for 12min. to sediment the chloroplasts. The pellet was resuspended in 20ml. of Stumpf & James (1963) buffer and recentrifuged at 1000*g* for 10min. In the second method the density-gradient technique of James & Das (1957) was used. Chloroplast fragments were prepared either by suspending the pellet in 0·5mM-MgO_2 for 5min., recentrifuging at 1000*g* and resuspending in distilled water, or by ultrasonic treatment at 2° (two 15sec. bursts) followed by the usual centrifugation procedure.

Radioactive materials. L-[^{35}S]Cystine, DL+*meso*-[3-^{14}C]-cystine hydrochloride and $^{35}SO_4^{2-}$ (carrier-free) were obtained from The Radiochemical Centre, Amersham, Bucks. L-[^{35}S]Cysteic acid and DL+*meso*-[3-^{14}C]cysteic acid were synthesized by the oxidation of the corresponding cystines with Br_2 in the presence of 0·25% HCl (Friedmann, 1903). Purification was achieved by thin-layer chromatography on Kieselgel G plates with butanol–propionic acid–water (6:3:4, by vol.) as solvent.

RESULTS

Effect of L-*cysteine on incorporation of* $^{35}SO_4^{2-}$ *into sulpholipid.* It is well known that cysteine is metabolized by animal tissues via cysteic acid and that the sulphur is eventually released as SO_4^{2-}. If cysteine sulphur could be oxidized by *Euglena* in this way then the incorporation of $^{35}SO_4^{2-}$ into sulpholipid should be reduced in the presence of L-cysteine.

A 6-day culture (1l.) of *E. gracilis* grown autotrophically was harvested by centrifugation, and the cells were resuspended in 150ml. of fresh medium. To three Erlenmeyer flasks (250ml.) each containing 50ml. of medium were added 50ml. of the suspension; $^{35}SO_4^{2-}$ was added to each flask (0·2mc/flask) and to two of the flasks were added 0·05g. and 0·5g. of DL-cysteine respectively. The flasks were incubated for 18hr. The cells were harvested and washed with 4 vol. of water to remove any free $^{35}SO_4^{2-}$. Each batch of cells was suspended in a known volume of water and samples were counted on a planchet with a Nuclear–Chicago D47 gas-flow detector. From this the uptake of $^{35}SO_4^{2-}$ in each flask could be calculated. The amount of [^{35}S]sulpholipid formed by the cells in each flask was determined as described in the Experimental section. The results (Table 1) indicate that although the higher concentration of DL-cysteine inhibits the uptake of $^{35}SO_4^{2-}$ by the cells the relative incorporation into the sulpholipid is very considerably increased; it changes from 1·53% in the absence of DL-cysteine to 27·62% in the presence of 0·5g. of DL-cysteine. It is evident from this experiment that, whatever the explanation of the effect of cysteine on $^{35}SO_4^{2-}$ uptake, there is no indication that the sulphur of cysteine is converted into SO_4^{2-} in sufficient amounts to dilute out the incorporation of $^{35}SO_4^{2-}$ into sulpholipid.

Effect of L-*cysteic acid on incorporation of* $^{35}SO_4^{2-}$ *into sulpholipid.* In animal tissue L-cysteic acid undergoes transamination with α-oxoglutarate and oxaloacetate to form β-sulphonylpyruvate (Cohen, 1940). The latter could be an intermediate in sulphoquinovose biosynthesis, and if so L-cysteic acid might by virtue of its conversion into β-sulphonylpyruvate dilute out the incorporation of $^{35}SO_4^{2-}$ into sulpholipid. On the other hand, the same result would be expected if SO_4^{2-} was liberated from L-cysteic acid. An experiment similar to that described for DL-cysteine in the previous section was set up and it will be seen (Table 2) that, in contrast with DL-cysteine, L-cysteic acid had no

Table 1. *Effect of* DL-*cysteine on the uptake and incorporation of* $^{35}SO_4^{2-}$ *into the sulpholipid in* Euglena gracilis

Autotrophic cultures (6 days) were incubated for 18hr. with $^{35}SO_4^{2-}$ (0·2mc/flask) in the presence or absence of DL-cysteine.

Amount of DL-cysteine added (g./flask)	$^{35}SO_4^{2-}$ taken up by cells (disintegrations/min.)	$^{35}SO_4^{2-}$ in sulpholipid (disintegrations/min.)	Incorporation into sulpholipid of $^{35}SO_4^{2-}$ taken up (%)
0	$7·2\times10^5$	$1·1\times10^4$	1·53
0·05	$6·7\times10^5$	$1·9\times10^4$	2·84
0·5	$2·1\times10^4$	$5·8\times10^3$	27·62

Table 2. *Effect of* L-*cysteic acid on the uptake and incorporation of* $^{35}SO_4^{2-}$ *into the sulpholipid in* Euglena gracilis

Autotrophic cultures (6 days) were incubated for 18 hr. with $^{35}SO_4^{2-}$ (0·5 mc/flask) in the presence or absence of L-cysteic acid.

