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Steryl Glucosides in *Phaseolus aureus*. Use of Gas–Liquid Chromatography and Mass Spectrometry for Structural Identification*

Roger A. Laine† and Alan D. Elbein

ABSTRACT: Chloroform–methanol extracts of a membrane-containing particulate enzyme fraction from *Phaseolus aureus* were concentrated to a small volume and subjected to thin layer chromatography in three solvent systems. An α-naphthol–H_2SO_4-staining band was purified and analyzed by gas–liquid chromatography and by combined gas–liquid chromatography–mass spectrometry. Two major components were identified as stigmasteryl and β-sitosteryl glucosides by mass spectra of the intact molecules as trimethylsilyl derivatives.

During studies on the biosynthesis of cell wall polysaccharides in mung bean seedlings, we found that a particulate enzyme preparation incorporated radioactivity from UDP-D-glucose-^{14}C into chloroform–methanol-soluble products. Since some types of glycolipids have been shown to be intermediates in bacterial cell wall synthesis (Anderson *et al.*, 1965; Dankert *et al.*, 1969; Lennarz and Talamo, 1966), we undertook to examine the endogenous glycolipids in the mung bean particulate enzyme fraction to determine whether similar intermediates occur in the synthesis of the plant cell wall.

This report concerns the identification of components in one lipid band isolated by thin-layer chromatography of crude lipid extracts. During the isolation, radioactivity associated with the lipid bands was monitored for related enzymatic investigations not considered here. Although only 400 μg of the lipid band was obtained for analysis, its constituents were easily identified as a series of steryl glucosides by the use of combined gas–liquid chromatography–mass spectrometry. The methods employed in this inquiry should be generally applicable to some types of glycolipids and may be useful in the investigation of membrane components.

Experimental Section

Preparation of the Membrane Particulate Fraction. A particulate enzyme fraction was prepared by grinding mung bean shoots with sand (Elbein, 1969). Material sedimenting between 1000*g* and 30,000*g* was resuspended in buffer and used as the enzyme fraction. Large-scale incubation mixtures

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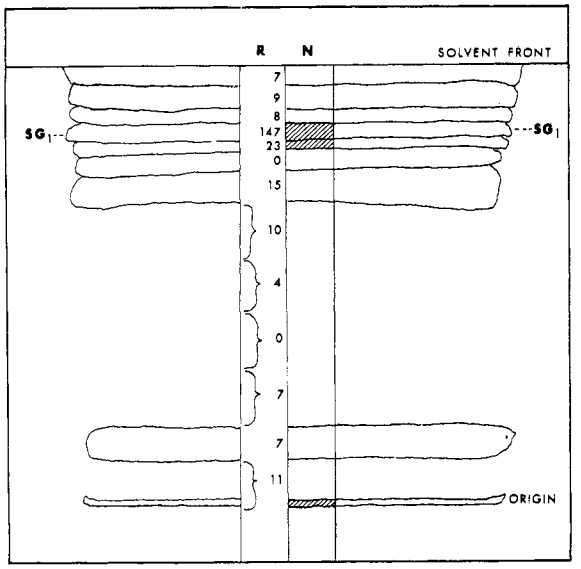

FIGURE 1: Thin-layer chromatography of crude chloroform–methanol extracts. After streaking these extracts on plates of silica gel G (500 μ), they were chromatographed in solvent system A. Plates were subsequently exposed to iodine vapors to visualize lipid bands as indicated by the outlined areas. The vertical strip R was assayed for ^{14}C; numbers indicate counts per minute in the iodine absorbing bands outlined and the intervening areas indicated. Shaded areas in vertical strip N indicate α-naphthol staining. Band SG_1 was removed for further purification.

contained the following (in a final volume of 5 ml): $MgCl_2$, 50 μmoles; Tris buffer (pH 7.0), 250 μmoles; UDP-D-glucose-^{14}C, 0.01 μmole (300,000 cpm); and 2.5 ml of enzyme particulate suspension (25 mg of protein). UDP-D-glucose-^{14}C (3×10^7 cpm/μmole), uniformly labeled in the glucose moiety, was obtained from New England Nuclear, Inc. Incubations were carried out at 37° for 30 min after which the reaction was stopped by the addition of two volumes of 2:1 chloroform–methanol, followed by vigorous mixing. The layers were separated by centrifugation and the lower, chloroform layer

