Metabolism of sulfoquinovosyl diglyceride in *Chlorella pyrenoidosa* by sulfoquinovosyl monoglyceride:fatty acyl CoA acyltransferase and sulfoquinovosyl glyceride:fatty acyl ester hydrolase pathways

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Abstract Cell-free preparations of *Chlorella pyrenoidosa* catalyze the transfer of the fatty acyl moiety of fatty acyl CoA derivatives to sulfoquinovosyl monoglyceride to form sulfoquinovosyl diglyceride. This reaction is stimulated by Triton X-100 concentrations of up to 0.6 mg/ml and has a pH optimum of 7.7. Similar *Chlorella* preparations catalyze the stepwise removal of both fatty acyl groups from sulfoquinovosyl diglyceride to form sulfoquinovosyl monoglyceride and then sulfoquinovosyl glycerol. This reaction is inhibited by both calcium and magnesium. The nonionic surfactant Triton X-100 inhibits the enzymatic deacylation at concentrations of less than 0.5 mg/ml but stimulates it at higher concentrations. The pH optimum for the deacylation of sulfoquinovosyl glycerides is 8.2, with little activity observed below pH 8. The enzymatic activities for both the transacylation and deacylation reactions are associated with a 30,000 *g* particulate fraction of *Chlorella*. Sulfoquinovosyl glycerol was found not to be an acceptor of the fatty acyl moiety of fatty acyl CoA derivatives. Methods are described for the preparation of sulfoquinovosyl monoglyceride, sulfoquinovose, and 3-sulfo-1,2-propanediol.

Supplementary key words algae · fatty acyl CoA · lipase · sulfolipid · sulfoquinovose · sulfoquinovosyl glycerol · sulfoquinovosyl monoglyceride · 3-sulfo-1,2-propanediol

Sulfoquinovosyl diglyceride (1,2-di-*O*-acyl-3-*O*-[6-deoxy-6-sulfo-α-D-glucopyranosyl]-*sn*-glycerol) is found in photosynthetic bacteria, algae, and higher plants (1, 2) and makes up as much as 10% of the total amount of plant glycolipid (1). This sulfolipid is localized in the chloroplast (3) and is greatly increased in concentration by illumination (4). The major fatty acids associated with sulfoquinovosyl diglyceride of several species of algae and higher plants are palmitic and linolenic acids (5, 6).

Although previous investigations have described the occurrence, abundance, and structure of the plant sulfolipid, there is a surprising dearth of information pertaining to the metabolism of this lipid. As one attempt to fill this gap we initiated studies on the biosynthesis and degradation of sulfoquinovosyl diglyceride by acyltransferase and deacylase enzymes found in cell-free extracts of *Chlorella pyrenoidosa*. This paper reports our work on these two enzyme systems.

MATERIALS AND METHODS

All reagents and solvents were reagent grade unless otherwise specified. Triton X-100 was a gift from Rohm and Haas Co. (Philadelphia, Pa.). Palmityl CoA was purchased from P-L Biochemicals (Milwaukee, Wis.). Other fatty acyl CoA derivatives were prepared by the method of Pieringer, Bonner, and Kunnes (7).

Infrared spectra were obtained with a Perkin-Elmer (Norwalk, Conn.) model 457 infrared grating spectrophotometer. Measurements of ultraviolet and visible absorption were obtained with either a Zeiss model PMQ II (Carl Zeiss, Inc., New York) or a Cary model 15 (Cary Instruments, Monrovia, Calif.) spectrophotometer.

Melting points were obtained with a Thomas-Hoover capillary melting point apparatus (Arthur H. Thomas Co., Philadelphia, Pa.) and are uncorrected.

The following solvent systems (all v/v) were used for chromatography of lipid-soluble compounds on Whatman no. SG 81 silicic acid-impregnated paper: A, diisobutyl ketone–acetic acid–water 24:16:3; B, diisobutyl ketone–methanol–water 100:25:4; C, diisobutyl ketone–acetic acid–water 8:5:1; D, diisobutyl ketone–pyridine–water

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27:20:3; E, diisobutyl ketone–acetic acid–water 10:5:1; F, hexane–diisobutyl ketone 16:1. The following solvent systems (all v/v) were used for chromatography of water-soluble compounds on Whatman no. 1 paper: G_1, pyridine–ethyl acetate–water–acetic acid 5:5:3:1; H, *n*-propanol–ammonium hydroxide–water 6:3:1; J, pyridine–ethyl acetate–water 4:10:10 (upper phase); K, methyl Cellosolve–3 N ammonium hydroxide–methyl ethyl ketone 7:3:2.

High-voltage electrophoresis was performed on a Camag HVE 60-650 (Camag, Inc., Milwaukee, Wis.) system.

Culture conditions

A culture of *Chlorella pyrenoidosa* was obtained from the Department of Biology, Temple University. The cells were grown in a medium consisting of 0.05 g KNO_3, 0.21 g $MgSO_4$, 0.08 g KCl, 0.07 g Na_2HPO_4, 0.07 g NaH_2PO_4, 0.04 g $CaCl_2$, 0.01 g $FeSO_4$, 5.2 mg H_3BO_3, 3.6 mg $MnSO_4$, 0.13 mg $CuSO_4$, 0.098 mg $Co(NO_3)_2$, and 0.026 mg $ZnSO_4$ per liter of distilled water. The pH was adjusted to 6.3 with HCl. Cultures were grown at room temperature with constant stirring; illumination was from two 15-W fluorescent bulbs backed by reflectors approximately 12 cm from opposing sides of the culture flask. A mixture of 5% CO_2 in compressed air was continuously bubbled through the mixture.

Radioactively labeled cells were produced by growing cultures as described above in a medium to which was added sufficient $Na_2{}^{35}SO_4$ (New England Nuclear, Boston, Mass.) to give a specific activity of about 15 mCi/nmole $SO_4{}^{2-}$.

Enzyme preparation

Chlorella cells (approximately 1 g wet wt) were collected by centrifugation at 4000 *g* for 10 min at 4°C in a Sorvall refrigerated centrifuge. The cells were disrupted by adding 10 ml of polystyrene beads to a suspension of about 2 g of cells in 10 ml of 0.1 M phosphate buffer, pH 7.4, and shaking the mixture successively for 10- and 7.5-min intervals at −10°C in the device of Shockman, Kolb, and Toennies (8). The beads were removed by filtration through a cold sintered-glass funnel, and the filtrate was centrifuged at 500 *g* for 1 min. Enzyme was routinely prepared from the supernate of this low-speed centrifugation by centrifuging it at 30,000 *g* for 30 min in the Sorvall refrigerated centrifuge (9). The pellet from this centrifugation was suspended in 5 ml of either 0.1 M sodium phosphate buffer, pH 7.4, or Tris–HCl buffer, pH 8.2, and used immediately. In a typical experiment the amount of protein in this fraction was 10 mg and the concentration of chlorophyll was 3 mg. In experiments dealing with fractionation of the enzyme, the 500 *g* supernate was first centrifuged at 15,000 *g* for 15 min and then the resulting supernate was centrifuged at 30,000 *g* as above. The 30,000 *g* supernate was centrifuged at 100,000 *g* for 30 min in a Beckman model L ultracentrifuge. All residues were suspended in half the volume of the original homogenate of the appropriate buffer with the aid of a hand-operated Potter-Elvehjem homogenizer. In experiments dealing with various pH values, the 30,000 *g* residue was suspended in 0.05% KCl in 1 mM dithiothreitol.

