product were carried out as described above for the corresponding derivatives of thioinosine. The crude barium salt so obtained (72.4 mg.) was found by paper chromatography (solvent B) and electrophoresis at *p*H 7.1 to consist principally of material with the properties expected of 2-amino-6-mercaptopurine nucleoside 5'-phosphate; minor amounts of three unidentified products were also present. Reprecipitation of the barium salt did not effect purification. A portion (15.3 mg.) of the crude product was dissolved in water (10 ml.) and applied to a column (height 7.5 cm.) of 5 ml. of Dowex-1 (Cl^-) ion-exchange resin (200–400 mesh, 8% cross linkage). The column was washed in turn with water (100 ml.), 0.003 *N* HCl (450 ml.), 0.004 *N* HCl (200 ml.), 0.007 *N* HCl (100 ml.), 0.01 *N* HCl–0.0025 *N* $CaCl_2$ (100 ml.), 0.01 *N* HCl–0.005 *N* $CaCl_2$ (90 ml.) and 0.01 *N* HCl–0.0075 *N* $CaCl_2$ (150 ml.); 9 ml. fractions were collected. With 0.003 *N* HCl, two small peaks, the first exhibiting ultraviolet end-absorption, and the second of λ_{max} 250 mμ, were eluted successively. 0.004–0.007 *N* HCl eluted 40 O.D. units of material (λ_{max} 256 mμ, A_{250}/A_{260}, 1.00, A_{290}/A_{260}, 0.45) which from its position on the elution diagram could represent 1.2 mg. of guanosine 5'-phosphate (λ_{max} 256 mμ, A_{250}/A_{260}, 0.99, A_{290}/A_{260}, 051).[52] Elution of the desired nucleotide commenced with 0.01 *N* HCl–0.005 *N* $CaCl_2$ and was completed in a volume of 180 ml. These fractions were combined, adjusted to *p*H 6 with $Ca(OH)_2$ suspension and lyophilized to dryness. The residue was suspended in 12 ml. of 1:1 ethanol–diethyl ether (peroxide-free) and the solid collected by centrifugation and washed with the ethanol–ether. The dried calcium nucleotide was stirred with HCl (5 ml.) at *p*H 2, insoluble solid was centrifuged down and the solution percolated through a column of 2 ml. of Dowex-50 (Li^+) resin. The resin was washed with 10 ml. of water and the combined eluates evaporated *in vacuo* to 2.5 ml., adjusted to *p*H 7 with LiOH and treated with 2 *M* barium acetate (0.1 ml.). Addition of ethanol (5 ml.) yielded a white precipitate of the barium nucleotide. This was collected by centrifugation, washed with 80% ethanol and dried. A solution of this salt in water (1.1 ml.) was applied across five strips of Whatman 3MM filter paper (width 8 inches) and subjected to electrophoresis in 0.05 *M* ammonium formate–formic acid buffer, *p*H 3.50, for 9 hr. at a gradient of 18 volts/cm. The papers were partially dried in a current of warm air, then at 100°, 10 mm. pressure, for 3 hr. to remove ammonium formate. In ultraviolet light, monoammonium 2-amino-6-mercapto-9-β-D-ribofuranosylpurine 5'-phosphate was visible as a green fluorescent band 14 cm. from the origin; smaller amounts of a dark band (λ_{max} 254 mμ at *p*H 4.9) and a blue fluorescent band (λ_{max} 275 mμ at *p*H 4.9) were located 19 and 30 cm., respectively, from the origin. The portions of paper containing the 2-amino-6-mercaptopurine nucleotide were extracted at room temperature with 80 ml. of water.[53] The extract was concentrated at 10 mm. pressure to *ca.* 20 ml. and clarified with Celite. The spectroscopic properties of the solution were: at *p*H 4.9, maxima at 257 and 342 mμ, minimum at 292–294 mμ, absorbancy ratios 342 mμ/257 mμ, 2.80, and 342 mμ/292 mu, 21; at *p*H 12, maxima at 252 and 319 mμ, shoulder at 270 mμ, minimum at 286 mμ, absorbancy ratios 319 mμ/252 mμ, 1.40, and 319 mμ/286 mμ, 5.0. The absorption maxima and minima of this nucleotide are identical with those reported[33] for the corresponding nucleoside, and the absorbancy ratios follow a similar pattern, the values for the nucleoside being: *p*H 4.9, 342 mμ/257 mμ, 2.81, 342 mμ/292 mμ, 13; *p*H 12, 319 mμ/252 mμ, 1.43, 319 mμ/286 mμ 4.3.