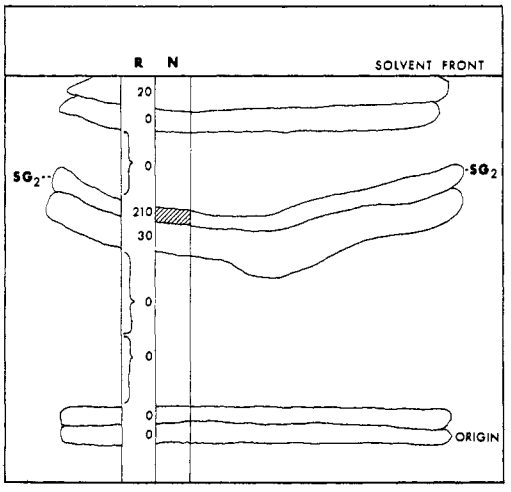

FIGURE 2: Thin-layer chromatogram of band SG_1 from Figure 1. Solvent system B was used on 500-μ thick plates of silica gel G, and the plates were exposed to idoine vapors. As in Figure 1, the vertical strip R contains the counts per minute in the indicated areas, while the shaded zone in strip N reveals sugar-containing areas. The band labeled SG_2 was removed for further chromatography.

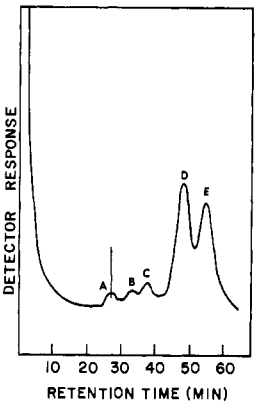

FIGURE 3: Gas–liquid chromatogram of the trimethylsilyl derivatives of the intact purified glycolipid mixture. Gas–liquid chromatography was performed isothermally on a 0.3 m × 4 mm column of SE-30 at 265° and flow rate of 50 cm³/min. The instrument was a Barber-Colman Model 500 equpped with a flame ionization detector and radioactivity monitoring system.

was removed. The extraction procedure was repeated twice and the combined chloroform–methanol fractions were concentrated to a small volume.

Chromatographic Procedures. Thin-layer plates were prepared with either silica gel G or silica gel HR (Brinkman, Inc.) to a thickness of either 250 or 500 μ using a Reeve Angel plate leveling apparatus and spreader. These plates were activated at 110° for 30 min and stored in a desiccator over KOH until used. Thin-layer chromatographic systems were as follows: (A) chloroform–methanol–water (65:25:4, v/v), (B) chloroform–methanol–water (85:15:0.5, v/v), and (C) chloroform–methanol–water (95:5:0.2, v/v).

Lipid bands were located by iodine vapor absorption and sugar-containing bands were detected by spraying with 5% α-naphthol in ethanol and then with 95% sulfuric acid and heating at 110° for 2–4 min. The iodine-absorbing bands were scraped from the plate except for two narrow vertical strips in the center of the plate. The iodine-absorbing bands and areas between these bands were scraped from the vertical strip labeled R (Figures 1 and 2) and transferred to a scintillation vial for radioactivity determination. The remaining strip of silica gel labeled N (Figures 1 and 2) was sprayed with the α-naphthol–H_2SO_4 reagents for detection of glycolipids.

Gas–Liquid Chromatography and Mass Spectrometry. Gas–

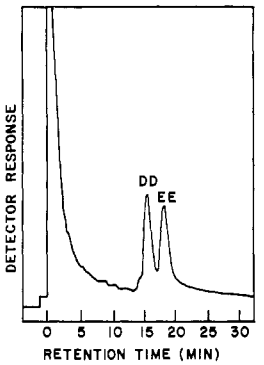

FIGURE 4: Gas–liquid chromatogram of the hexane fraction from a methanolysis of purified glycolipid. The aglycon peaks eluted at 220° in 15 and 17.5 min, respectively. Other conditions were as in Figure 3.

