Sulfur Metabolism in Algae. I. Synthesis of Metabolically Inert Chloroform-Soluble Sulfate Esters by Two Chrysomonads and *Chlorella pyrenoidosa**

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SYNOPSIS. *Ochromonas danica* and *O. malhamensis* were grown in the presence of $Na_2S^{35}O_4$. Four chloroform-soluble S^{35}-compounds were separated from them, one of which was also found in the chloroform extract of *Chlorella pyrenoidosa*. All four S^{35}-compounds were hydrolyzed by acid to yield S^{35}-sulfate. Two of the S^{35}-compounds were fed to *O. danica* and incorporated into the organism without reduction of the S^{35}-sulfate. Evidence is presented suggesting a structural relationship between these chloroform-soluble S^{35}-compounds.

SURPRISINGLY little is known of the biochemistry of sulfolipids despite their presence in the brain and central nervous system and apparent universal occurrence in plants. Not until 1958 was a sulfolipid reported in plants(2). This compound, isolated from chloroplasts, was shown to be a stearic ester of glycerol containing glycoside-6-sulfonate(1). Although the same compound was not found in runner bean leaves, another sulfonic acid-containing sulfolipid was observed by Kates(6). Recent evidence indicates the presence of sulfolipid in the cytoplasm as well as the chloroplasts of beets(13). A sulfolipid containing a sulfonic acid has also been found in the tubercle bacillus(9). These plant sulfolipids where investigated contain, not sulfuric acid, but sulfonic acid. They appear to be long-chain fatty acid esters of glycerol as shown by deacylation with methanolic KOH(1,6) or by infrared spectrophotometry(9).

* Aided by U. S. Public Health Service Grant RG-4987 (C3,4).

† Deceased February 4, 1962.

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J. PROTOZOOL. 9(1), 33-38 (1962).

TABLE 1. *Ochromonas defined medium (g/100 ml final medium)*

L-Arginine · HCl	0.06
L-Asparagine · H_2O	0.08
L-Glutamic acid	0.4
L-Histidine HCl-H_2O	0.08
N-Acetylglutamic acid	0.04
Na_2 glycerophosphate · $5H_2O$	0.04
K_3 citrate · H_2O	0.08
$Fe(NH_4)_2$ citrate	0.0005
$(NH_4)_2H$ citrate	0.08
Thiamine · HCl	0.0005
KH_2PO_4	0.01
Sucrose	1.0
$CaCO_3$	0.003
$MgCO_3$ (basic)	0.08
"A_5 metals"	0.2

O. malhamensis also requires 0.001 mg B_{12}. "A_5 metals" consists of the following metals as sulfur-free salts: 500 p.p.m. boron, 500 p.p.m. manganese, 50 p.p.m. zinc, 20 p.p.m. copper, 50 p.p.m. molybdenum, and 10 p.p.m. cobalt. pH adjustment is unnecessary (4.8 to 5.2).

The present communication describes the isolation of chloroform soluble sulfur compounds[1] from *O. danica, O. malhamensis,* and *Chlorella pyrenoidosa.* They do not appear to be deacylated by methanolic KOH and yield sulfate readily on acid hydrolysis.

MATERIALS AND METHODS

Cultures. *O. malhamensis* and *O. danica,* originally isolated by E. G. Pringsheim, were obtained from Dr. S. H. Hutner of Haskins Laboratories, N. Y. Stock cultures were maintained on a medium used by Hutner and colleagues. The stock medium consisted of: K_3 Citrate · H_2O—0.25, Na Acetate · $3H_2O$—0.5, $MgSO_4$ · $7H_2O$—0.049, cane sugar—2.0, starch—3.0, trypticase—6.0, yeast autolysate—2.0, liver L—0.2, yeast cells—0.5, agar—0.9 g per liter. All media were prepared as dry mixtures. When a liter of this stock medium was to be autoclaved, 0.3 ml 85% (w/v) (DL) lactate and 1.0 ml glycerol were added. The pH of the medium was 6.7-7.2. Experimental cultures were grown on a modification of the defined medium developed by Hutner et al.(5) (Table 1).