Chlorophyll was determined by the method of Arnon (10). Protein was determined on the residues, after chlorophyll extraction, by the method of Lowry et al. (11) for insoluble proteins. A linear relationship between chlorophyll and protein content was found for the 30,000 *g* enzyme preparations.

Preparation of sulfoquinovose and 3-sulfopropanediol

2,3,4-Triacetyl-6-tosyl-α-methyl glucoside was prepared by the method of Compton (12). This compound was dissolved in methyl isobutyl ketone and refluxed with one equivalent of NaI for 3 hr. The mixture was cooled and filtered, and the filtrate was concentrated to dryness in vacuo. The residue was dissolved in chloroform and washed with water, 5% sodium thiosulfate, and water. The solvent was removed from the washed organic phase in vacuo and the residue was dissolved in boiling ethanol. After standing at 4°C overnight, the product separated as a white solid. This compound was suspended in dry methanol to which was added 0.03 vol of 10% sodium methoxide. The resulting mixture was stirred at room temperature until a clear solution was produced. Then 0.27 vol of water was added and the resulting mixture was neutralized with Dowex 50 hydrogen ion-form resin. The solvent was removed in vacuo, leaving a white solid that was recrystallized from acetone as fine white needles. This compound gave a positive Beilstein test for halogen and had a melting point of 144–146°C; lit. mp 146–147°C (13). The IR spectrum was compatible with that predicted for 6-deoxy-6-iodo-α-methyl glucoside. The overall yield from α-methyl glucoside was 62%.

Sulfoquinovose was prepared from this compound by a modification of the method of Helferich and Ost (14). To 10 mmoles of the above compound was added a solution of 11 mmoles of sodium sulfite in 40 ml of water. The resulting mixture was refluxed for 20 hr. The solvent was evaporated in vacuo and the residue was dried over P_2O_5 in a vacuum desiccator. The dry glass-like residue was extracted into methanol and filtered. The residue resulting after evaporation of the methanol in vacuo was dissolved in water. The solution was freed of sodium ion by ion exchange chromatography (Dowex 50, hydrogen ion form) and then neutralized with cyclohexylamine (Eastman Organic Chemicals, Rochester, N.Y.). A portion of the cyclohexylamine salt was crystallized as hygroscopic white leaflets by addition of ethyl acetate to a concentrated ethanolic solution of the compound. The crystals were washed with hot toluene, and their melting point was de-

termined to be 157–160°C (dec). The compound gave a negative test for halogen and had an IR spectrum compatible with that predicted for the cyclohexylamine salt of 6-deoxy-6-sulfo-α-methyl glucoside. This compound was converted to the free sulfo-sugar by refluxing for 20 min in 12% aqueous HCl. The sulfoquinovose produced in this reaction gave a positive Benedict's test and ran as a single spot on paper chromatography.

3-Sulfo-1,2-propanediol was prepared from 3-iodo-1,2-propanediol (Eastman Organic Chemicals) by the above procedure used to prepare α-methyl sulfoquinovose from 6-iodo-α-methyl quinovose. The product migrated as a single spot on electrophoresis and had an IR spectrum compatible with the proposed structure.

Isolation of [^{35}S]sulfoquinovosyl diglyceride

Cells were grown in radioactive medium as described above and harvested by centrifugation. The lipids were extracted by the technique of Roughan and Batt (15) and fractionated by the method of Rouser, Kritchevsky, and Yamamoto (16). Approximately 95% of the total radioactivity applied to the DEAE cellulose (acetate form) column was recovered in the fraction eluted with 20 mM ammonium acetate in chloroform–methanol–ammonium hydroxide 40:10:1 (v/v). This fraction contained sulfoquinovosyl diglyceride of at least 96% radiochemical purity as determined by thin-layer chromatography according to Roughan and Batt (15) or silicic acid-impregnated paper chromatography according to Kates (17).

The identity of this lipid was further established by chromatography of the water-soluble radioactive product from acid hydrolysis of the deacylated lipid with chemically synthesized sulfoquinovose in solvent system J (descending) or K (R_F 0.57) as well as by electrophoresis in pyridine–acetic acid–water 1:10:89 (v/v) buffer, pH 3.6.

When this radioactive lipid was subjected to mild alkaline hydrolysis (0.1 M KOH for 30 min at 37°C) and the resulting water-soluble radioactive product was treated with 0.1 M neutral sodium metaperiodate in the dark for 16 hr followed by hydrolysis in 2 N HCl at 100°C for 15 min, a radioactive compound was produced which after sodium borohydride reduction had the same electrophoretic mobility as 3-sulfo-1,2-propanediol.

Preparation of [^{35}S]sulfoquinovosyl monoglyceride

The method for hydrolyzing [^{35}S]sulfoquinovosyl diglyceride to sulfoquinovosyl monoglyceride and free fatty acid was similar to that previously published for the hydrolysis of monogalactosyl diglyceride (18, 19) and used porcine pancreatic lipase (Mann Research Laboratories, Inc.) pretreated according to Safford and Nichols (18) to ensure monodeacylation. A standard reaction mixture contained 0.1 mM [^{35}S]sulfoquinovosyl diglyceride (15 mCi/nmole) and 0.25 M calcium chloride sonicated in 0.5 ml of 0.1 M Tris–HCl, pH 8.2, to which was added 25 mg of lipase powder. Incubations were conducted at 38°C for 1 hr with shaking. Reactions were stopped by the addition of 7.5 ml of chloroform–methanol 2:1 (v/v) followed by 4.5 ml of water. After thorough mixing, the phases were separated by centrifugation. The organic phase was removed with a capillary pipette, and the aqueous phase was extracted with 5 ml of chloroform. Solvent was removed from the combined organic phases in vacuo, and the residue was applied to Whatman no. SG 81 silicic acid-impregnated paper. The chromatogram was developed for 18 hr with solvent system A. The position of the product was determined with a model 363 radiochromatogram scanner (Atomic Accessories, Inc., Valley Stream, N.Y.). The amount of product formed was determined by quantitatively eluting the spot with chloroform–methanol 3:2 (v/v) and counting a dried aliquot on a planchet in a gas-flow Geiger counter (Nuclear-Chicago).