Amount of L-cysteic acid/flask (g.)	$10^{-9} \times$ $^{35}SO_4^{2-}$ taken up by cells (disintegrations/min.)	$10^{-6} \times$ $^{35}SO_4^{2-}$ in sulpholipid (disintegrations/min.)	Incorporation into sulpholipid of $^{35}SO_4^{2-}$ taken up (%)
0	4·15	4·16	0·100
0·01	4·20	4·15	0·098
0·03	4·45	3·69	0·083
0·05	4·38	3·09	0·075
0·10	3·65	1·85	0·050

Table 3. *Comparison of the uptake of* $^{35}SO_4^{2-}$, L-[^{35}S]*cysteic acid* and DL + meso-[3-^{14}C]*cysteic acid into the sulpholipid in* Euglena gracilis

Experimental conditions were as outlined in Table 2.

Label added	Total activity in cells (disintegrations/min.)	Activity present in sulpholipid (disintegrations/min.)	Incorporation into sulpholipid of label taken up (%)
$^{35}SO_4^{2-}$	$1·15 \times 10^9$	$3·08 \times 10^6$	0·26
L-[^{35}S]Cysteic acid*			
(*a*)	$1·27 \times 10^7$	$2·91 \times 10^5$	2·29
(*b*)	$1·79 \times 10^7$	$5·16 \times 10^5$	2·88
DL + *meso*-[3-^{14}C]-Cysteic acid	$9·93 \times 10^5$	$2·64 \times 10^4$	2·66

* Separate experiments.

effect on the uptake of $^{35}SO_4^{2-}$ by *Euglena* except to a slight extent at the very highest concentration; it did, however, markedly reduce the incorporation of $^{35}SO_4^{2-}$ into the sulpholipid and this reduction increased with increased concentration of L-cysteic acid.

Incorporation of L-[^{35}S]*cysteic acid and* DL + meso-[3-^{14}C]*cysteic acid into sulpholipid.* To distinguish between the two possible explanations suggested above, of the ability of L-cysteic acid to dilute out the incorporation of $^{35}SO_4^{2-}$ into sulpholipid, an experiment was carried out to compare the incorporation of L-[^{35}S]cysteic acid and DL + *meso*-[3-^{14}C]cysteic acid with that of $^{35}SO_4^{2-}$. The results, collected in Table 3, demonstrate clearly that, when the cysteic acid is labelled with ^{35}S or ^{14}C in C-3, it is ten times more effectively incorporated into sulpholipid than is $^{35}SO_4^{2-}$ under the same conditions. Two samples of the sulpholipid produced in the presence of DL + *meso*-[3-^{14}C]cysteic acid were degraded and the activity was determined in the 6-sulphoquinovose and in the diglyceride residue. In both cases almost all the activity recovered was in the sulphoquinovose (Table 4).

Table 4. *Distribution of radioactivity in the sulpholipid of* Euglena gracilis *grown in the presence of* DL + meso-[3-^{14}C]*cysteic acid*

The sulpholipid from the experiment reported in Table 3 was degraded as described in the Experimental section.

	Total activity in sample (disintegrations/min.)	Activity in 6-sulphoquinovose (disintegrations/min.)	Activity in diglyceride residue	Activity recovered in 6-sulphoquinovose (%)
(*a*)	1240	922	171	84·5
(*b*)	1000	757	33	95·7