Chlorella pyrenoidosa Van Niel, obtained through the courtesy of Drs. Sorokin and Krauss, Botany Dept., University of Maryland, was maintained on Difco potato dextrose agar slants. Experimental cultures were grown on a modification of the defined medium of Sorokin & Krauss(11). The medium contained H_3BO_3—0.112, $CaCO_3$—0.073, $CoCO_3 \cdot 3Co(OH)_2$—0.0017, $Cu(NO_3)_2 \cdot 2H_2O$—0.0149, $FeCl_3 \cdot 6H_2O$—0.098, KNO_3—1.01, $MgCl_2 \cdot 6H_2O$—0.817, MnO_2—0.0061, $Na_2MoO_4 \cdot 2H_2O$—0.0117, $K_2HPO_4 \cdot 3H_2O$—2.52, $2ZnCO_3 \cdot Zn(OH)_2$—0.033, sucrose—10.0, ethylenediamine tetraacetic acid (EDTA)—0.5 g per liter.

All cultures were grown in either 100 ml of autoclaved medium in a 250-ml Erlenmeyer flask or in a liter of medium in a 3-liter Fernbach flask on a rotary shaker at 20-25° illuminated with 250 to 500 f.c. for 12 hr a day. To insure maximum utilization of the $Na_2S^{35}O_4$, the cultures were incubated until the medium approached sulfur deficiency. In a typical case, a 3-liter Fernbach flask containing 1 liter of medium (with 0.004% Na_2SO_4) was autoclaved. At the same time, 5 test tubes containing 10 ml of medium each were autoclaved. The test tubes contained the following concentrations of Na_2SO_4 in per cent:

Tube 1	0.000 (sulfur-deficient medium)
Tube 2	0.002
Tube 3	0.003
Tube 4	0.004
Tube 5	0.005

Growth was measured by optical density. The inoculum consisted of 5 drops of stock culture in 10 ml of distilled water. Of this mixture, 1 drop was inoculated into each 10 ml of medium. Tube 1 contained sulfur due to contamination from the original salts used in the medium (estimated by growth as well as stated impurities to be ca. 2.25 mg %). It served as a control and was the first to level off in growth. Tube 2 helped estimate the time necessary for the utilization of 0.001% Na_2SO_4. When Tube 3 leveled off in optical density, carrier-free $Na_2S^{35}O_4$ was added to the flask (equivalent to Tube 4 in Na_2SO_4 concentration). The flask was incubated for 2 to 24 hr, care being taken not to let Tube 4 level off with respect to Tube 5 in optical density. Should this happen, the sulfur metabolism of the cells would be seriously altered(10, p. 330).

Extraction procedures. The cells were centrifuged from the medium at 12,000 *g*. The clear supernatant solution was removed and designated "medium." The pellet was rinsed with deionized water and the rinse, which contained very little radioactivity, was discarded. The cells were taken up in water to wash the "water space"(10, p. 63) and centrifuged again. The supernatant solution was discarded. The pellet was frozen and thawed three times to break up the cells. In preliminary screening of the compounds in the cell, the extraction procedure of Roberts et al.(10, p. 13) was used. Upon the discovery of S^{35}-sulfolipids the following procedure was used for their extraction:

The cells were extracted 4 times with 40 times their volume of hot chloroform:methanol (2:1, v/v). The procedure was repeated with hot chloroform:methanol: 12N HCl (200:100:1, v/v/v)(7). Originally the extractions had been carried out at 4°(7), but as the same compounds were found in the subsequent frac-

[1] Sulfolipid is used for convenience in this paper to denote organic soluble tetravalent-sulfur containing compounds. The word itself is inexact because the biochemistry of the class is largely unknown.

tions at boiling temperature, the extractions at 4° were omitted.

Chromatographic detection and isolation. Samples were concentrated *in vacuo* and stored at −5°. The radioactive compounds were identified by means of 1- and 2-dimensional chromatography on Whatman No. 3 paper. Two-dimensional systems(8) employing different combinations of 3 solvents were used. In the first system the chromatograms were developed in the first direction with ethanol:*t*-butyl alcohol:58% (w/v) NH_4OH:H_2O (60:20:5:15, v/v/v/v) (Solv. 1) and in the second direction with *t*-butyl alcohol:88% (w/v) formic acid: H_2O (14:3:3, v/v/v) (TBF). In the second system, TBF was used in the initial development followed by phenol:water (5:1, v/v) containing 15 mg of 8-hydroxyquinoline per 100 ml of solvent (PW).

Autoradiograms of chromatograms were prepared utilizing Kodak no-screen X-ray film with exposures appropriate for the detection of the radioactive spots (1 to 4 weeks). Strips of 1-dimensional chromatograms were scanned by passing them under a Geiger-Muller counter (D-47) attached to an automatic recording device.