This product (R_F 0.2) was slightly less mobile than sulfoquinovosyl diglyceride (R_F 0.3) in the silicic acid-impregnated paper chromatography system of Kates (17) (see also lane B, Fig. 1) or the thin-layer system of Roughan and Batt (15). The water-soluble mild alkaline hydrolysis product of this lipid chromatographed with sulfoquinovosyl glycerol in system H (R_F 0.43) or G (Fig. 2). Treatment of this radioactive mild alkaline hydrolysis product with 0.1 M HCl at 100°C for 2 hr produced a new radioactive compound that had the same electrophoretic mobility as sulfoquinovose and chromatographed with standard sulfoquinovose in several solvent systems. Based on the work of Noda and Fugiwara (19), the product of this lipase reaction is assumed to be predominantly the 2-acyl isomer of sulfoquinovosyl monoglyceride.

Assay for the acylation of sulfoquinovosyl monoglyceride

The formation of sulfoquinovosyl diglyceride from sulfoquinovosyl monoglyceride and fatty acyl CoA was measured as follows. Enzymatic reactions were started by the addition of enzyme and stopped by the addition of 4.5 ml of chloroform–methanol 2:1 (v/v). 3 ml of water was added to each sample and, after thorough mixing, the phases were separated by centrifugation. The upper (aqueous) layer was removed with a Pasteur pipette, and an aliquot of the lower (organic) phase was taken and evaporated under reduced pressure. The lipid residue from the organic phase was applied to silicic acid-impregnated paper. The chromatogram was developed for 18–20 hr with solvent systems A or C, and the positions of the radioactive compounds were determined with a radiochromatogram scanner. The amount of sulfoquinovosyl diglyceride synthesized was determined by cutting out the radioactive spot of the chromatogram with the same mobility as standard sulfoquinovosyl diglyceride and counting it in a vial of a scintillation spectrometer (Packard Instrument Company, Downers Grove, Ill.). A scintillation fluid of 4

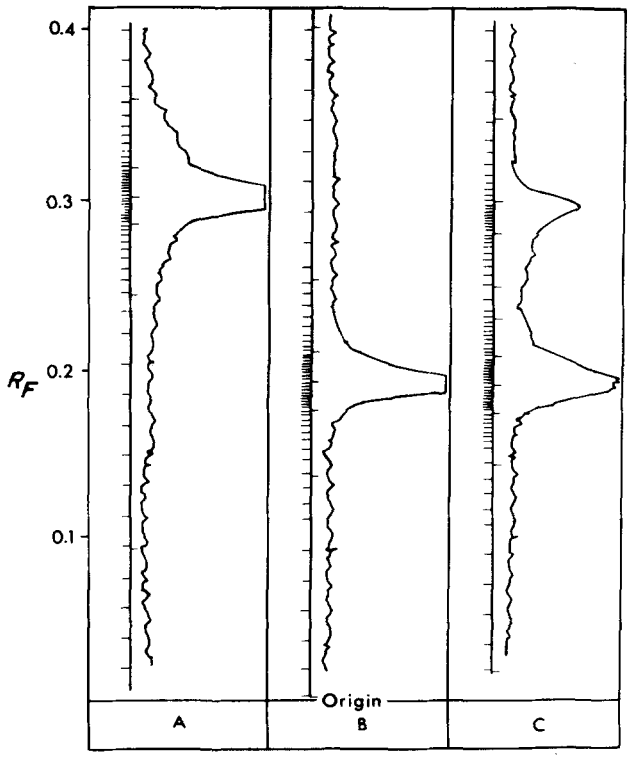


Fig. 1. Chromatography of the lipids extracted from the incubation of [^{35}S]sulfoquinovosyl monoglyceride and fatty acyl CoA in the presence of a *Chlorella pyrenoidosa* enzyme preparation with standard sulfolipids. Lane A, [^{35}S]sulfoquinovosyl diglyceride isolated from *C. pyrenoidosa*; lane B, [^{35}S]sulfoquinovosyl monoglyceride produced by treatment of [^{35}S]sulfoquinovosyl diglyceride with pancreatic lipase as described in the text; lane C, lipid extract from incubation of [^{35}S]sulfoquinovosyl monoglyceride and fatty acyl CoA under conditions described in Table 1. Chromatography was carried out using solvent system C. Radioactive spots were detected with a radiochromatogram scanner equipped with an integrator pen as well as a peak pen. No radioactivity was detected above R_F 0.4.

g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene per liter of toluene was used. This assay technique gave predictable and reproducible results. Duplicate samples had values usually within 5–8% of each other.

Assay for the deacylation of sulfoquinovosyl glycerides

The hydrolysis of [^{35}S]sulfoquinovosyl glycerides by *Chlorella* enzyme preparations was routinely measured as follows. After stopping the enzymatic reaction by addition of 4.5 ml of chloroform–methanol 2:1 (v/v), 3 ml of water was added and the mixture was thoroughly agitated. The phases were separated by centrifugation. The quantity of [^{35}S]sulfoquinovosyl glycerol produced in the reaction was measured by counting an aliquot of the aqueous phase in a gas-flow Geiger counter.

The amount of [^{35}S]sulfoquinovosyl monoglyceride formed by the enzymatic deacylation of [^{35}S]sulfoquinovosyl diglyceride was determined by separating the two

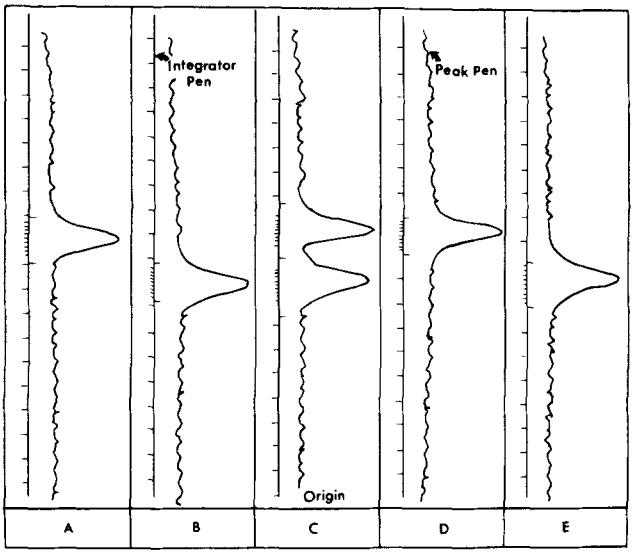


Fig. 2. Identification of product formed from sulfoquinovosyl monoglyceride and fatty acyl CoA. Lane A contains the water-soluble mild alkaline hydrolysis deacylation product of the lipid formed from [^{35}S]sulfoquinovosyl monoglyceride and fatty acyl CoA under conditions described in Table 1. Lane B contains the acid hydrolysis ^{35}S-labeled product of the compound in Lane A. Lane C contains the water-soluble radioactive products formed by ethanolic HCl hydrolysis of the lipid product formed in vitro from [^{35}S]sulfoquinovosyl monoglyceride and [^{14}C]palmityl CoA. Lane D contains [^{35}S]sulfoquinovosyl glycerol formed by mild alkaline hydrolysis of standard [^{35}S]sulfoquinovosyl diglyceride. Lane E contains sulfoquinovose formed by acid hydrolysis of the compound in Lane D. Chromatography was carried out in system G. The positions of the radioactive compounds were detected with a radiochromatogram scanner equipped with an integrator pen as well as a peak pen. The entire chromatogram from origin to solvent front is shown.

