nerve conduction produced by the azulenecarboxamides II-1 and III-1. Although the prototype compound for these carboxamides, procainamide, is without effect on frog sciatic nerve, both azulenoid compounds inhibited conduction. Compared with lidocaine, these compounds are about half as active.

The 3-nitro compounds (II-2 and III-2) consistently produced an irreversible inhibition of conduction graded with concentration. However, in contrast with the other compounds, the dose–effect relationships produced by the nitro derivatives were extremely variable from nerve to nerve.

The 3-acetamidocarboxamides are devoid of nerve conduction-inhibiting activity at concentrations up to 50 mmoles.

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Chromatography on Lipophilic Dextran Gels for Fractionation of Low Molecular Weight Compounds I: Steroid Digitonides

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Abstract □ A method for the separation of 3β-hydroxysterols from other sterols is presented. The method involves precipitating 3β-hydroxysterols as the digitonides. The digitonide is then decomposed and separated into components using chromatography on highly cross-linked lipophilic polysaccharide gels. The digitonin in the mother liquor is separated from other sterols using the same chromatographic procedure.

Keyphrases □ Dextran gel chromatography—decomposition of steroid digitonides □ 3β-Hydroxysterols—separation from other sterols by dextran gel chromatography □ Chromatography—separation of 3β-hydroxysterols from other sterols, dextran gel columns

The ability of digitonin to form a sparingly soluble complex with an equivalent amount of 3β-hydroxysteroids was first noted in 1910 (1). The manipulation of concentration (2) as well as the use of gathering agents such as aluminum chloride and aluminum hydroxide was later introduced to permit the rapid and quantitative precipitation of steroid digitonides (3–5). Specificity of digitonide formation to 3β-hydroxysterols (6) and 3β-hydroxysteroidal sapogenins (7) has been shown. Haslam and Klyne (6) showed that 3β-hydroxysterols of the 5α series (A/B *trans*) and Δ^5-3β-hydroxysterols are precipitated at a higher dilution than 3β-hydroxysterols of the 5β series (A/B *cis*). This property can be used to fractionate the 3β-hydroxysterols further (6).

DISCUSSION

Digitonide formation has been used in the quantitative estimation of 3β-hydroxysteroids. A gravimetric assay was proposed (1) while more recently a photometric assay was used for this purpose (2, 4, 8, 9). A radioisotopic assay was also proposed (10).

Decomposition of steroid digitonides has afforded a means for the isolation of 3β-hydroxysteroids. Windaus (1) proposed prolonged boiling with xylene. Schöenheimer and Dam (11) decomposed the digitonides in pyridine. Ether was then added to precipitate digitonin and the steroids were obtained by evaporation of the filtrate.

Mikhelovich (12) showed that the Schöenheimer and Dam (11) method results in less decomposition of either the steroid or digitonin than the Windaus (1) method. Bergmann (13) modified the Schöenheimer and Dam procedure by heating the digitonide with anhydrous pyridine at 70–100°, evaporating pyridine, and extracting the steroid with ether in a soxhlet apparatus. The digitonin was then recovered by extraction with hot 90% alcohol.

Sobel *et al.* (9) extracted the steroids with petroleum ether after digitonide decomposition and demonstrated that pyridine was superior to acetic acid for digitonide decomposition. Issidorides *et al.* (14) used dimethyl sulfoxide to decompose the digitonide. Digitonin remained in solution, while the precipitated steroids were extracted with hexane. The digitonin was subsequently obtained by evaporation of dimethyl sulfoxide.

Digitonide formation has been used to isolate digitonin-like compounds by precipitation with cholesterol. Chanley *et al.* (15) used this procedure in the isolation of holothurin A from the sea cucumber, *Actinopygo agassiza*, while Issidorides *et al.* (14) used this procedure for the recovery of tomatine and honothurin.

^{3}H-Digitonin has been employed to determine the subcellular location of sterols by autoradiography (16). Digitonin has also been used to fragment chloroplasts. The enzymes associated with fragments of various particle sizes can then be examined (17).

Several sources of errors may be encountered when using digitonin to precipitate sterols quantitatively (18). Terpenoids and other products are also precipitated, while excess digitonin cannot be readily removed from the precipitate (18).

Occasionally, the separation of 3β-hydroxysterols from 3α-, 3-dehydro-, or other sterols is required. This report describes a method for effecting such fractionation using digitonide preparations and lipophilic, highly cross-linked dextran gel[1] chromatography. The procedure involves precipitation of 3β-hydroxysterols as the digitonides. The digitonides, in solution, are applied to this column, using benzene–methanol (1:1) as the eluting solvent,

whereby the digitonides decompose and the components become separated at the same time. The mother liquor from the digitonide preparation, containing excess digitonin and other sterols, can also be separated into its components using the same type of column.