The solution contained 2-amino-6-mercaptopurine,[54] pentose and total phosphorus in the ratio 1:1.08:[55] 1.04; paper chromatography (solvent B) and electrophoresis (22 volts/cm., 0.04 *M* phosphate buffer, *p*H 7.15) revealed only one component (R_f 0.73; mobility 9.3 cm./hour); this component reacted positively in the spray test for *cis*-glycol groups. Spectrophotometric assay[54] showed that the solution contained 3.8 mg. of the free nucleotide, corresponding to a yield of 23% based on the isopropylidene 2-amino-6-mercaptopurine nucleoside.

Acknowledgments.—The authors are indebted to Dr. George Bosworth Brown for continued interest and encouragement and to Dr. Bernard R. Baker for a generous gift of 6-chloro-9-β-D-ribofuranosylpurine.

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(53) An equal area of the paper which was free of ultraviolet-absorbing material was extracted with water and the solution used as a blank in the spectrophotometric and pentose determinations.

(54) Determined spectrophotometrically at 342 mμ, *p*H 4.9, with the assumption that the extinction coefficient of 2-amino-6-mercaptopurine nucleoside 5'-phosphate is the same as the value (24.8×10^3) reported[33] for the corresponding nucleoside.

(55) Sodium guanosine 5'-phosphate (Pabst Laboratories) was used as a pentose standard. Substitution of water for the "blank" solution[53] gave a pentose value of 1.18.

[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL AND BIOLOGICAL CHEMISTRY, THE PENNSYLVANIA STATE UNIVERSITY, UNIVERSITY PARK, PENNSYLVANIA]

The Plant Sulfolipid. II. Isolation and Properties of Sulfoglycosyl Glycerol[1]

BY M. LEPAGE,[2] H. DANIEL[3] AND A. A. BENSON

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The sulfolipid found in photosynthetic tissues is readily deacylated to give a sulfodeoxyhexopyranosyl glycerol. This sulfo glycoside has been isolated from the deacylation products of alfalfa leaf lipids by anion exchange resin chromatography. Elution of the product was followed by assay of the radioactivity of added sulfoglycoside-S^{35} and the major anionic deacylation products of glycerolphosphatides-P^{32}. The infrared spectrum and physical properties of the sulfoglycosyl glycerol cyclohexylammonium salt are reported.

The sulfolipid which occurs in all photosynthetic tissues yet investigated[4] appears to be a sulfonic acid analog of the major choloroplast lipid, β-D-galactosyl diglyceride.[5,6] It failed to yield sulfate ion under any hydrolytic conditions and did so only in small yield upon periodate or biological oxidation. Further evidence for occurrence of sulfosugars in Nature is based upon isolation and properties of sulfoglycosyl glycerol which are reported in this paper.

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(2) This paper forms part of a Thesis to be submitted by M. Lepage to the Graduate School of the Pennsylvania State University in partial fulfillment of the requirements for the Ph.D. degree.

