[9] Bergmann, F., in *Advances in Catalysis*, **10**, 130 (1958).

[10] Di Carlo, F. J., A. S. Schultz, and A. M. Ken, *Arch. Biochem. Biophys.*, **43**, 468 (1953).

[11] Unpublished calculations by the authors.

[12] Pullman, A., and B. Pullman, *Bull. Soc. Chim. France*, 594 (1959).

[13] See, e.g., Porter, G. R., H. N. Rydon, and J. A. Schofield, *Nature*, **182**, 927 (1958); Rydon, H. N., *Nature*, **182**, 928 (1958).

[14] Ronwin, E., *Enzymologia*, **16**, 81, 179 (1953–1954), *J. Amer. Chem. Soc.*, **75**, 4026 (1953); see also Ferguson, L. N., *Enzymologia*, **17**, 95 (1954); **18**, 273 (1957).

[15] See, e.g., Wilson, I. B., in *The Mechanism of Enzyme Action* (Baltimore: Johns Hopkins Press, 1954).

A SULFOLIPID IN PLANTS†*

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A sulfolipid-S^{35} was isolated from photosynthetic microorganisms and higher plants after culture in media containing radiosulfate. Alcohol extracts of *Chlorella*, *Scenedesmus*, *Rhodospirillum rubrum*,[1] barley, clover, New Zealand spinach (*Tetragonia expansa*) chloroplasts, chive, and green sections of coleus were chromatographed on paper and found to contain varying amounts of a radioactive compound with high R_f values (Fig. 1). Its concentration in *Chlorella* was $4 \cdot 10^{-3}$ *M*, which exceeds those of the major plant phosphatides. The radiochromatographic method has revealed its chemical composition and aspects of its molecular structure.

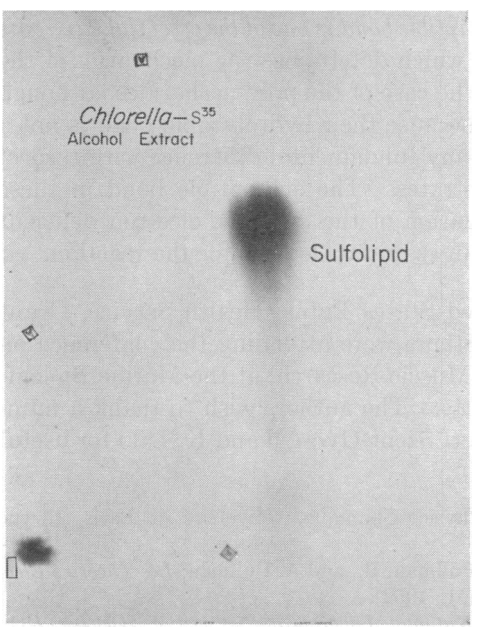

FIG. 1.—Radiogram of alcohol extract of *Chlorella*-S^{35}. Chromatograms are developed from the origin at the lower left with phenol-water (1) to the right and with butanol-propionic acid-water (2) upwards.

Materials and Methods.—Isolation of sulfolipid-S^{35}: A suspension of approximately 100 mg of *C. pyrenoidosa* or of *Scenedesmus* D_3 in 30 ml of low-sulfur (*ca.* 10^{-5} *M* total sulfates) culture medium containing 1 mc carrier-free sulfate-S^{35} in a glass cylinder was perfused with 5 per cent carbon dioxide-enriched air and illuminated with a 20-watt fluorescent lamp. During one week the cells increased fivefold. They were centrifuged from the medium and washed three times with water. The cells were extracted twice with 5-ml portions of hot absolute ethanol and twice with hot 25 per cent chloroform in ethanol. The extract of 20-mg cells was chromatographed two-dimensionally on Whatman No. 1 paper in phenol-water (PW) and

butanol-propionic acid-water (BPAW)[2] solvents. The sulfolipid had an R_f of 0.8 in PW and 0.7 in BPAW (Fig. 1). This allowed separation from the slower moving phosphatidyl inositol in subsequent experiments. The sulfolipid contained 90 per cent of the S^{35} activity of the chloroform-ethanol extract. It was eluted from chromatograms with 25 per cent chloroform in ethanol or with pyridine.

In analogous experiments the leaves of higher plants were labeled during 10–20 hours' illumination while their petioles were immersed in radiosulfate solutions. Extractions and isolation of the lipid gave in each case a product with identical R_f values and chemical properties.