The procedure is especially suitable for heat-labile sterols, since a minimum of heat is utilized. This method is particularly suited for small amounts of materials but can be easily adapted to a larger scale. It can also be adapted to the isolation of the digitonin-like compounds by precipitation with cholesterol and subsequent fractionation.

The procedure was first successfully tested on an artificial mixture of the cholesterol and progesterone. It was then applied with equal success to an experimental mixture of stigmasterol and $\Delta^{4,22}$-stigmastadien-3-one obtained from the oxidation of stigmasterol. The oxidation of stigmasterol does not readily go to completion, and the mixture cannot be completely separated by recrystallization or column chromatography on silica gel.

EXPERIMENTAL

Δ^{4-22}-Stigmastadien-3-one from Stigmasterol—$\Delta^{4,22}$-Stigmastadien-3-one was synthesized by the Oppenauer (19) oxidation process. Stigmasterol (2 g) was dissolved in a dry mixture of 30 ml benzene and 15 ml acetone with 8 g aluminum *tert*-butoxide, and the mixture was refluxed for 8 hr. Upon cooling, 4 ml of water was added, followed by 10 ml of 10% H_2SO_4 with vigorous shaking and an additional 30 ml of water. The organic phase was removed, and the aqueous phase was reextracted with benzene. The benzene fractions were pooled, dried over anhydrous sodium sulfate, and concentrated *in vacuo*.

The reaction products were tested on silica gel H plates using isopropyl ether–petroleum ether–acetic acid (70:30:1) (20). The spots, when visualized by spraying with sulfuric acid and heating at 110° for 10 min, indicated a $\Delta^{4,22}$-stigmastadien-3-one to stigmasterol ratio of about 3:1. The mixture was recrystallized once from ethanol before being used for digitonide preparation.

Preparation of Digitonide—The oxidation product (20 mg) was dissolved in 1 ml of hot 90% ethanol. To this solution was added 4 ml of 1% digitonin in 90% ethanol. The solution was concentrated under nitrogen to 3 ml and cooled, and the crystals that formed (22 mg) were filtered. The mother liquor was then concentrated to 1 ml and cooled to obtain a second crop of the digitonide. The combined stigmasterol digitonide obtained was recrystallized once from ethanol to obtain 16 mg of the product, which was used in the chromatography.

The mother liquor was tested in TLC, using the system silica gel H plates with isopropyl ether–petroleum ether–acetic acid (70:30:1). $\Delta^{4,22}$-Stigmastadien-3-one and digitonin were detected with no trace of stigmasterol.

Dextran Gel Chromatography—A column (130 × 2 cm) of lipophilic dextran gel[1] was used for the fractionation of both the steroid digitonide and its mother liquor. A column was prepared using a slurry of the gel in benzene–methanol (1:1), eluting with the same solvent under a head of 150 cm at a flow rate of 1.0 ml/min, and collecting 5-ml fractions. Variation of flow rate between 0.5 ml and 1.3 ml/min did not hinder the separation. The relative concentration of each fraction was determined by means of TLC on silica gel H plates with isopropyl ether–petroleum ether–acetic acid (70:30:1). The relative concentrations were determined according to the size of each spot and density of the color obtained by spraying with 20% H_2SO_4 and heating the plates at 110° for 10 min.

Stigmasterol Digitonide—The digitonide, 10 mg, was dissolved in 1 ml of methanol–benzene (1:1) and applied to the described column. The digitonide simply decomposes on the column without the use of xylene, pyridine, acetic acid, or heat. In these investigations, digitonin was eluted in fractions 57–63 with the peak in fraction 60, while stigmasterol was obtained in fractions 65–74 with the peak in fraction 70.

The fractions containing stigmasterol were combined and evaporated to obtain stigmasterol, which was purified by recrystallization. Greater than 90% of the initially chromatographed amounts of both products were routinely recovered by this procedure. Larger amounts of materials can be separated effectively using larger columns.

Mother Liquor—The mother liquor was evaporated to dryness and dissolved in 1 ml of benzene–methanol (1:1). A 0.5-ml aliquot of this solution was applied to a dextran gel column. Digitonin was eluted in fractions 56–63 with the peak fraction at 61, while $\Delta^{4,22}$-stigmastadien-3-one was obtained in fractions 63–78 with the peak fraction at 68.

The fractions containing only $\Delta^{4,22}$-stigmastadien-3-one were combined and evaporated *in vacuo*. The residue was recrystallized from ethanol to obtain $\Delta^{4,22}$-stigmastadien-3-one in a pure form (R_f in TLC and mass spectroscopy).

[1] Sephadex LH-20, Pharmacia Fine Chemicals, Inc., Piscataway, NJ 08854

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