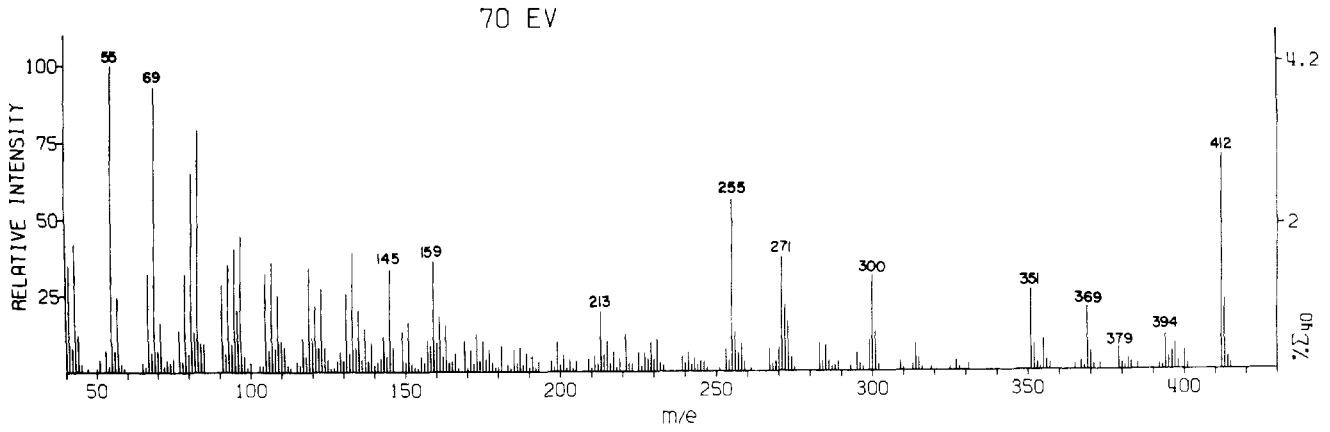

FIGURE 5: The mass spectrum of component DD (an aglycon peak) from Figure 4. Mass spectra were recorded on an LKB-9000 combined gas–liquid chromatograph–mass spectrometer. Columns were 3 m long, and used a 1% liquid phase of SE-30. The ionizing electron energy was 70 eV. Spectra were scanned in 3–5 sec on the apex of the gas–liquid chromatographic peak represented on the total ionization monitor. Temperatures were as follows: ion source, 250°, helium separator, 250° and gas–liquid chromatographic column 220–280°.

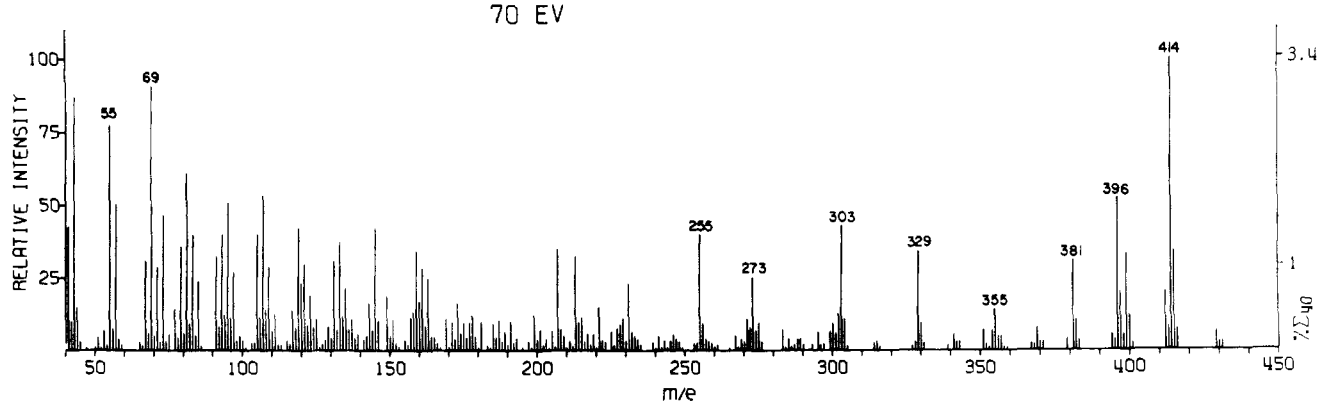

FIGURE 6: The mass spectrum of component EE from Figure 4. Instrumental conditions were the same as indicated in Figure 5.

liquid chromatography was performed on a Barber-Coleman Series 500 instrument equipped with U-shaped columns and hydrogen flame ionization detectors. Liquid phases used were SE-30 and DEGS (Supelco, Inc.) on a support of Chromosorb W.

Trimethylsilyl ethers of the methyl glycosides were prepared by the addition of equal amounts of acetonitrile and bistrimethylsilyltrifluoroacetamide (Regisil, Regis Chemical Co.). Samples were allowed to react for 30 min at 110° in tubes stoppered with Teflon-lined caps. Standard compounds were treated in the same manner. The trimethylsilyl ethers of the intact glycolipids were prepared in the same way except that the reaction time was increased to 60 min.

The permethyl ethers of the intact glycolipids were prepared as described by Hakomori (1964) and then dissolved in acetonitrile for gas–liquid chromatography.

Mass spectra were recorded on an LKB 9000 gas–liquid chromatography–mass spectrometer. Gas–liquid chromatography columns were 1 or 3 m long, as indicated, and used a 1% SE-30 liquid phase. The ionizing electron energy was 70 eV except as stated otherwise. Mass spectra were scanned in 3–5 sec on the apex of the gas–liquid chromatography peak represented on the total ionization monitor. Temperatures were as follows: ion source 250 and 270°; carrier gas was helium, carrier gas separators 250°; gas–liquid chromatographic column 220–280°.