Isolation of sulfolipid-C^{14}: A suspension of 100 mg of *Chlorella* in 30 ml of culture medium in a glass cylinder was connected in a closed system to a gas-generating flask and a valveless circulating pump.[3] $C^{14}O_2$ was introduced by addition of 0.5-mc portions of barium carbonate-C^{14} to dilute lactic acid at two-day intervals. After eight days the weight of the algae was 400 mg. They were separated from the growth medium and extracted with 10 ml of boiling 80 per cent ethanol. Further extracts with 10-ml portions of hot 95 per cent ethanol and 10 ml of hot 25 per cent ethanol-chloroform were combined and concentrated *in vacuo.* Chromatography in PW and BPAW effected complete separation of the sulfolipid-C^{14} from the other labeled components. Phosphatidyl inositol occupied an adjacent area and was recognized by conversion to fatty acids and glycerophosphoryl inositol in the anticipated C^{14} activity ratio. No other phosphatides or sulfolipids occupy this region of the chromatograms of *Chlorella* extracts.

The identity of the sulfolipid-C^{14} with the sulfolipid-S^{35} was confirmed by conversion by acid hydrolysis and by deacylation to the readily recognizable sugar-6-sulfonate and glyceryl glycoside-6-sulfonate. In all cases the rates of hydrolysis of the C^{14}- and S^{35}-labeled lipids were identical.

Experiments and Results.—Structural aspects of the sulfolipid were deduced from the chromatographic properties and chemical reactivities of its components. Their chemical composition was revealed by the relative C^{14} radioactivity in each moiety derived from uniformly labeled sulfolipid-C^{14}.

Acid hydrolysis of sulfolipid-S^{35}: A solution of 10,000 cpm of sulfolipid-S^{35} in 0.10 ml 50 per cent ethanol was added to 0.05 ml of 6 *N* hydrochloric acid and heated at 100° for 30 minutes. Chromatography of the hydrolysate revealed but one sulfur-containing product with R_f values of 0.05 in PW and BPAW. Rapid paper electrophoretic migration with lactic acid-hydrochloric acid at pH 1 and with boric acid-sodium hydroxide buffer at pH 9 indicated its strong anionic nature. No inorganic sulfate was produced by heating at 100° for ten hours in normal hydrochloric acid or for two hours in 50 per cent nitric acid.

Base-catalyzed deacylation[4,5] of sulfolipid: To 10,000 cpm of sulfolipid-S^{35} in 0.05 ml toluene-methanol was added 0.05 ml of 0.2 *N* methanolic potassium hydroxide. After 20 minutes at 37° a drop of water was added followed by equivalent Dowex-50·H^+ for neutralization. The resin was centrifuged. Chromatograms of the solution revealed one product. Its R_f was 0.1 in both PW and BPAW and it readily eluted with water. Paper electrophoresis demonstrated its strong anionic nature Acid hydrolysis produced the $R_f = 0.05$ compound. A sample of the sulfolipid-C^{14} was deacylated similarly and gave 14,200 cpm fatty acid and 5,850 cpm of the $R_f = 0.1$ product, a ratio of 21.7 to 9.

Presence of free hydroxyl groups: Pyridine solutions of the S^{35}-labeled hydrolysis products were treated with acetic anhydride and chromatographed. The presence of free hydroxyl groups was revealed by the high R_f values of the products. Free hydroxyl groups in the lipid-S^{35} were established by benzoylation with benzoyl chloride in dry pyridine during two hours at room temperature. Chromatography of the solution revealed formation of a new compound with $R_f = 0.9$ in both PW and BPAW. This compound was deacylated by treatment with 0.1 *N* methanolic potassium hydroxide at 37° for 45 minutes. The single product had R_f values of 0.6 in PW and 0.5 in BPAW. Treatment with 2 *N* methanolic potassium hydroxide at 75° for two hours gave a product with $R_f = 0.1$ in both solvent systems. Its properties were identical with that directly derived by deacylation of the lipid-S^{35}.