lipids in the organic phase of the chloroform–methanol–water 2:1:2 extract with solvent system C and counting the isolated sulfoquinovosyl monoglyceride spot in a liquid scintillation spectrometer.

EXPERIMENTAL RESULTS

Product of the acyltransferase reaction

Incubation of [^{35}S]sulfoquinovosyl monoglyceride with fatty acyl CoA in the presence of a cell-free preparation of *Chlorella pyrenoidosa* produced a chloroform-soluble radioactive compound that chromatographed with sulfoquinovosyl diglyceride (Fig. 1) in lipid solvent system C or on thin-layer plates developed with chloroform–methanol–acetic acid–water 85:15:10:3 (v/v). When this compound was deacylated (0.1 M KOH in toluene–methanol 1:1 [v/v]) for 15 min at 37°C, diluted with 2 ml of water and 2 ml of $CHCl_3$, and the aqueous phase passed over Dowex 50-H^+, all of its radioactivity was found in the aqueous phase of the chloroform–water extract of the reaction mixture. This water-soluble mild alkaline hydrolysis product chromatographed with standard sulfoquinovosyl glycerol in solvent system H or G (lane A, Fig. 2).

When this water-soluble radioactive compound was treated with 0.1 M HCl at 100°C for 2 hr, a new radioactive compound was produced that had the same electrophoretic mobility as sulfoquinovose and chromatographed with standard sulfoquinovose in both of the above solvent systems (lane B, Fig. 2).

The incubation of [^{14}C]palmityl CoA (New England Nuclear) with a 30,000 *g* particulate preparation (containing endogenous lipids as well as enzyme) of *Chlorella pyrenoidosa* resulted in the formation of several chloroform-soluble radioactive compounds, one of which chromatographed with standard sulfoquinovosyl diglyceride in system C (R_F 0.30), B (R_F 0.05), A (R_F 0.35), or D (R_F 0.15). This ^{14}C-labeled lipid was eluted from the paper and either deacylated with mild alkali as previously described or hydrolyzed with 2 N ethanolic HCl according to Davies, Mercer, and Goodwin (20). When these hydrolysis reaction mixtures were extracted with chloroform and then with water, the radioactivity was found in the chloroform phase of the extract. If [^{35}S]sulfoquinovosyl monoglyceride was added to the [^{14}C]palmityl CoA and 30,000 *g* enzyme preparation, nearly twice as much product was detected at the R_F of standard sulfoquinovosyl diglyceride (Table 1). When the above alkaline and acid hydrolysis procedures were applied to this doubly labeled lipid, radioactivity was found in both the organic and the aqueous phases of the extraction mixture. The aqueous phase of the extract from mild alkaline hydrolysis of this compound contained only one radioactive product that chromatographed with sulfoquinovosyl glycerol (similar to lane A, Fig. 2). After acid hydrolysis of the doubly labeled lipid, the aqueous phase of the extract contained two radioactive compounds, one of which chromatographed with sulfoquinovosyl glycerol while the other chromatographed with sulfoquinovose (lane C, Fig. 2).

The possibility that the fatty acyl group from fatty acyl CoA became attached to a free hydroxyl of the sulfoquinovose moiety rather than to the free hydroxyl of the glycerol moiety of sulfoquinovosyl monoglyceride was eliminated by periodate degradation of the doubly labeled lipid. The doubly labeled product isolated from incubation of [^{14}C]palmityl CoA and [^{35}S]sulfoquinovosyl monoglyceride in the presence of a 30,000 *g* particulate fraction of *Chlorella* was allowed to react with 0.05 M sodium metaperiodate (adjusted to pH 7) in the dark at room temperature for 18 hr. The reaction mixture was then made 1 N in HCl and heated in a boiling water bath for 15 min. Radioactivity was found in both the aqueous and the organic phases of a chloroform–methanol–water 2:1:2 (v/v) extract of the hydrolysis mixture. The radioactivity in the aqueous phase of this extract chromatographed with standard 3-sulfolactaldehyde in solvent system G (R_F 0.59). The radioactivity in the organic phase of this extract was found to chromatograph with standard dipalmitin in lipid solvent system E (R_F 0.92), F (R_F 0.02), or B (R_F 0.85).

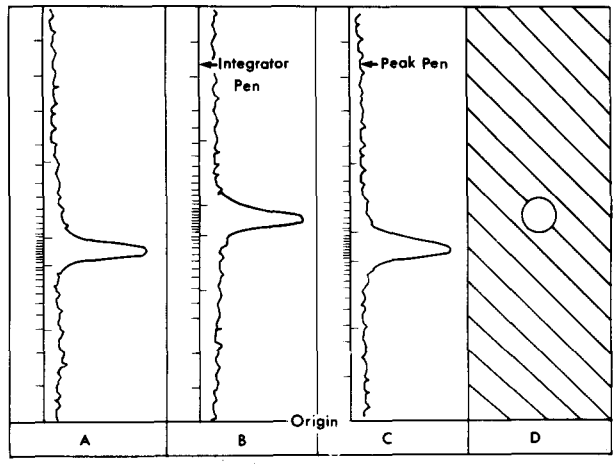


Fig. 3. Chromatography of sulfoquinovose and sulfoquinovosyl glycerol. Lane A contains the water-soluble radioactive product isolated from incubation of [^{35}S]sulfoquinovosyl mono- or diglyceride with a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *Chlorella pyrenoidosa*. Lane B contains the acid hydrolysis product of the ^{35}S-labeled compound in Lane A. Lane C contains standard [^{35}S]sulfoquinovosyl glycerol. Lane D contains standard chemically synthesized sulfoquinovose. These compounds were chromatographed in system H. The lanes containing radioactive compounds were counted with a radiochromatogram scanner equipped with an integrator pen as well as a peak pen. The standard sulfoquinovose was detected by periodate–starch stain. The entire chromatogram from origin to solvent front is shown.