Other Procedures. Methanolysis was accomplished with 1 N methanolic HCl (Carter and Koob, 1969) at 80° for 4 hr. Water (10 μl) and hexane (2 ml) were added to the methanolysate in 1 ml. After mixing, the upper, hexane layer was removed. This procedure was repeated and the combined hexane fractions were dried under a stream of dry nitrogen at 40°, and redissolved in 100 μl of hexane for injection into the gas–liquid chromatograph. The lower or methanol–HCl layer contained the sugar moiety. Silver acetate was added to the methanol fraction to remove chloride (Windeler and Feldman, 1969). After centrifugation, the supernatant fraction was removed and dried under a stream of dry nitrogen at 40°. Subsequently, 50 μl of acetonitrile and 50 μl of bis(trimethylsilyl)trifluoroacetamide were added to the residue after drying to prepare the Me_3Si ethers.

For infrared spectrophotometry, 50 μg of glycolipid mixture was dissolved in chloroform–methanol (2:1, v/v) and dispersed on powdered pure KBr, and compressed into a 1.5-mm micropellet with a Perkin-Elmer micropellet press. Using a beam condenser with a KBr lens on a Perkin-Elmer Model 247 infrared spectrophotometer, an infrared spectrum was recorded.

Results

Characterization of the Products. The endogenous glycolipids were purified by repeated thin-layer chromatography.

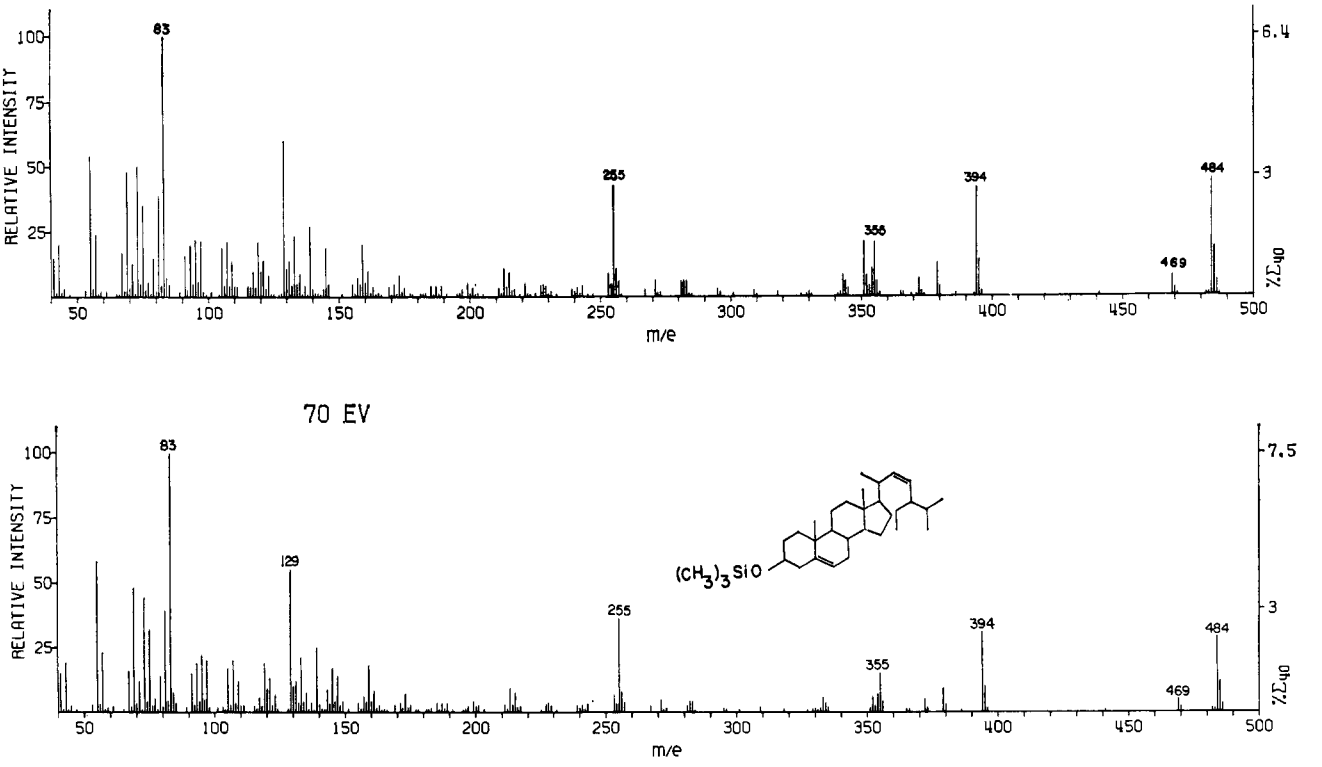

FIGURE 7: The mass spectrum of the trimethylsilyl ether of the aglycon component DD from Figure 4 (top), as compared with the mass spectrum of the trimethylsilyl ether of an authentic sample of stigmasterol (lower figure). The spectra were recorded under the same conditions as listed for Figure 5.