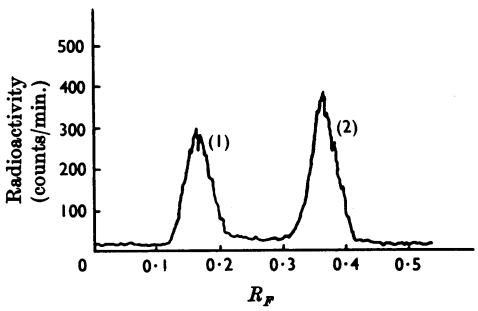


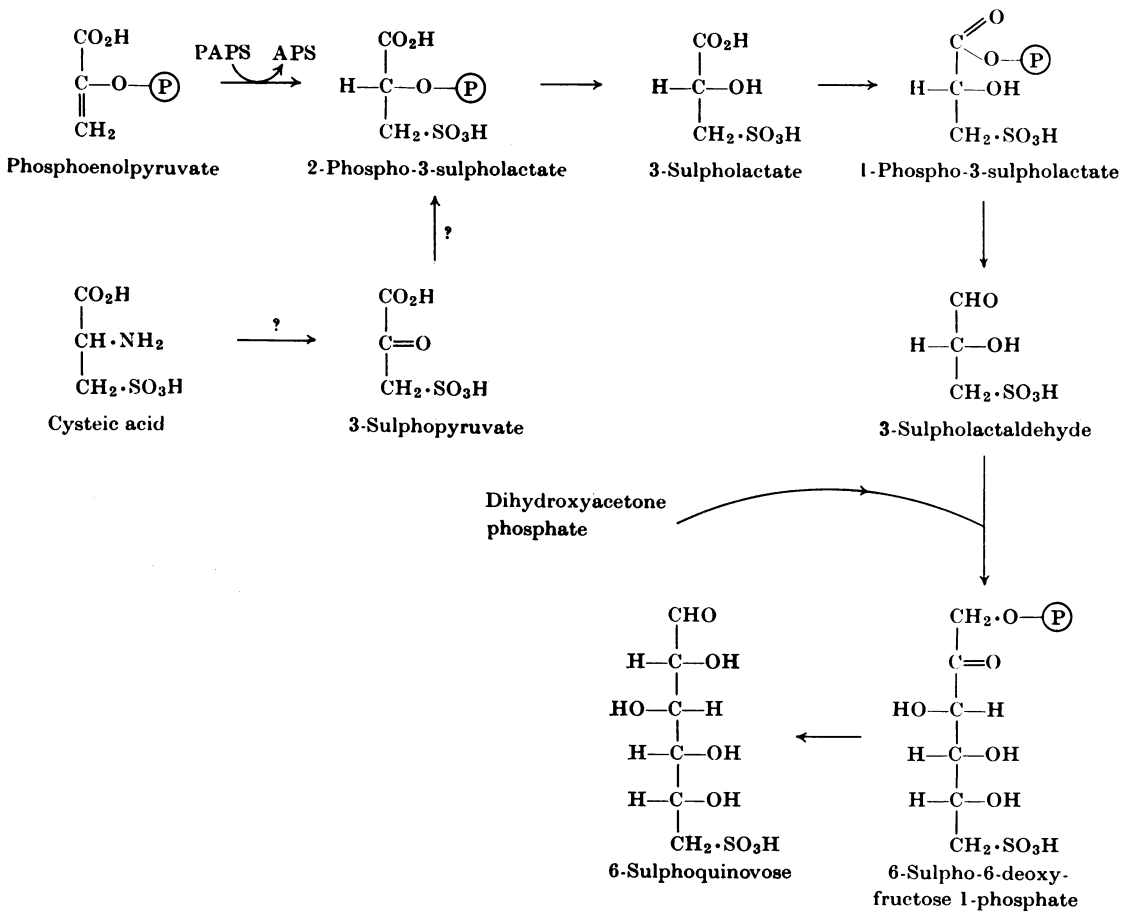
Fig. 1. Formation of adenosine 3′-phosphate 5′-sulphatophosphate (1) from ATP and $^{35}SO_4^{2-}$ (2) by *Euglena* chloroplast fragments. Reaction mixture (Asahi, 1964) was incubated with fragments of chloroplasts prepared by density-gradient centrifugation. Nucleotide extract was run on paper with propanol–ammonia–water (6:3:1, by vol.) as solvent and scanned for radioactivity.

Table 5. *Effect of* MoO_4^{2-} *on the uptake of* $^{35}SO_4^{2-}$ *by* Euglena gracilis *and its incorporation into sulpholipid*

Experimental conditions were as described in Table 2.

Concn. of MoO_4^{2-} (mM)	$10^{-6} \times$ Total uptake of $^{35}SO_4^{2-}$ (disintegrations/min.)	$10^{-4} \times$ Total activity in sulpholipid (disintegrations/min.)	Incorporation into sulpholipid of $^{35}SO_4^{2-}$ taken up (%)
0	3·78	5·78	1·53
0·03	3·52	4·40	1·25
0·3	3·97	3·61	0·91
3	4·67	3·58	0·77