The following procedure was used to separate the radioactive compounds from the greater part of the non-radioactive compounds in the lipid fraction: Mallinckrodt silicic acid (100 mesh) A. R. was graded in MeOH at 5-min intervals (to remove the fines) until the supernatant was clear. The graded resin was taken up in methanol and packed in a column 2 cm in diam. to a height of 7 cm. It was washed with 2 vol. methanol, 3 vol. acetone, 3 vol. ether, and 3 vol. petroleum ether. The lipid extract was concentrated nearly to dryness and taken up in petroleum ether with enough chloroform to solubilize the lipids. (Total volume not more than 750 μl.) The following fractions were taken according to an adaptation of the procedure of Fillerup & Mead(3): (50 ml each) petroleum ether, 2% (v/v) ether in petroleum ether, 8% ether in petroleum ether, 20% ether in petroleum ether, 50% ether in petroleum ether, ether, 2% methanol in ether, 8% methanol in ether, 20% methanol in ether, 50% methanol in ether, methanol, 1.5% 12N HCl in methanol.

Although this procedure did not effectively separate the radioactive compounds from each other, it did remove most of the other lipids and pigments from the S^{35}-compounds. These radioactive fractions, which contained little lipid material, were combined and chromatographed on paper.

The S^{35}-compounds were separated by paper chromatography (Fig. 1A). After development in the appropriate solvent, they were eluted from the paper with methanol.

Each sample was concentrated to a small volume

TABLE 2. *Distribution of S^{35} in* O. danica *after 24 hr incubation with $Na_2S^{35}O_4$*

Material analyzed	Distribution of S^{35}	
	Medium and cells	Within cells
Culture medium	23.4	
Lipid fraction of cells*	27.8	36.3
HCl lipid fraction of cells†	8.3	10.8
Hydrolyzed residue of cells	40.6	52.9
	100.1	100.0

* Forty volumes of hot chloroform:methanol (2:1, v/v) 4 times.

† Forty volumes of hot chloroform:methanol:12N HCl (200:100:1, v/v/v) 4 times.

and rechromatographed in the 3 solvents to give a single radioactive peak. They were then hydrolyzed with 1N HCl. The hydrolyzates were treated as follows to identify the S^{35}-sulfate(12): 100 μl of the hydrolyzate (10,000 cpm) were added to 100 μl of 20 per cent (w/v) trichloroacetic acid (TCA) containing 100 μg of Na_2SO_4. To this mixture were added 500 μl of freshly prepared 1% (w/v) benzidine in ethanol. After 1 hr at 4° the mixture was centrifuged. The supernatant solution was removed with a pipette and the precipitate was washed 3 times with ethanol (4°). After the final wash, 5 drops of 6N HCl were added to the precipitate and both the supernatant solution and the precipitate were co-chromatographed with $Na_2S^{35}O_4$ for further identification.

RESULTS

Ochromonas sulfolipids. Table 2 shows the distribution of S^{35} in a typical lipid extraction of *O. danica*. After 24-hr incubation, approximately 47% of the S^{35}-sulfur of the cells was in the lipid fraction. This constitutes 36% of the total S^{35} added. Further amounts of chloroform-soluble sulfur compounds were obtained by digesting the residual tissues with 0.2N methanolic KOH for 18 hr at 37° followed by refluxing the residue in 0.2N methanolic KOH for 24 hr. These findings indicate that a considerable part of the sulfolipids are firmly bound to the insoluble cell components. The relative amounts of the four S^{35}-compounds varied in different experiments, probably due to changes in the extraction procedure and subsequent handling. The major component, however, was always Unknown No. 1, ranging from 8 to 10 times the quantity of the other 3 compounds combined.

The medium of actively growing cultures also contained a considerable amount of S^{35}-Unknown No. 1. This compound may result from excretion by the organisms or autolysis of dead cells. The only other S^{35}-compounds in the medium were S^{35}-sulfate and a water-soluble unidentified S^{35}-compound not found in extracts of the cells.