Figure 1 shows the results of chromatography of the crude chloroform–methanol extracts in system A. The lipid bands outlined were revealed by exposure to iodine vapors. A narrow vertical strip near the center of the plate labeled R was removed in sectors for radioactivity determination. Numbers within this strip indicate the level of radioactivity for the areas indicated. The vertical strip marked N was left on the plate while the iodine-absorbing bands were carefully removed by scraping. The shaded areas in Figure 1 indicate α-naphthol–H_2SO_4-positive reactions (rose-red color). The band labeled SG_1 was eluted from the silica gel with 2:1 chloroform–methanol and rechromatographed in system B as indicated in Figure 2. Again a small strip was removed for ^{14}C determination, and the iodine-positive staining areas were removed from the plate leaving another vertical strip for analysis with α-naphthol. Numbers in the strip marked R indicate the radioactivity for the area designated, and the shaded area in strip N was positive for α-naphthol–H_2SO_4 staining. An iodine-absorbing band (labeled SG_2) was eluted from the silica gel as before, and the product was rechromatographed in system C. Two ^{14}C-positive, α-naphthol–H_2SO_4-positive bands were resolved in the latter system. One had an R_F of 0.25 and contained 80% of the ^{14}C and sugar-staining properties while the other band had an R_F of 0.15 and contained 20% of the ^{14}C and α-naphthol–H_2SO_4 staining. The former band was eluted from the silica gel as in the other steps and referred to as purified glycolipid.

The purified glycolipid fraction was chromatographed as the trimethylsilyl derivative on an 0.6 m × 4 mm column of 3% SE-30 at 265°. This procedure resolved the material into 5 peaks as shown in Figure 3. The major peaks D and E were eluted in 50 and 55 min. Permethyl derivatives of the glycolipid fraction gave only two peaks which were eluted in 29 and 31 min using the same gas–liquid chromatographic column and conditions as above. These data suggested that the purified fraction contained several glycolipids having molecular weights in the order of 500–600. In order to examine the aglycon portion of the molecule, the purified glycolipid fraction was acid methanolysed and the aglycons were extracted into hexane. The hexane fraction contained no radioactivity as measured by scintillation spectrometry. Figure 4 shows the results of gas–liquid chromatography of the hexane fraction. Utilizing the 0.6 m × 4 mm column of 3% SE-30 at 220°, two peaks were detected eluting in 15 and 17.5 min. The presence of these peaks indicated that the purified glycolipid fraction contained at least two components. The sugar released by acid methanolysis was identified by gas–liquid chromatography of its trimethylsilyl derivatives. This product had identical retention times to the trimethylsilyl derivatives of the methyl glycosides of authentic D-glucose on both a polar (DEGS) and nonpolar (SE-30) liquid phase. In addition, after aqueous hydrolysis of the methyl glycosides, the product reacted with D-glucose oxidase, indicating that it was D-glucose.

The infrared spectrum of the purified glycolipid fraction did not contain a strong absorption band in the region of 1750 cm^{-1}, indicating the absence of an ester carbonyl group and suggesting that the sugar was glycosidically linked to the aglycon moiety. The glycolipid fraction thus appeared to be a mixture of glucosides, differing in their aglycon moieties. Further identification was obtained by combined gas–liquid chromatography–mass spectrometry.

Mass spectra were first recorded on the two aglycon components obtained from the glycolipid (see Figure 4, DD and EE). As shown in Figures 5 and 6, component DD has an apparent molecular ion at m/e 412, while the molecular ion for EE was 414. These molecular weights suggested plant