These hydrolysis data confirm the conclusion that the product formed from sulfoquinovosyl monoglyceride and fatty acyl CoA in the presence of a *Chlorella* enzyme preparation is sulfoquinovosyl diglyceride.

Products of the enzymatic hydrolysis of sulfoquinovosyl glycerides

When [^{35}S]sulfoquinovosyl mono- or diglyceride was incubated with 30,000 *g* particulate preparations of *Chlorella pyrenoidosa,* radioactivity was found in the aqueous phase of a chloroform–methanol–water extract of the reaction mixture. Chromatography of this water-soluble radioactive material on Whatman no. 1 paper showed the radioactivity to be localized in a single spot that had the same mobility as standard sulfoquinovosyl glycerol (Fig. 3). When this ^{35}S-labeled compound was heated at 100°C for 1 hr in 3 N HCl, a radioactive compound was formed that chromatographed with standard sulfoquinovose in system G, K, or H, as shown in Fig. 1. Chromatography on Whatman SG 81 silicic acid-impregnated paper of the organic phase of the chloroform–methanol–water extract from incubation of [^{35}S]sulfoquinovosyl diglyceride with a *Chlorella* enzyme preparation showed in addition to unhydrolyzed starting material a small amount of radioactivity associated with a compound that had the same chromatographic mobility as sulfoquinovosyl monoglyceride (Fig. 4). Thus, sulfoquinovosyl glycerol and sulfoquinovosyl monoglyceride were found to be the only detectable radioactive products of the enzymatic hydrolysis of [^{35}S]sulfoquinovosyl diglyceride.

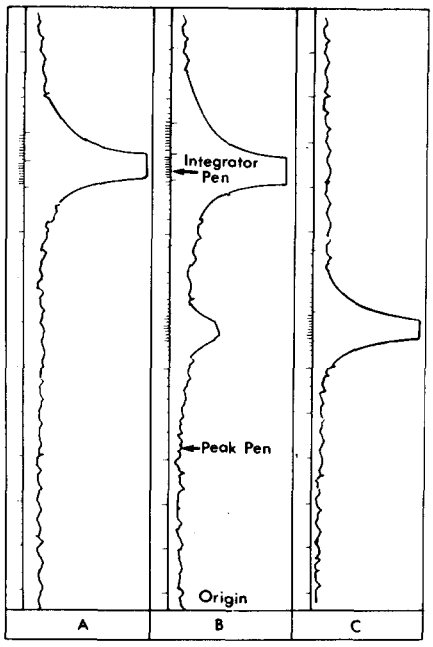


Fig. 4. Identification of the lipid product formed enzymatically from sulfoquinovosyl diglyceride. Lane A contains sulfoquinovosyl diglyceride isolated from *Chlorella pyrenoidosa* grown on a medium containing sodium [^{35}S]sulfate. Lane B contains the chloroform phase of a chloroform–methanol–water 2:1:2 extract from incubation of [^{35}S]sulfoquinovosyl diglyceride with a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *C. pyrenoidosa*. Lane C contains [^{35}S]sulfoquinovosyl monoglyceride prepared by treatment of sulfoquinovosyl diglyceride with pancreatic lipase. These compounds were chromatographed in system C. The positions of the compounds were detected with a radiochromatogram scanner equipped with an integrator pen as well as a peak pen. Only the first 40% of the chromatogram scan is shown because no radioactivity was found at greater R_F values.

TABLE 1. Conditions required for synthesis of sulfoquinovosyl diglyceride

	Sulfoquinovosyl Diglyceride
	nmoles/mg chlorophyll/hr
Complete system	2.58
−Sulfoquinovosyl monoglyceride	1.51
−Palmityl CoA	0.01
−Enzyme	0

The complete system contained 25 μM [^{35}S]sulfoquinovosyl monoglyceride (45,750 dpm/nmole), 0.5 mg/ml Triton X-100, 10 μM [^{14}C]palmityl CoA (111 dpm/pmole), and 37 μg of chlorophyll of a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *Chlorella* suspended in 0.2 ml of 0.1 M sodium phosphate buffer, pH 7.5, in a final volume of 0.4 ml. Incubation was for 20 min at 30°C with shaking. The amount of product formed in the complete system was based on the amount of [^{14}C]palmitate incorporated into the product (rather than [^{35}S]sulfoquinovosyl monoglyceride incorporation) because the reaction was almost completely dependent on exogenous palmityl CoA. The radioactivity of the [^{14}C]palmitate moiety of the isolated sulfoquinovosyl diglyceride product was readily separated from the radioactivity of the ^{35}S-labeled moiety by complete mild alkaline hydrolysis. The [^{14}C]palmitate released by alkali is soluble in organic solvents after adjusting the pH to less than 5. The [^{35}S]sulfoquinovosyl glycerol moiety is soluble in water.

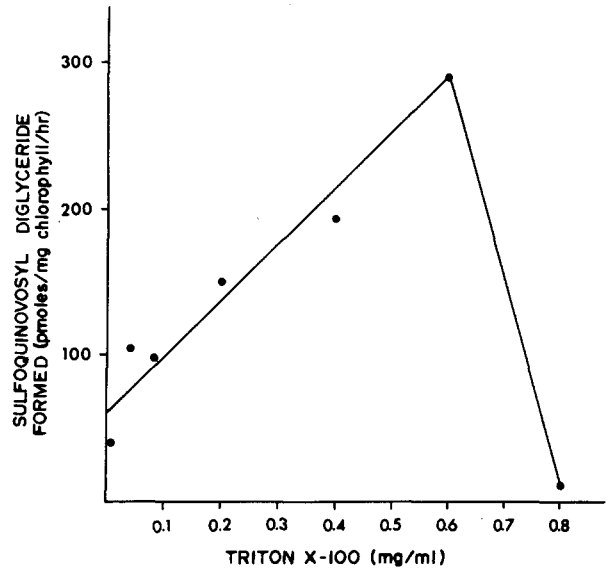


Fig. 5. Effect of Triton X-100 on the biosynthesis of sulfoquinovosyl diglyceride. Each incubation tube contained 20 μM sulfoquinovosyl monoglyceride (27,144 cpm/nmole), 20 μM palmityl CoA, 2 mM dithiothreitol, 80 μg of chlorophyll of a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *Chlorella pyrenoidosa* suspended in 0.1 ml of 0.1 M sodium phosphate buffer, pH 7.4, and the indicated concentration of Triton X-100 in a final volume of 0.25 ml. The incubation was carried out as in Table 1.