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Results and Discussion

A survey of the readily available leaf lipids indicated that those of alfalfa contain considerable concentrations of sulfolipid. It was recognized by paper cochromatography of its deacylated derivative with sulfodeoxyhexosyl glycerol-S^{35}, readily prepared by culture of *Chlorella* or *Scenedesmus* in radiosulfate.[4] Alfalfa leaf lipids were deacylated in 0.1 *N* solution of potassium hydroxide in pyridine and methanol at 37° during ninety minutes. The sulfoglycoside was isolated from the other anionic products by gradient elution from Dowex-1 formate resin. Elution of each of the interfering anionic lipid derivatives was studied and a procedure was chosen for direct separation of all of them. The elution of anionic components of the mixture was followed using P^{32}-labeled glycerophosphoryl glycerol, glycerophosphoryl inositol, glycerophosphate and cyclic 1,2-glycerophosphate[7] isolated by two-dimensional paper chromatography from deacylated algal lipids. Separation of glycerophosphoryl inositol has been reported previously.[8] The zwitterionic glycerophosphoryl esters of choline and ethanolamine, which are the major sources of cyclic glycerophosphate, are not absorbed by the resin. Although tracer amounts of cyclic glycerophosphate were separable from sulfoglycosyl glycerol on the same columns, as shown in Fig. 1, the large scale

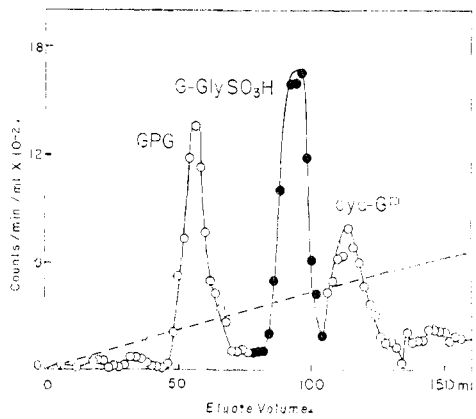

Fig. 1.—Chromatographic separation of sulfodeoxyhexosyl glycerol-S^{35} from cyclic 1,2-glycerophosphate-P^{32} and glycerophosphoryl glycerol-P^{32} by elution from Dowex-1 formate. Concentration of ammonium formate eluting agent is given by broken line.

separations yielded products contaminated with the cyclic phosphate ester produced under the deacylation conditions. Hence the cyclic ester was hydrolyzed under mild acid conditions prior to column chromatography. By passing the column effluent through a spiral of capillary plastic scintillator before collection of fractions it was possible to record S^{35} and P^{32} radioactivity peaks directly.

The fraction containing the sulfoglycosyl glycerol as defined by its S^{35} tracer radioactivity, was crystallized as its cyclohexylamine salt. Elemental analyes were in accord with the structure proposed on the basis of the previous radiochromatographic investigation. It possessed infrared absorption bands, Fig. 2, at 1035 and 1170 cm.$^{-1}$ characteristic of S→O stretching and at 771 or 791 cm.$^{-1}$ characteristic of C–S stretching in aliphatic sulfonic acids.[9] The cyclohexylamine salt of 1-sulfo-2,3-propanediol was prepared and its absorption spectrum is compared in Fig. 2. The sulfolipid of *Mycobacterium*

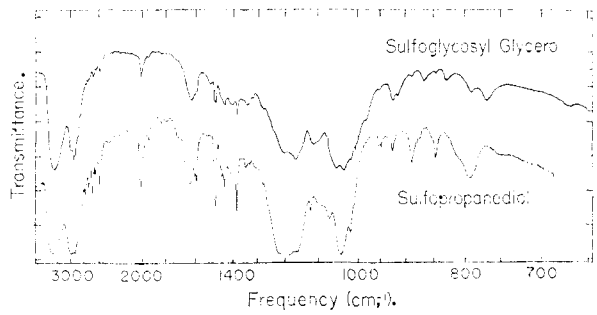

Fig. 2.—Infrared spectra of cyclohexylamine salts of sulfodeoxyhexopyranosyl glycerol and 1-sulfo-2,3-propanediol in potassium bromide. The transmittance scale for the lower curve is displaced 40%.

tuberculosis was reported to have absorption bands at 1035 and 1150 cm.$^{-1}$ and was therefore suspected to be a sulfonic acid.[10] The widespread occurrence of sulfosugar in Nature suggests participation of sulfite fixation products in certain phases of carbohydrate metabolism. It is possible that the plant sulfolipid acts as a storage reservoir for partially-reduced sulfate.