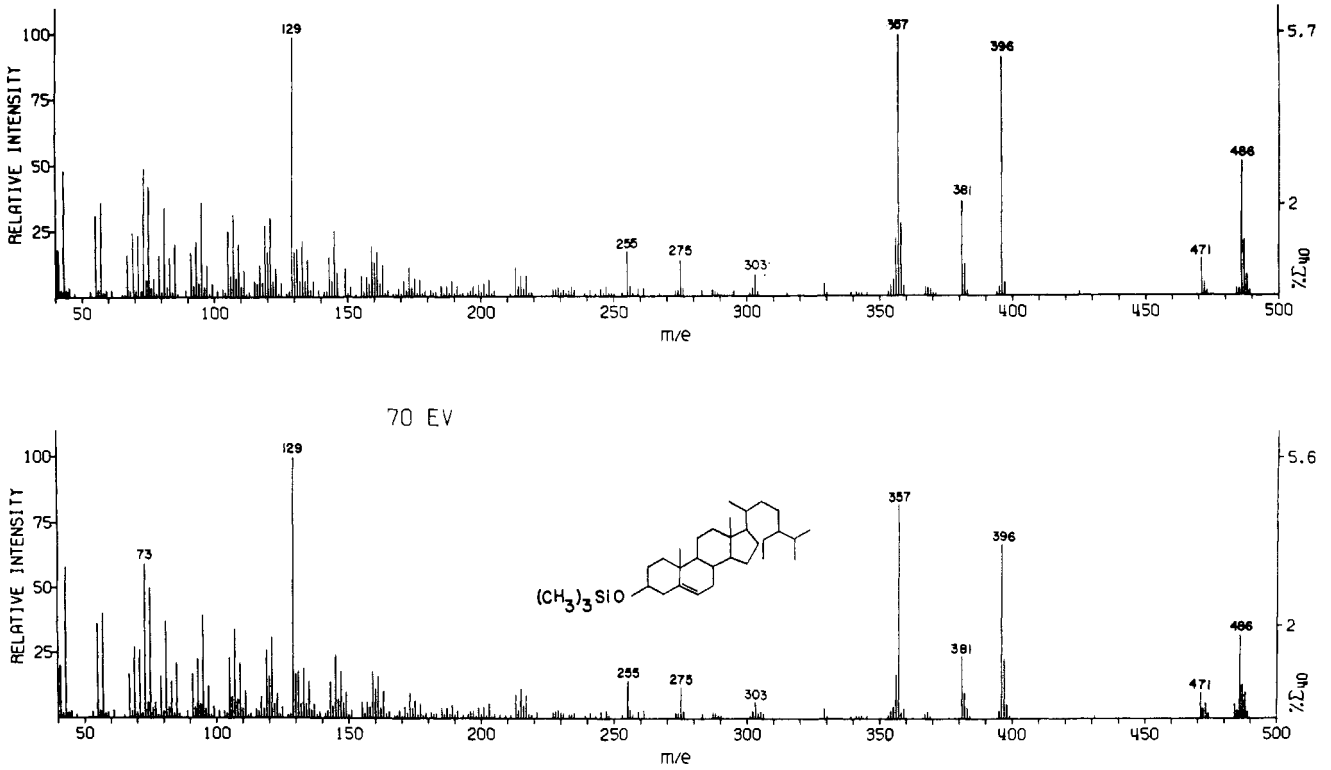

FIGURE 8: The mass spectrum of the trimethylsilyl ether of the aglycon component EE from Figure 4 (top), as compared with the spectrum of the trimethylsilyl ether of an authentic sample of β-sitosterol (lower figure). Spectra were recorded under the same conditions listed under Figure 5.

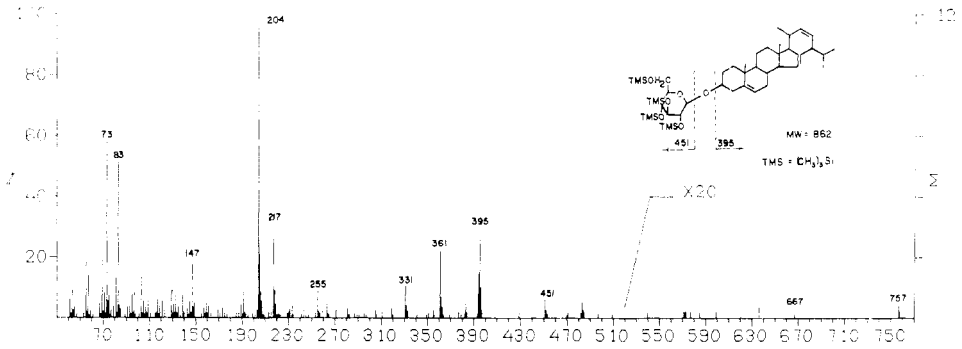

FIGURE 9: The mass spectrum of the intact steryl glucoside as its trimethylsilyl ether. The spectrum is taken on component D from Figure 3 and represents the trimethylsilyl ether of stigmasteryl glucoside. Recording conditions were the same as that for Figure 5, except that a 1-m column of SE-30 was used for gas–liquid chromatography to facilitate the elution of higher molecular weight components.

sterols. Comparative spectra were also taken at lower ionizing electron energies (14 and 16 eV) to confirm the molecular ion. Mass spectra were then taken of the trimethylsilyl derivatives of the aglycon portion and these spectra were compared to the trimethylsilyl derivatives of known plant sterols β-sitosterol and stigmasterol as shown in Figures 7 and 8. The molecular ions had increased 72 mass units, indicative of one replaceable hydrogen, and the ion at *m/e* 129 suggested that these compounds were Δ5-3-ol steroids (Diekman and Djerassi, 1967). The ions at *m/e* 255 indicated one double bond in the steroid nucleus. Cholestane exhibits an ion at *m/e* 257, whereas ergosterol exhibits an *m/e* 253 ion, indicative of the number of unsaturations in the steroid nucleus (Jaureguiberry *et al.*, 1965).