Although the data on *O. malhamensis* are not quan-

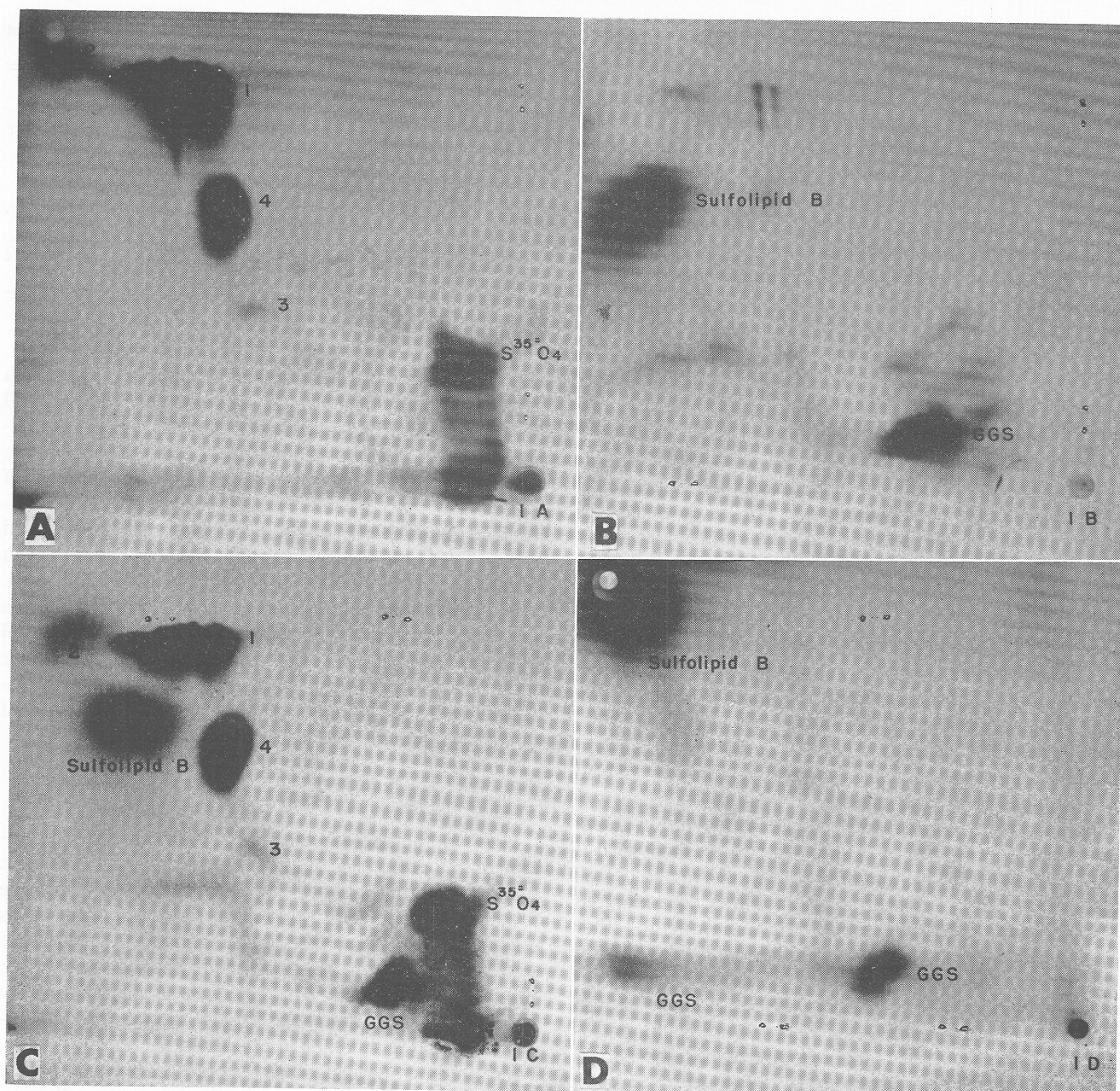

Fig. 1. Autoradiograms of 2-dimensional chromatograms of lipid extracts of *O. danica* and *C. pyrenoidosa* grown in the presence of $Na_2S^{35}O_4$. All four chromatograms were developed upward in TBF. A, B, and C were developed to the left in PW. D was developed to the left in Solv. 1. A. Lipid extract of *O. danica*. B. Water wash of a lipid extract of *C. pyrenoidosa*. C. A and B co-chromatographed. D. Same as B in different solvents showing breakdown of sulfolipid B to glycerol glycoside-6-sulfonate (GGS).

titative, the autoradiograms of extracts of the 2 organisms were similar.