Experimental Part

Lipid Extraction and Deacylation.—Five kilograms of dry alfalfa leaves were extracted with hot 95% ethanol. The extract was concentrated *in vacuo* and taken up in chloroform. The chloroform solution was washed thoroughly with water and evaporated to incipient dryness. The residue was taken up in 600 ml. of pyridine and 400 ml. of ethanol and treated with 1 l. of 0.2*N* methanolic potassium hydroxide at 37° for ninety minutes. The deacylated products were decationized by Dowex-50.H^+ and washed with chloroform and petroleum ether to remove fatty acids and unsaponifiable materials. Two-dimensional paper chromatography[4] revealed the presence of the major lipid derivatives, β-galactosyl glycerol, α-galactosyl-(1→6)-β-galactosyl glycerol and sulfoglycosyl glycerol. Smaller but appreciable amounts of at least five other polygalactosyl glycerols were separated in chromatographic positions indicative of their membership in a homologous series of galactosyl glycerols. Low concentrations of the plant glycerolphosphatides were observed. Labeled glycerolphosphatides from *Chlorella*-P^{32} and labeled sulfolipid from *Scenedesmus*-S^{35} were deacylated and chromatographed two-dimensionally. The eluted products were used as tracers in following the succeeding column elutions.

Anion Exchange Resin Chromatography.—Qualitative separations of P^{32}- and S^{35}-labeled tracers were made on 0.8 cm.^{2} × 12 cm. columns of Dowex-1 formate (400 mesh). Preparative separations utilized columns of 16 cm.^{2} cross section with corresponding increases in elution rate and collected fraction volume. The columns were treated with excess 2.0 *M* ammonium formate and washed thoroughly with water before addition of the deacylated lipids.

Elutions were performed at room temperature and *p*H 4 using 300 ml. of 0.01 *M* formic acid–0.05*M* ammonium formate in the mixing chamber and 0.04*M* formic acid–0.20*M* ammonium formate in the reservoir at 1.0 ml. per minute for the 0.8 cm.^{2} columns. The effluent passed through a spiral of 1.5 mm. o.d. capillary plastic scintillator and into an automatic fraction collector adjusted for 2.0

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ml. fractions. The phosphor[11] was attached to an RCA 6810A photomultiplier tube operated at 1800 volts. With suitable discriminator settings, both S^{35} and P^{32} radioactivity peaks were sensitively recorded using a linear count rate meter and strip chart recorder.

The deacylated alfalfa lipids were fractionated on the larger columns in the presence of glycerophosphoryl glycerol-P^{32} and sulfoglycosyl glycerol-S^{35} as described above. Phosphate-containing contaminant was found in the product and found to be due to the cyclic 1,2-glycerophosphate. Subsequent separations were performed after treating the lipid deacylate with Dowex-50·H^+ and traces of hydrochloric acid which effected hydrolysis of the cyclic ester. Fractions containing S^{35} activity were decationized with Dowex-50·H^+ and concentrated *in vacuo*. Neutralization of the concentrate with cyclohexylamine and crystallization from ethanol–toluene gave colorless prisms, m.p. 191–192°, $[\alpha]^{25}D$ + 74.5° in water (*p*H 4, *c* 18), $[\alpha]^{25}_{436}$ + 127° in water (*p*H 4, *c* 1.75), $[\alpha]^{25}_{436}$ + 38° in cupra B (*c* 0.706). The yield from 2 kg. of dry leaves was 180 mg. of crystalline salt. Products from independent separations were analyzed.