Because of the reported spectra for glycosides and steroids, we decided to record the spectra of the trimethylsilyl ethers of the intact steryl glucosides. These spectra proved to be very informative, having characteristic peaks for both the hexose and sterol moieties. A molecular weight of 862 would be expected for stigmasteryl 2,3,4,6-tetra(trimethylsilyl)glucoside. Figure 9 shows the spectrum of component D from Figure 3. No molecular ion was observed. $(M - 15)^+$ was not a clear ion in the spectrum, but M − 90 − 15 (corresponding to M − trimethylsilanol − CH_3 at *m/e* 757) was observed as was M − 90 − 90 − 15 (at *m/e* 667). These ions were used for molecular weight determination (Chizov *et al.*, 1967). Loss of the sugar (tetratrimethylsilyl) with charge retention on the sugar portion and cleavage of the glucose C–O glycosidic bond, produces an ion mass 451, which could lose trimethylsilanol = 90 producing an ion of mass 361 (DeJongh *et al.*, 1969), both of which are prominent ions in this spectrum. Cleavage

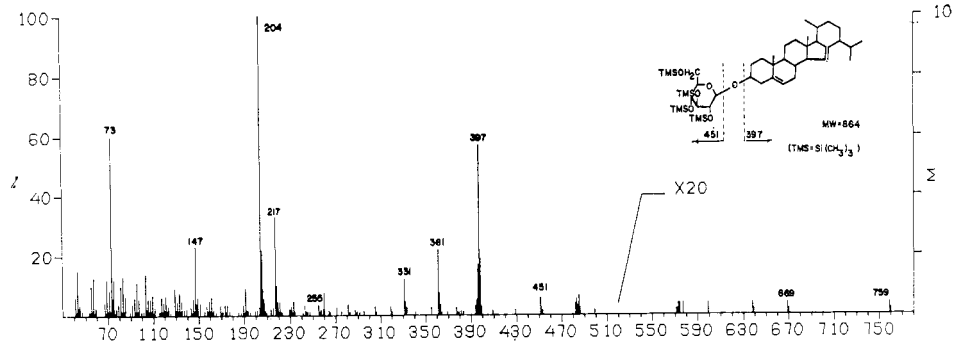

FIGURE 10: The mass spectrum of component E from Figure 3, which represents the trimethylsilyl ether of β-sitosteryl glucoside. The spectrum was recorded under the same conditions as that for Figure 5 except that a 1-m column of SE-30 was used for gas–liquid chromatography.

of the sterol-3-(C–O) bond with charge retention on the steroid would produce an ion in this case of *m/e* 395, which is apparent in the spectrum. Characteristic ions for hexoses at *m/e* 147, 204, 217, 305, 361, and 451 are prominent and *m/e* 204 is the base peak, an indication of hexose pyranoside configuration (DeJongh *et al.*, 1969).

The analogous spectrum for component E from Figure 3 corresponding to β-sitosteryl 2,3,4,6-tetra(trimethylsilyl)-*O*-glucoside (Figure 10) contains the same saccharide ions mentioned above. Ions corresponding to M^+ and M − 15 again were not observed, but *m/e* 759 (M − 90 − 15) and 669 (M − 90 − 90 − 15) are apparent. The *m/e* value corresponding to retention of charge on the steroid moiety at 397 is analagous to the 395 in Figure 9. In addition, a partial spectrum was obtained for component A from Figure 3, and although the spectrum was not clear, peaks at *m/e* 253 and 393 indicate that this compound may be a stigmastatrienyl glucoside, with two unsaturations in the steroid nucleus (Jauréguiberry *et al.*, 1965), and one in the C-17 side chain. A greater amount of component A will have to be accumulated for further analysis.

The *m/e* value 83 visible in the trimethylsilylstigmasterol spectrum (Figure 7) and the stigmasteryl trimethylsilylglucoside spectrum (Figure 9) may be derived from side-chain rearrangement with subsequent allylic cleavage (Figure 11).

Discussion

Incubation of UDP-D-glucose-^{14}C with a particulate enzyme fraction from *P. aureus* incorporated radioactivity into chloroform–methanol extracts. This prompted a study of the endogenous glycolipids in the particulate fraction.

Part of the investigation demonstrated the occurrence of a series of steryl glucosides in these extracts. Two of the components have been identified as stigmasteryl glucoside and β-sitosteryl glucoside. A third has been tentatively assigned the structure of stigmastatrienyl glucoside, having two of the unsaturations in the steroid nucleus.

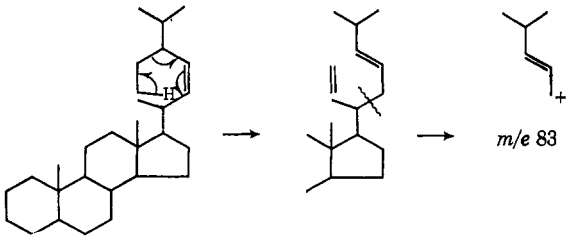

FIGURE 11: Illustration of a possible electron-impact-induced, six-membered rearrangement in the carbon-17 side chain of stimasterol. Subsequent allylic cleavage could give the indicated ion of *m/e* 83. Cleavage is indicated by a wavy line (homolytic fission), while rearrangement is indicated by fishhook arrows.