Conditions required for the acylation of sulfoquinovosyl monoglyceride

The formation of sulfoquinovosyl diglyceride from [^{35}S]sulfoquinovosyl monoglyceride is dependent upon fatty acyl CoA and enzyme (Table 1). The incorporation of [^{14}C]palmityl CoA into sulfoquinovosyl diglyceride is dependent on enzyme and is stimulated by addition of sulfoquinovosyl monoglyceride (Table 1). Sulfoquinovosyl diglyceride formation is stimulated by the nonionic surfactant Triton X-100 at concentrations of up to 0.6 mg/ml (Fig. 5). The pH optimum for the enzymatic acylation of sulfoquinovosyl monoglyceride is approximately 7.7 in either Tris–HCl or sodium phosphate buffer. Half-maximal

TABLE 2. Subcellular fractionation of acyltransferase

Fraction	Total Protein	Total Chlorophyll	Sulfoquinovosyl Diglyceride	
	mg	*mg*	*nmoles/per mg protein/hr*	*nmoles/per total protein/hr*
15,000 *g*	9.20	3.65	0.062	0.570
30,000 *g*	3.83	0.35	0.141	0.540
100,000 *g*	1.68	0	0.038	0.063
Cytosol	7.92	0	0.076	0.602

Each tube contained 20 μM [^{35}S]sulfoquinovosyl monoglyceride (17,706 cpm/nmole), 0.4 mg/ml Triton X-100, 20 μM palmityl CoA, and an enzyme preparation (containing 1/50 of total protein of fraction except cytosol, which contained 1/200 of total protein) suspended in 0.1 ml of 0.1 M sodium phosphate buffer, pH 7.4, in a final volume of 0.24 ml. Incubation was at room temperature for 30 min with shaking. Product was isolated and measured in the usual manner.

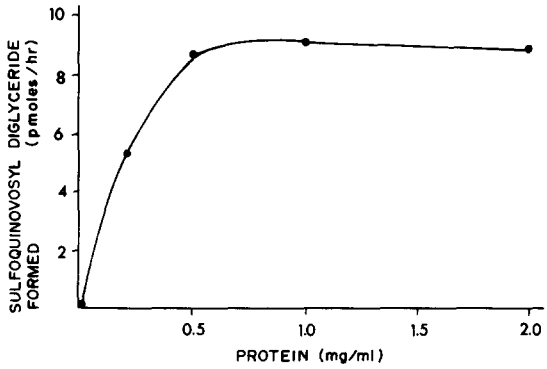


Fig. 6. Proportionality of transacylation to added enzyme. The incubation mixtures consisted of 20 μM sulfoquinovosyl monoglyceride (16,529 cpm/nmole) (counting efficiency 88%), 0.4 mg/ml Triton X-100, 26 μM linoleyl CoA, 40 mM sodium phosphate buffer, pH 7.4, and the indicated amount of protein of a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *Chlorella pyrenoidosa* in a final volume of 0.25 ml. The incubations were carried out at room temperature for 30 min with shaking.

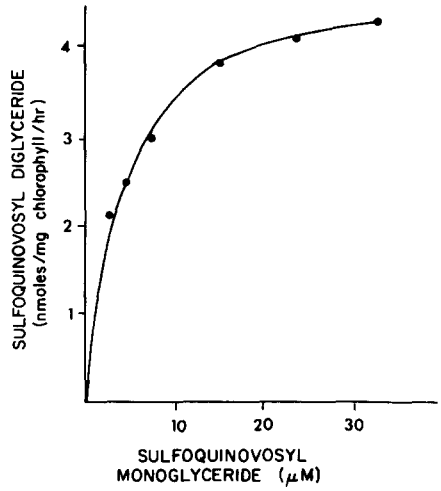


Fig. 7. Effect of concentration of sulfoquinovosyl monoglyceride on transacylation reaction. Incubation mixtures consisted of 0.4 mg/ml Triton X-100, 8.8 μM linoleyl CoA, 40 mM sodium phosphate buffer, pH 7.5, the indicated concentration of [^{35}S]sulfoquinovosyl monoglyceride (19,906 cpm/nmole), and 43 μg of chlorophyll of a 30,000 *g* enzyme (derived from 500 *g* supernate) preparation of *Chlorella pyrenoidosa* suspended in 0.1 ml of 0.05% KCl in a final volume of 0.24 ml. Incubation was carried out at 30°C for 25 min with shaking.

synthesis was obtained at pH 6.5 and pH 8.9. Incorporation of sulfoquinovosyl monoglyceride into sulfoquinovosyl diglyceride is proportional to both added enzyme for protein concentration of not more than 0.5 mg/ml (Fig. 6) and time up to 30 min.

Fig. 7 shows the effect of sulfoquinovosyl monoglyceride concentration on sulfoquinovosyl diglyceride formation. A K_m value for sulfoquinovosyl monoglyceride of 8 μM was calculated from these data. It should be pointed out that this value must be considered as an apparent K_m because the complete solubility of the lipid substrate in the incubation medium even in the presence of the surfactant cannot be assured. The optimum concentrations for the palmityl CoA, linoleyl CoA, and linolenyl CoA substrates used in these experiments were approximately 10–25 μM.

Subcellular localization of enzyme activities

The 15,000 and 30,000 *g* particulate fractions were always found to contain the bulk of the acyltransferase activity as well as nearly all of the cellular chlorophyll (Table 2). However, the 30,000 *g* fraction had the highest specific activity. Variable amounts of enzyme activity were associated with the 100,000 *g* particulate and cytosol fractions presumably depending upon the extent of fragmentation of cellular organelles during the disruption procedure. Table 3 shows the results of incubation of sulfoquinovosyl diglyceride with various subcellular fractions. It is seen that most of the lipase activity was associated with the 30,000 *g* particulate fraction.

TABLE 3. Fractionation of lipase activity

Fraction	Total Protein	Total Chlorophyll	Sulfoquinovosyl Glycerol	
	mg	*mg*	*nmoles/mg protein/hr*	*nmoles/total protein/hr*
Homogenate	16.6	2.59	2.85	47.3
15,000 *g* sediment	7.5	2.30	1.15	8.6
30,000 *g* sediment	1.3	0.47	14.42	18.8
100,000 *g* sediment	0.8	trace	8.62	6.9
100,000 *g* supernate	5.2	0	0.83	4.3

Each incubation mixture contained 35 μM [^{35}S]sulfoquinovosyl diglyceride (4875 cpm/nmole), 40 mM Tris–HCl buffer, pH 7.8, and an aliquot of the indicated enzyme preparation in a total volume of 0.25 ml. The amount of protein used in each assay was 0.12 mg (homogenate), 0.15 mg (15,000 *g*), 0.026 mg (30,000 *g*), 0.016 mg (100,000 *g* sediment), and 0.012 mg (100,000 *g* supernate).