Anal. Calcd. for $C_{15}H_{31}O_{10}NS$: C, 43.2; H, 7.44; N, 3.36; S, 7.66. Found: C, 43.22, 43.15; H, 7.44, 7.37; N, 3.20, 3.26; S, 7.44, 7.37.

1-Sulfo-2,3-propanediol.—The reaction of allyl alcohol with sulfuric acid according to the method of Friese[12] gave 1-sulfo-2,3-propanediol which was converted to its cyclohexylammonium salt in the usual manner. The infrared absorption spectrum of the salt revealed strong absorption bands at 1042 and 1200 cm.$^{-1}$ and a moderately strong band at 792 cm.$^{-1}$.

(11) Plastic phosphor, NE 501, was obtained from Nuclear Enterprises, Ltd., 1750 Pembina Highway, Winnipeg, Canada.

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[CONTRIBUTION FROM THE CHEMISTRY DIVISION OF THE BRITISH COLUMBIA RESEARCH COUNCIL, VANCOUVER 8, B. C., CAN.]

2-Cyanoethyl Phosphate and its Use in the Synthesis of Phosphate Esters[1]

BY G. M. TENER[2]

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A new method for the synthesis of phosphomonoesters has been developed. 2-Cyanoethyl phosphate (V) is coupled to an alcohol using dicyclohexyl carbodiimide (DCC) as the condensing agent and then the cyanoethyl group is removed from the resulting phosphodiester by very mild alkaline hydrolysis to give the desired phosphate ester. The method has been applied to the synthesis of a number of nucleotides in good yield. An improved method for the synthesis of 2-cyanoethyl phosphate is reported as well as a method for the synthesis of P^{32}-labeled 2-cyanoethyl phosphate and its application to the synthesis of P^{32}-labeled phosphate esters.

Many reagents[3,4] have been employed for the synthesis of phosphate esters but none has proved to be completely general and, indeed, for the phosphorylation of certain compounds no satisfactory reagent exists. The limitations of existing reagents are particularly evident in the synthesis of nucleotides where such considerations as acid lability of purine glycosidic bonds, alkaline lability of the 6-amino group of cytosine[5] and the catalytic reduction of pyrimidine rings[6] must be taken into account. Multifunctional reagents such as phosphorus oxychloride[7–9] and polyphosphoric acid[10] are of limited value because of the complex mixture of products they produce and their use is practical only in those cases where the reaction products can withstand hydrolysis. The most successful reagent so far employed in the nucleotide field is dibenzyl phosphorochloridate.[11] It readily phosphorylates most primary alcoholic functions and the benzyl groups can be removed from the intermediate phosphotriesters by mild catalytic hydrogenolysis using a palladium catalyst. The reagent is, however, unstable and very sensitive to traces of water and during the prolonged reaction time required to phosphorylate secondary alcoholic functions the reaction solvent (pyridine) causes some debenzylation[12] of the intermediates thus giving lower yields of the desired products. Further, dibenzyl phosphorochloridate is not extremely powerful as shown by its inability to phosphorylate guanosine nucleosides.[8] Tetra-*p*-nitrophenyl pyrophosphate[9] was developed to fill this latter need, but in this case the *p*-nitrophenyl protecting groups must be removed from the intermediate phosphotriester by drastic alkaline hydrolysis or by a specific enzymic procedure. Likewise, *O*-benzylphosphorous-*O,O*-diphenyl phosphoric anhydride[13] was developed to provide a more powerful phosphorylating agent but because of the difficulties encountered[14] in removing benzyl groups from cytosine-containing nucleotides by hydrogenolysis, even with palladium catalysts, alternate reagents were sought.

In view of the above limitations, it is apparent that any new phosphorylating agent for use in the nucleotide field should satisfy the following requirements; it should be very powerful; and it should be a monofunctional reagent from which the protecting groups can be removed by very mild and specific methods. In addition, it is desirable to have a simple procedure for both the preparation of

(1) This work has been supported by a grant from the National Research Council of Canada, Ottawa.

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