Acid hydrolysis products of sulfolipid-C^{14}: The C^{14}-labeled sulfolipid was hydro-

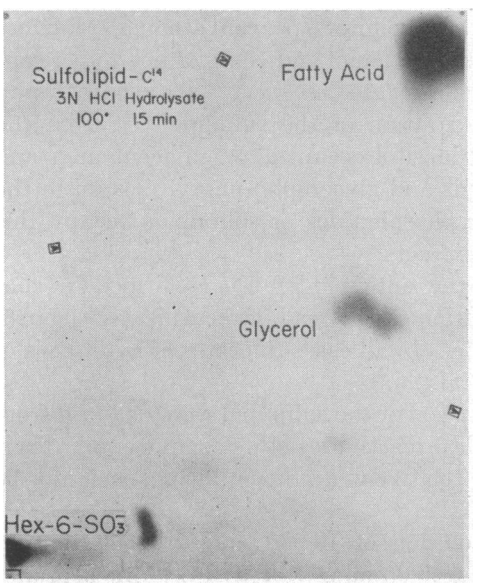

FIG. 2.—Radiogram of acid hydrolysis products of sulfolipid-C^{14}.

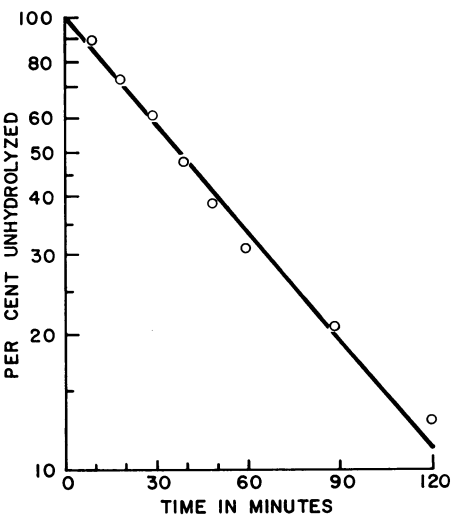

FIG. 3.—Acid hydrolysis rate of 1′-glyceryl glycoside-6-sulfonate in *N* hydrochloric acid at 100°.

lyzed in 2 *N* hydrochloric acid for 30 minutes at 100°. The hydrolysate was chromatographed to provide the radiogram shown in Figure 2. The products were 2,100 cpm glycerol, 13,600 cpm fatty acid, and 4,370 cpm of the $R_f = 0.05$ compound. This is a ratio of 3:18.7:6.2.

The glycerol-C^{14} produced was identified by R_f value and by periodate oxidation which yielded formic acid and formaldehyde. These were isolated as barium formate and formaldimedone in a C^{14} activity ratio of 0.95 to 2.0.

The $R_f = 0.05$ product was then cochromatographed with synthetic galactose-6-sulfate prepared according to the method of Percival and Soutar.[6,7] It cochromatographed closely with the unknown in PW, BPAW, and butanol-pyridine-water solvents.

Identification of aldohexose‡ structure: With the presence of six carbon atoms and hydroxyl groups indicated, the $R_f = 0.05$ compound was treated with 2,4-dinitro-

phenylhydrazine to produce two products with increased R_f values corresponding to a hydrazone and an osazone.

Treatment of the sugar sulfonate with 3 per cent concentrated hydrochloric acid in ethanol at 100° for 15 hours produced an ethyl glycoside sulfonate with R_f = 0.35 in PW. The glycoside was completely hydrolyzed in normal hydrochloric acid at 100° in thirty minutes. After ten hours' heating without apparent decomposition the sugar sulfonate could be reconverted to the R_f = 0.35 ethyl glycoside sulfonate. Tritylation of the R_f = 0.05 compound or its derivatives was unsuccessful.

Properties of glyceryl glycoside-6-sulfonate: The deacylated lipid was resistant to bromine oxidation during six hours at room temperature in the presence of barium carbonate. Under these conditions, the sugar-sulfonate-S^{35} was oxidized to form a new compound with R_f = 0.1 in PW. Further, the sulfolipid-C^{14} was deacylated to give fatty acid and water-soluble product in a ratio of 18 to 8. The same water-soluble product hydrolyzed in acid to give 70 cpm of glycerol and 147 cpm of sugar-6-sulfonate, a ratio of 2.8 to 6.0.

Acid hydrolysis rate of glyceryl glycoside-6-sulfonate: The acid hydrolysis rate of the sulfur-labeled compound was determined by chromatography of aliquot portions as a function of time in mineral acid. Its rate of acid hydrolysis ($T_{1/2}$ = 30 minutes in *N* hydrochloric acid at 100°) is shown in Figure 3.