Conditions for the enzymatic hydrolysis of sulfoquinovosyl glycerides

The deacylation of sulfoquinovosyl glycerides is dependent only upon the addition of enzyme (Table 4). Triton X-100 concentrations of greater than 0.5 mg/ml stimulate the hydrolysis of sulfoquinovosyl diglyceride. However,

TABLE 4. Conditions for deacylation of sulfoquinovosyl diglyceride

	Sulfoquinovosyl Glycerol
	nmoles/mg chlorophyll/hr
Complete system	10.75
+0.8 mg/ml Triton X-100	21.75
+5 mM $MgCl_2$	1.29
+0.16 M $CaCl_2$	2.36

The complete system contained 35 μM [^{35}S]sulfoquinovosyl diglyceride (4307 cpm/nmole), 60 mM Tris–HCl buffer, pH 8.2, 50 μg of KCl, and 59 μg of chlorophyll of a 30,000 *g* particulate (derived from 500 *g* supernate) fraction of *Chlorella pyrenoidosa* in a final volume of 0.25 ml. Incubation was for 20 min at 30°C with shaking. The reactions were started by addition of enzyme and stopped by the addition of 4.5 ml of chloroform–methanol 2:1 (v/v) followed by 2.75 ml of water. After thorough mixing, the phases were separated by centrifugation, and product formation was measured by counting an aliquot of the aqueous phase dried on a planchet with a gas-flow Geiger counter.

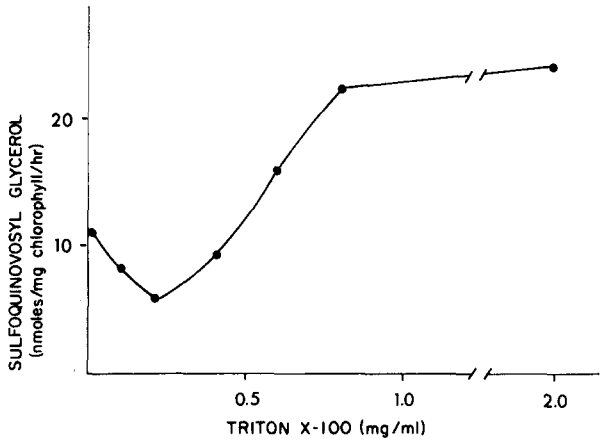


Fig. 8. Effect of Triton X-100 concentration on deacylase. Each incubation tube contained 35 μM [^{35}S]sulfoquinovosyl diglyceride (4300 cpm/nmole), 60 mM Tris–HCl buffer, pH 8.2, the indicated concentration of Triton X-100, and 50 μg of chlorophyll of a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *Chlorella pyrenoidosa* suspended in 0.1 ml of 0.05% KCl in a final volume of 0.25 ml. After incubation at 30°C for 20 min, reactions were stopped and product was measured as in Table 4.

lower concentrations of this surfactant inhibit sulfoquinovosyl glycerol formation (Fig. 8). Addition of calcium or magnesium inhibited the hydrolysis of sulfoquinovosyl glycerides at all concentrations tested (Table 4). The formation of sulfoquinovosyl glycerol from sulfoquinovosyl diglyceride is linear with time for at least 1 hr, whereas the amount of sulfoquinovosyl monoglyceride in the reaction mixture reaches a steady state value in 5 min or less (Fig. 9).

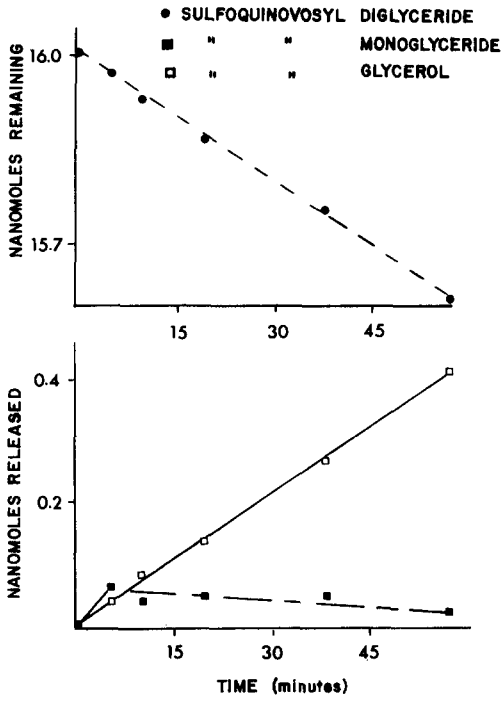


Fig. 9. Time course of deacylase. Each incubation tube contained 16 nmoles of [^{35}S]sulfoquinovosyl diglyceride (10,690 cpm/nmole), 30 mM Tris–HCl buffer, pH 8.2, and 55 μg of chlorophyll of a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *Chlorella pyrenoidosa* suspended in 0.1 ml of 0.05% KCl in a final volume of 0.25 ml. The samples were incubated at 30°C with shaking. At the indicated times, reactions were stopped and product was measured as described in Materials and Methods.

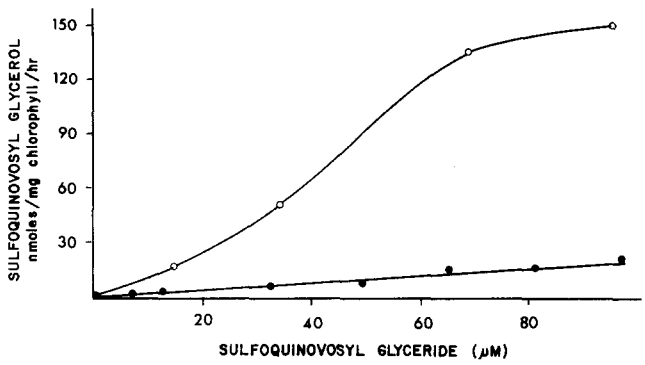


Fig. 10. Sulfoquinovosyl glycerol produced from either sulfoquinovosyl monoglyceride or diglyceride. ○, sulfoquinovosyl monoglyceride as substrate: Each incubation tube contained 63 μg of chlorophyll of a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *Chlorella pyrenoidosa* suspended in 0.1 ml of 0.1 M Tris–HCl buffer, pH 8.35, and the indicated concentration of [^{35}S]sulfoquinovosyl monoglyceride (8330 cpm/nmole). Incubation was at 30°C for 20 min with shaking. ●, sulfoquinovosyl diglyceride as substrate: Each tube contained 60 mM Tris–HCl buffer, pH 7.9, 60 μg of chlorophyll of a 30,000 *g* particulate preparation of *C. pyrenoidosa* suspended in 0.1 ml of 0.05% KCl and the indicated concentration of [^{35}S]sulfoquinovosyl diglyceride (4307 cpm/nmole). Incubation was at 30°C for 15 min with shaking. The final volume of all incubation mixtures was 0.25 ml. Reactions were stopped and product was measured as described in Table 4.