Steroid glycosides have long been known to occur in a variety of plant tissues (Aylward and Nichols, 1958; Carter *et al.*, 1961; Wright *et al.*, 1962; Nielson and Kofod, 1963). Very little data are available on possible functions of these compounds in plants. Eichenberger and Newman (1968) found evidence for steryl glucoside synthesis from incubation of lettuce leaf punches with UDP-D-glucose-^{14}C. Hou *et al.* (1968) demonstrated this pathway in enzyme fractions from immature soy bean seeds. After incubation with UDP-D-glucose-^{14}C, radioactivity in the steroid glucoside band from the mung beans can be followed through three steps of purification on thin-layer chromatography. This suggests that a similar pathway for synthesis of steryl glucosides in mung beans exists although detailed enzymatic studies will be required to confirm this.

Isolations of steroid glycoside mixtures in the past have been hydrolyzed and the steroid and sugar moieties analyzed separately. By preparing the trimethylsilyl ethers of the intact glycolipids, we have separated the molecular species of the mixture from a biological source and obtained direct evidence for structures from mass spectrometry.

The molecular species of one other type of glycolipid have been separated and identified by combined gas–liquid chromatography–mass spectrometry. Samuelsson and Samuelsson (1969) separated cerebrosides on the basis of their fatty acid and sphingosine base content.

Compounds which are structurally related to steryl glucosides, such as steryl glucuronides and cardiac glycosides may lend themselves to this kind of analysis. To our knowledge no mass spectra have been published on steryl glucuronides, although VandenHeuvel (1967) reported their elution and separation by gas–liquid chromatography. Combined gas–liquid chromatography–mass spectrometry may be useful for some urinalysis procedures. Also, few sensitive assays are available for cardiac glycosides in the microgram range. Such applications of gas–liquid chromatography–mass spectrometry might be very useful for assays of these kinds of compounds in biological fluids and tissues.

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Properties of Some Steroid Glycosyl Transferases from Rabbit Tissues*

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ABSTRACT: The steroid *N*-acetylglucosaminyl and glucuronyl transferases in Triton-solubilized preparations of rabbit liver and kidney microsomes were not effectively separated by chromatography on a variety of ion-exchange and gel filtration columns or by centrifugation or electrophoresis in sucrose density gradients. The *N*-acetylglucosaminyl transferase was, however, selectively inhibited by UTP and by thiol reagents, and was not, in contrast to the glucuronyl transferase, solubilized by cetyltrimethylammonium bromide. The *N*-acetylglucosaminyl transferase was more stable than the glucuronyl transferase to treatment with snake venom and to heating at 55°. Selective use of these procedures, together with chromatography on Sepharose 2B, can be used to obtain preparations of either transferase essentially free of the other.

There is now a great deal of evidence that the UDP-glucuronic acid:steroid glucuronyl transferase in animal tissues consists of a group of enzymes with similar physical properties and different substrate specificities (Rao and Breuer, 1969). Two UDP-*N*-acetylglucosamine:steroid *N*-acetylglucosaminyl transferases have been investigated. That described by Collins *et al.* (1968) in the rabbit has a strict specificity for the 17α-hydroxyl group of phenolic steroids which carry an acidic conjugating group on the 3 position. This enzyme therefore differs from the transferase described by Levitz and his collaborators (Cable *et al.*, 1970) in the human, which seems to be specific for the 15α-hydroxyl group on phenolic steroids, and will transfer *N*-acetylglucosamine to this group in the presence or absence of a sulfate radical at the 3 position of the steroid. The physiological role of these steroid *N*-acetylglucosaminyl transferases is unknown, and this work was undertaken to investigate means for their purification and further study.

Experimental Section

Materials. Sephadex preparations were obtained from Pharmacia Fine Chemicals, Montreal. Snake venoms, CM-cellulose, DEAE-cellulose, Sepharose preparations, and nucleotides were purchased from Sigma Chemical Co. Steroids and steroid conjugates were obtained and purified as described by Jirku and Layne (1965) and Collins *et al.* (1968). Calcium phosphate gel was prepared according to the procedure of Swingle and Tiselius (1951). Rabbits were mature virgin female New Zealand whites.

Assay of Transferase Activities. Glucuronyl transferase and *N*-acetylglucosaminyl transferase activities were determined as detailed by Collins *et al.* (1968, 1970) using 17α-estradiol-*6,7-t* and 17α-estradiol-*6,7-t* 3-glucuronide as substrates, respectively, with UDP-glucuronic acid and UDP-*N*-acetylglucosamine as the donor nucleotides. The only change from

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