Chemistry of the sulfolipid fraction in O. danica. An autoradiogram of a 2-dimensional chromatogram of a crude fraction of the chloroform-soluble S^{35}-compounds is shown in Fig. 1A. Four unidentified chloroform-soluble sulfur-containing components are seen. The Rf values of these compounds in the 3 solvent systems are listed in Table 3.

Each of the 4 compounds was hydrolyzed in 1N HCl at 100°. Aliquots were taken at 10-min intervals for a period of 2 hr and rate hydrolysis curves drawn for each compound. The curve for Unknown No. 4 is shown in Fig. 2. S^{35}-Unknown No. 3, when hydrolyzed under these conditions, yielded S^{35}-sulfate and S^{35}-Unknown No. 4 within 10 min, with subsequent aliquots showing the same rate of hydrolysis as that given by Unknown No. 4 (Fig. 2). S^{35}-Unknown No. 1 and S^{35}-Unknown No. 2 were each hydrolyzed in 5% (w/v) trichloroacetic acid for 30 min at 100°

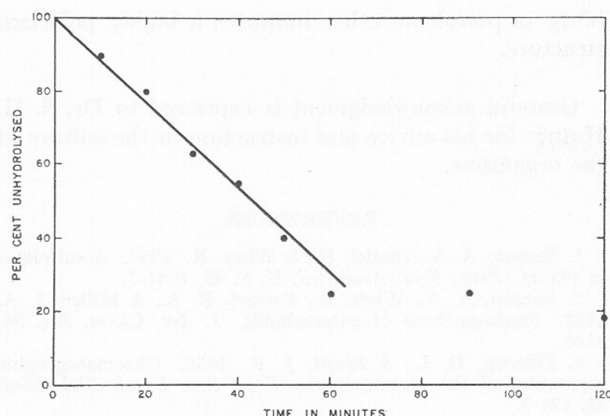

Fig. 2. Hydrolysis rate of S^{35}-Unknown No. 4 in 1 N HCl at 100°.

to yield S^{35}-sulfate and S^{35}-Unknown No. 4.

The hydrolyzate of each of the S^{35}-Unknowns was analyzed for S^{35}-sulfate by the benzidine method and by co-chromatography as described in the *Methods* section.

Attempts to deacylate all four S^{35}-compounds by 0.2N methanolic KOH at 37° for 45 min(1) were unsuccessful. Unknowns No. 1 and 2 are soluble in chloroform, methanol, and ether, but are insoluble in petroleum ether and slightly soluble in water. Unknowns No. 3 and 4 are soluble in chloroform, methanol, ether, and water, but insoluble in petroleum ether. These 4 compounds are quite stable to alkali as shown by their resistance to hydrolysis in 0.2N KOH at 45° for 18 hr. They can be stored at room temperature for weeks without change, provided they are kept dry or are in anhydrous methanol.

None of the sulfolipids or their decomposition products gives a positive test with ninhydrin or phosphate reagent. They do not co-chromatograph with choline sulfate.

Feeding experiments with O. danica *sulfolipids.* S^{35}-Unknown No. 1 and S^{35}-Unknown No. 3 were extracted from *O. danica* and purified as described above. Each S^{35}-compound was added to the medium of an actively growing culture. After 8 hr incubation the cells were extracted according to the procedure of Roberts et al.(10, p. 13). S^{35}-Unknown No. 3 remained in the medium. S^{35}-Unknown No. 1, however, was taken into the cells. Extraction of the cells yielded only S^{35}-Unknown No. 1, S^{35}-Unknown No. 3, and S^{35}-sulfate. The insoluble cellular residue, after hydrolysis in 3N HCl for 18 hr, yielded ca. 40% of the administered S^{35} as sulfate. This implies that the values given in Table 2 are only a minimal estimate of the percentage of S^{35} present as sulfolipid. Probably 50% or more of the S in the cells is present in the organic soluble sulfate fractions.

TABLE 3. *Approximate Rf values of the chloroform-soluble unknown S^{35}-compounds in 3 solvent systems*

Unknown	Solvent 1	TBF	PW
No. 1	0.88	0.90	0.75
No. 2	0.88	0.91	0.8-0.9
No. 3	0.71	0.49	0.60
No. 4	0.85	0.71	0.55

Solvent 1 = ethanol:*t*-butyl alcohol:58% (w/v) NH_4OH: H_2O = 60:20:5:15 (v/v).
TBF = *t*-butyl alcohol:88% (w/v) formic acid: H_2O = 14:3:3 (v/v).
PW = phenol:water = 5:1 (v/v) containing 15 mg 8-hydroxyquinoline per 100 ml solvent.