Nature of the glycosidic linkage: The glycoside-C^{14} was stable to the α-galactosidase of bottom yeast invertase,[8] an enzyme capable of hydrolyzing the isomeric 2-glyceryl α-D-galactoside (floridoside) which occurs in red algae.[9] Solution of glyceryl glycoside-6-sulfonate-S^{35} and 2 mg. of β-galactosidase-containing emulsin in 0.1 ml of water at pH 5 with a drop of toluene produced a 70 per cent yield of sugar-6-sulfonate-S^{35} after five days at room temperature.

Periodate oxidation of glyceryl glycoside-6-sulfonate: The position of the glycosidic linkage on a primary hydroxyl of glycerol was established by periodate oxidation of the glyceryl glycoside-6-sulfonate-C^{14} to yield one mole of formaldehyde-C^{14}. To 12,500 cpm of carbon-labeled glyceryl glycoside-6-sulfonate in 0.10 ml of water were added 3.0 mg. of glycerol in 1.5 ml of water and 0.25 ml of 0.5 *N* periodic acid. The solution was allowed to stand five hours at room temperature and was then treated by the procedure used in the glycerol degradation to produce formaldimedone. After correction for the yield from 3.0 mg. glycerol, the C^{14} activity of the formaldimedone was 1,550 cpm. The theoretical yield of formaldehyde from 12,500 cpm of a 1′-glyceryl glycoside-6-sulfonate is 1,400 cpm. The compound, therefore, differs from the 2-glyceryl galactoside of red algae and is related to those of the galactolipids, recently isolated by Carter, McCluer, and Slifer,[10] which are 1-glyceryl β-D-galactoside esters.

Glyceryl glycoside-6-sulfonate-C^{14} (90,400 cpm) and 31.4 mg glycerol in 2.4 ml water were oxidized with 1.0 mmol. sodium periodate at pH 6 for two hours.[11] The volatile products were distilled *in vacuo*. The distillate was neutralized with barium hydroxide to give barium formate with a specific activity of 180 cpm/mg after recrystallization. This indicated that 0.9 mole of formic acid was produced from one mole of the glycoside sulfonate. This result suggests an aldopyranoside-6-sulfonate structure.

Fatty acid of the Chlorella sulfolipid: It appeared from the results of hydrolyses of the sulfolipid-C^{14} that there is only one fatty acid present. The fatty acid-C^{14}

has been shown[12] to be largely oleic acid by paper cochromatography with oleic acid and hydrogenation to stearic acid-C^{14}. The location of the oleoyl ester on the 3-position of glycerol was established in the following manner. A sample of the sulfolipid-C^{14} was benzoylated in pyridine and the product isolated by chromatography in PW and BPAW. It was deacylated by treatment with 0.1 *N* methanolic potassium hydroxide at 37° for 30 minutes. Under these conditions benzoyl groups[13] are not removed. The hexose-6-sulfonate was removed by treatment with *N* hydrochloric acid at 100° for 90 minutes. Benzoyl glycerol was isolated by paper chromatography of the hydrolysate and subjected to periodate oxidation in the presence of glycerol carrier. No C^{14} activity was present in the formaldimedone produced.

Discussion.—The experimental results strongly suggest the structure shown in Figure 4 for the plant sulfolipid. The fact that it is a glycosyl monoglyceride establishes its relationship to the galactolipids recently identified among wheat flour lipids by Carter, McCluer, and Slifer.[10] It is clear from the present work that there is indeed only one fatty acid in the molecule and that the galactolipids are monoglyceride derivatives.

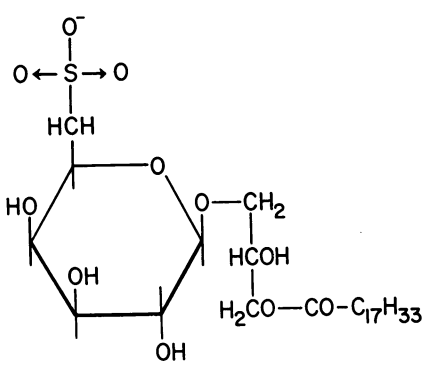

FIG. 4.—Proposed structure for the plant sulfolipid.

The resistance of the anionic S^{35}-labeled hexose derivative to acid hydrolysis suggests the presence of a sulfonic acid group. Production of formic acid by periodate oxidation of its glycoside implies that it may be a 6-sulfonic acid derivative of a 6-deoxyhexose. While R_f values are nearly identical, galactose 6-sulfate was readily hydrolyzed by acid whereas the unknown sugar sulfonate was not. Since no synthetic α-hydroxy sulfonic acids appear to have been reported, complete structural assignment must await isolation and synthesis.