Synthesis of adenosine 3′-phosphate 5′-sulphatophosphate by chloroplast fragments of Euglena gracilis. If 'active sulphur' (adenosine 3′-phosphate 5′-sulphatophosphate) were concerned in sulpholipid biosynthesis then one would expect it to be formed in the chloroplast, which is the major site of sulpholipids. Chloroplasts were prepared and fragmented as described in the Experimental section. The fragments were suspended in 0·5ml. of water and 0·5ml. of reaction mixture (tris, pH 7·5, 25μmoles; magnesium chloride, 5μmoles; $^{35}SO_4^{2-}$, 0·2mc) was added. After incubation for 90min. in the absence of light at 30° the reaction was stopped by boiling. The nucleotides were extracted and purified by column chromatography (Asahi, 1964) and then chromatographed together with $^{35}SO_4^{2-}$ as marker on paper with the solvent, propanol–ammonia–water (6:3:1, by vol.), recommended by Asahi (1964). The resulting chromatograph was scanned for radioactivity with the Nuclear–Chicago Automatic chromatogram scanner. The formation of adenosine 3′-phosphate 5′-sulphatophosphate, as indicated by the R_F value (Asahi, 1964) of the major radioactive zone, was clearly demonstrated. A scan obtained with chloroplast fragments prepared by density gradient is indicated in Fig. 1. A very similar pattern was obtained with chloroplast fragments prepared by disruption of chloroplasts by placing them in magnesium chloride (0·5mM) for 5min., although a small peak corresponding to adenosine 5′-sulphatophosphate was usually noted in these preparations. Although Asahi (1964) could demonstrate formation of adenosine 5′-sulphatophosphate in spinach chloroplasts, he was never able to obtain adenosine 3′-phosphate 5′-sulphatophosphate. He attributed this to the lability in plants of the kinase necessary for the phosphorylation of adenosine 5′-sulphatophosphate.



Scheme 1. Possible pathway for biosynthesis of 6-sulphoquinovose.

Abraham & Bachhawat (1963) demonstrated the formation of adenosine 3′-phosphate 5′-sulphatophosphate in the supernatant obtained by centrifuging disrupted *E. gracilis* cells at 20000*g*; they did not investigate the chloroplast fragment.

Effect of MoO_4^{2-} *on incorporation of* $^{35}SO_4^{2-}$ *into sulpholipid by* Euglena gracilis. Wilson & Bandurski (1958) have shown that molybdate inhibits the formation of adenosine 5′-sulphatophosphate by the enzyme ATP sulphurylase (ATP–sulphate adenylyltransferase, EC 2.7.7.4). If molybdate inhibited the uptake of $^{35}SO_4^{2-}$ into sulpholipid, then it would suggest that APS, and thus adenosine 3′-phosphate 5′-sulphatophosphate, are on the route from SO_4^{2-} to sulpholipid.

Table 5 gives the result of an experiment which shows that, whereas MoO_4^{2-} at concentrations of up to 3mM has no effect on the uptake of $^{35}SO_4^{2-}$ by *E. gracilis*, it does inhibit incorporation into sulpholipid; the inhibition amounts to about 50% at the highest concentration used.

DISCUSSION

It would appear from Table 1 that the sulphur from cysteine is not incorporated into sulpholipid by *E. gracilis*; that is the organism cannot oxidize the SH of cysteine to a sulphonic acid grouping such as exists in the sulpholipid and in cysteic acid. An indirect effect of added cysteine cannot be ruled out but it seems unlikely because Nissen & Benson (1964) showed that [3-^{14}C]cysteine is not incorporated into the sulpholipid in *Chlorella*. However, our experiments with [^{35}S]cysteic acid and [3-^{14}C]-cysteic acid show that cysteic acid can provide a C–S unit for sulpholipid biosynthesis. Benson & Shibuya (1961) have demonstrated the presence of sulpholactaldehyde, sulpholactic acid and sulphopropanediol in *Chlorella pyrenoidosa*. These observations suggest that the sulphur is added at a C_3 stage. The existence of adenosine 3′-phosphate 5′-sulphatophosphate in *Euglena* and the inhibition of $^{35}SO_4^{2-}$ uptake into sulpholipid by MoO_4^{2-} suggests that the compound may take part in sulpholipid biosynthesis, perhaps by forming a C–S linkage with phosphoenolpyruvate; the resulting 2-phospho-3-sulpholactate could eventually be converted into 3-sulpholactaldehyde, which could undergo an aldol condensation with dihydroxyacetone phosphate to form 6-sulpho-6-deoxyfructose 1-phosphate, which on isomerization would yield 6-sulphoquinovose (Scheme 1). Cysteic acid could then enter this pathway via 3-sulphopyruvate, which, as stated above, is known to be formed by transamination in animals (Cohen, 1940). It should be emphasized that cysteic acid is not envisaged as a normal intermediate in sulpholipid biosynthesis.

We thank the Agricultural Research Council for financial support for this project.

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