In another experiment a mixture of all the S^{35}-sulfolipids of *O. danica* was added to an actively growing culture of the phytoflagellate. After incubation for 8 hr the cells were centrifuged, washed, and hydrolyzed in 3N HCl for 18 hr. S^{35}-sulfate constituted over 99% of the radioactivity in the cells. Thus, the organism did not hydrolyze the administered sulfolipids to yield sulfate, for, if that had been the case, S^{35}-cystine and S^{35}-methionine would have been synthesized.

Chorella *sulfolipid.* A lipid extract of S^{35}-fed *C. pyrenoidosa* was taken up in ether and partitioned against water. The ether fraction was chromatographed and the autoradiogram is shown in Fig. 1D. The water fraction is shown in Fig. 1B. Glyceryl glycoside-6-sulfonate (GGS—Fig. 1B and 1D) was identified by co-chromatography in 3 solvent systems with the compound generously provided by Dr. Benson. The streaks on Fig. 1D show that GGS was formed, in part, from sulfolipid B during paper chromatography. Sulfolipid B, therefore, is presumably that isolated in Benson's laboratory(1). Sulfolipid B chromatographs in 2 solvents with Unknown No. 4 derived from *O. danica* but is effectively separated by solvent PW (Fig. 1C).

Glyceryl glycoside-6-sulfonate was extracted by cold (4°) 5% (w/v) TCA(10, p. 13). This extract normally contains compounds loosely bound or "free" in the cell. The presence of GGS in this fraction suggests its function as an intermediate in the synthesis of sulfolipid B.

DISCUSSION

The large quantity of the sulfolipids, together with the ability of *O. danica* to incorporate them rapidly from the medium, indicates their importance to the organism. The fact that the sulfur of Unknown No. 1 is not reduced to yield sulfur amino acids shows that Unknown No. 1 is not an intermediate in sulfur metabolism. Many compounds (including taurine and creatine) are metabolically inert. These S^{35}-sulfolipids appear to fall in this class. That 40% of the S^{35}-sulfolipid remained in the protein polysaccharide residue even after extensive chloroform:methanol:12N HCl (200:100:1, v/v/v) extraction indicates how

tightly bound it is in the phytoflagellate.[2]

Unknown No. 4 was identified by co-chromatography in 3 solvent systems as a component of the chloroform extract of the German cockroach, *Blattella germanica* L.(4). This observation, coupled with occurrence of Uknown No. 1 in quantity in *Chlorella,* indicates an apparent ubiquity of these chloroform-soluble sulfur-containing compounds. Sulfolipid B (Fig. 1B), isolated from chloroplasts by Benson et al.(1), was also found in *B. germanica* as identified in the 3 solvent systems. These 2 compounds, Unknown No. 4 and sulfolipid B, differ in that the first is a sulfate ester and the latter a sulfonic acid derivative of a fatty acid glyceride. They gave, however, the same hydrolysis curve under comparable conditions (Fig. 2)(1).

There are several indications of a structural relationship between the 4 unknown sulfolipids separated in this investigation. First, S^{35}-Unknown No. 4 is produced from the other S^{35}-Unknowns by hydrolysis with 5% (w/v) TCA at 100° (Nos. 1 and 2) or by 1N HCl (No. 3) at the same temperature. Second, the feeding of S^{35}-Unknown No. 1 to *O. danica* did not produce free S^{35}-sulfate, but rather Unknown No. 3. Had free S^{35}-sulfate been present in the cell, it would have been utilized for the synthesis of S^{35}-cystine or S^{35}-methionine, as was the case when S^{35}-sulfate was fed. Neither of these S^{35}-amino acids was found. The fact that *O. danica* did not cleave sulfate from Unknown No. 1 but converted it to Unknown No. 3 is strong evidence of their structural interrelationships.

The presence of a large organic moiety in the unknown sulfolipids is suggested by their insolubility in water and their solubility in chloroform. Their insolubility in petroleum ether indicates a highly polarized structure.

[2] No evidence has been obtained which indicates that the sulfolipids are bound to carbohydrate.

Grateful acknowledgment is expressed to Dr. S. H. Hutner for his advice and instruction in the culture of the organisms.

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