A sulfonic acid group in the sulfolipid would be unique in carbohydrate chemistry. The biochemical implications of such a compound are intriguing. The most reasonable mode of biosynthesis would be via peroxide oxidation of an -S-S- linked thioglycolipid. Of all metabolic structures the photosynthetic apparatus is the most likely to provide such an oxidative environment.

The apparent ubiquity of the sulfolipid within green plant tissues, its high concentration, and its rapid metabolism[14] imply that it performs physiological and chemical functions at least as important as those of the phosphatides. The strong anionic properties of the sulfonate ion could provide oriented polar interfaces potentially useful in the photosynthesis process. Its counterpart in nervous tissues, cerebroside sulfate,[15] has similar solubility properties and perhaps similar physico-chemical functions.

Summary.—A sulfolipid occurs in high concentrations in photosynthetic tissues. It is readily labeled with S^{35} or C^{14} during photosynthesis by *Chlorella*. Radiochromatographic examination of the sulfolipid molecule revealed an anionic aldohexopyranosyl monoglyceride structure. Acid hydrolysis gave an acid-stable anionic hexose containing the sulfur. Periodate oxidation of the ethyl glycoside-

sulfonate-C^{14} produced one mole of formic acid. The structure of the sulfolipid consistent with the observed properties is 1-O-(β-6′-deoxy-aldohexopyranosyl 6′-sulfonic acid)-3-O-oleoyl glycerol. The sulfonic acid structure suggests its origin by peroxidation of an -S-S- linked glycolipid in the photosynthetic apparatus.

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† Part of the material in this paper is included in the thesis submitted by R. Wiser to the Graduate School of The Pennsylvania State University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, August, 1958.

‡Available structural evidence is also consistent with an isomeric 1-deoxyketopyranoside 1-sulfonic acid structure.

[1] We are indebted to the generosity of Dr. John Ormerod, of the Technical College of Norway, Trondheim, for the sample of *Rhodospirillum rubrum*-S^{35}.

[2] Benson, A. A., J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka, *J. Am. Chem. Soc.*, **72**, 1710 (1950).

[3] Cole-Parmer Instrument and Equipment Co., Chicago, 10.

[4] Dawson, R. M. C., *Biochem. J.*, **59**, 5 (1955).

[5] Benson, A. A., and B. Maruo, *Biochim. et Biophys. Acta*, **27**, 189 (1958).

[6] Lloyd, A. G., *Nature*, **183**, 109 (1959).

[7] Percival, E. G. V., and T. H. Soutar, *J. Chem. Soc.*, **1940**, 1475.

[8] We are indebted to Dr. E. W. Putman for a gift of bottom yeast invertase capable of cleaving floridoside.

[9] Putman, E. W., and W. Z. Hassid, *J. Am. Chem. Soc.*, **76**, 2221 (1954).

[10] Carter, H. E., R. H. McCluer, and E. D. Slifer, *J. Am. Chem. Soc.*, **78**, 3735 (1956).

[11] Benson, A. A., and M. Calvin in *Methods in Enzymology*, vol. IV, ed. S. P. Colowick and N O. Kaplan (New York: Academic Press, 1957), p. 882.

[12] We are indebted to R. McCarl of this laboratory for paper chromatographic identification of the labeled fatty acid and its hydrogenation product.

[13] Benzoyl groups are known to migrate under such conditions. However, 2-benzoyl-glycerol would be expected to be the least stable of the two monobenzoates.

[14] Ferrari, R. A., and A. A. Benson, to be published; Benson, A. A., R. Wiser, R. A. Ferrari, and J. A. Miller, *J. Am. Chem. Soc.*, **80**, 4740 (1958).

[15] Thannhauser, S. J., J. Fellig, and G. Schmidt, *J. Biol. Chem.*, **215**, 211 (1955).

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*AN EFFECT OF AUXINS ON THE HEAT COAGULABILITY OF THE PROTEINS OF GROWING PLANT CELLS**

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Most current theories of auxin action are based on the discovery by Heyn[1] that the application of auxin to growing plant cells results in an increased plastic extensibility of the cell wall. While such a mechanism suffices to explain elongation induced by auxin, it is obviously inadequate to account for the known effects of