Sulfoquinovosyl glycerol formation increases with increasing sulfoquinovosyl glyceride concentration up to at least 70 μM (Fig. 10). An aliquot of a solution of sulfoquinovosyl diglyceride in methanol–benzene was routinely added to a dry incubation tube and the solvent was evapo-

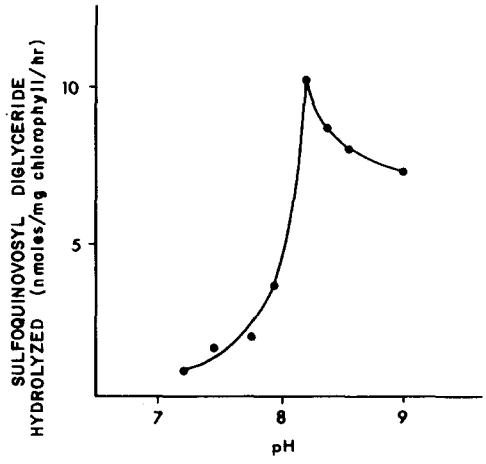


Fig. 11. pH curve of deacylase. Each incubation tube contained 32 μM [^{35}S]sulfoquinovosyl diglyceride (9600 cpm/nmole), 57 μg of chlorophyll of a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *Chlorella pyrenoidosa* suspended in 0.1 ml of 0.05% KCl, and 60 mM Tris–HCl buffer of the indicated pH in a final volume of 0.25 ml. The incubation was carried out for 20 min at 30°C. A zero-enzyme control was run at each pH and the nonenzymatic hydrolysis was subtracted from each sample.

rated with a stream of nitrogen. Aqueous solutions of the various reagents were then added to this film of dry lipid. It should be noted that in this enzyme system, as in most aqueous enzyme systems using water-insoluble substrates, it is impossible to state the actual concentration of the sulfoquinovosyl glyceride physically available to the enzyme. Thus, the concentrations of sulfoquinovosyl diglyceride and monoglyceride used under these conditions might best be expressed as apparent concentrations.

The hydrolysis of sulfoquinovosyl diglyceride is proportional to added enzyme up to a concentration of about 0.4 mg of enzyme protein/ml of reaction mixture. Higher enzyme concentrations did not result in significantly more or less sulfoquinovosyl glycerol formation. The pH optimum for the enzymatic deacylation of sulfoquinovosyl glycerides is 8.2 in Tris–HCl buffer (Fig. 11). Rather little hydrolysis occurs if incubations are carried out at a pH of less than 8.0 in either Tris–HCl or sodium phosphate buffer.

Incompetence of sulfoquinovosyl glycerol as a substrate

Incubation of [^{35}S]sulfoquinovosyl glycerol with fatty acyl CoA in the presence of a 30,000 *g* particulate preparation of *Chlorella pyrenoidosa* resulted in very little, if any, lipid formation (Table 5). Similar results were obtained if a whole homogenate was used instead of a particulate preparation. The amount of chloroform-soluble material formed was insufficient for identification.

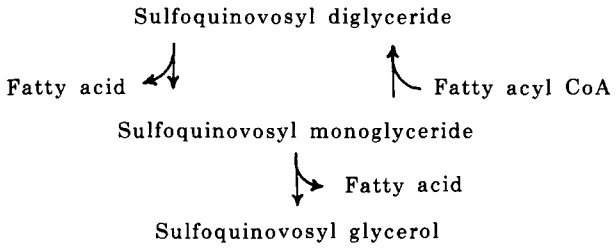
TABLE 5. Sulfoquinovosyl glycerol as a substrate for acyltransferase reaction

	Lipid Formed
	cpm
Complete system	0
+5 μM palmityl CoA	0
+10 μM palmityl CoA	0
+4.4 μM linolenyl CoA	10
+8.8 μM linolenyl CoA	0

The complete system contained 20 μM [^{35}S]sulfoquinovosyl glycerol (11,200 cpm/nmole), 50 mM sodium phosphate buffer, pH 7.5, and 0.1 mg of chlorophyll of a 30,000 *g* particulate (derived from 500 *g* supernate) preparation of *Chlorella pyrenoidosa* suspended in 0.15 ml of 0.5% KCl in a final volume of 0.5 ml. Palmityl CoA or linolenyl CoA was added to a final concentration as indicated. Incubation was at 30°C for 20 min with shaking. Reactions were started by addition of enzyme and stopped by addition of 7.5 ml of chloroform–methanol 2:1 (v/v). After addition of 5 ml of water and thorough mixing, the phases were separated by centrifugation. Lipid formation was measured by counting an aliquot of the chloroform phase with a gas-flow Geiger counter. The reported counts are corrected for background.

DISCUSSION

The data presented in this paper indicate that *Chlorella pyrenoidosa* contains enzymes metabolizing sulfoquinovosyl diglyceride by the following pathways:



The relative importance of these reactions in vivo would be quite dependent upon the pH and the lipophilic nature of the intracellular melange. Under optimal conditions the lipase activity is nearly 10-fold greater than acyltransferase activity, but at pH values between 7.4 and 8.0 they are nearly equal. Therefore, if conditions at the sites of sulfoquinovosyl glyceride metabolism should be in the pH range 7.4–8.0 and approximate the lipophilic properties of solutions containing 0–0.6 mg/ml Triton X-100, a delicate balance between deacylation and transacylation would exist.

It is not yet resolved whether the coenzyme A or the acyl carrier protein derivatives of fatty acids function as acyl group donors in acyltransferase reactions in photosynthetic tissues (21). Although our work does not rule out the participation of acyl carrier protein derivatives as a fatty acid transferring agent, it does demonstrate that fatty acyl CoA can function as a substrate in the formation of sulfoquinovosyl diglyceride. Whether it is the exclusive fatty acid transferring agent or shares this role with acyl carrier protein remains to be determined.

Water-soluble compounds other than sulfoquinovosyl glycerol were not detected as products of the enzymatic hydrolysis of sulfoquinovosyl glycerides, indicating that sulfoquinovosyl glycerol may be an end product of the enzymatic degradation of sulfoquinovosyl diglyceride. This is in agreement with reports that free sulfoquinovose is not found in nature (4). These findings, when taken in conjunction with the observations concerning the lack of acylation of sulfoquinovosyl glycerol, indicate that this compound may not be metabolized by *Chlorella pyrenoidosa*.

The skillful assistance of Mrs. Joanne K. Northrop, who prepared much of the sulfoquinovosyl monoglyceride used in this study, is gratefully acknowledged. This study was supported by grants from the National Science Foundation, the NIAID (AI-05730), and the NICHD (1-P01-HD-05874-01).

Manuscript received 27 October 1972 and in revised form 12 June 1973; accepted 28 August 1973.

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