A STUDY OF THE STEROLS, STEROLINS, AND CERTAIN ALCOHOLS OF SOME LEGUME SEED OILS

by

L. Carroll King

A THESIS

Submitted to the Graduate School of Michigan
State College of Agriculture and Applied
Science in partial fulfilment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

1942

I wish to thank Professor C. D. Ball

CONTENTS

Presentation of Methods and Data

Discussion

Introduction

The intent of this study was to compare the amount and identity of sterols occurring in plant seeds with variety, species and genus differences of plants. The sterol composition of seeds of two varieties of alfalfa (Hardigan alfalfa seed and Grim alfalfa seed) and of two species of clover (Medium Red clover seed and Dutch White clover seed) was examined. The results of this investigation, although limited in scope and as yet incomplete, indicate that sterol composition is the same for different varieties of single species and for different species of a single genus. The sterol composition of the two genera studied was distinctly different.

There have been previous attempts[1] to classify sterols as characteristic of certain orders or families of plants. In view of the occurrence of α-spinasterol[2,3,4,5,6,7] in very different plant types it is probable that such classifications are unjustified. There is need for a detailed characterization of the sterols of many more plants before any general relationship can be defined.

It is well known that sterols form a major portion of the unsaponifiable fraction of most seed oils. Accordingly the crude sterols were isolated from the unsaponifiable fraction of the oil from each of the seeds mentioned and some progress was made in separating the crude material into its component chemical individuals.

In each of the oils studied there was observed a characteristic phytosterolin which could be hydrolyzed into a mixture of sterols and a reducing substance. These compounds are resistant to alkaline hydrolysis and do not accompany the unsaponifiable fraction. They were, therefore, isolated and worked up separately. Phytosterolins probably occur very generally in plant seed oils and any study of sterols should provide for observation of them separate and apart from the sterols in the unsaponifiable fraction.

In order to present the experimental part clearly it has been written as a series of short Sections, I-X . In a special addendum, Section XI, the sterol composition of the four seed oils has been summarized.

I. The Sterols from the Unsaponifiable Fraction of Hardigan Alfalfa Seed Oil.

Three isomeric sterols, α-spinasterol, β-spinasterol and a new sterol of the same general type which for purposes of this thesis was designated δ-spinasterol, have been isolated from the unsaponifiable portion of Hardigan alfalfa seed oil.

In order to separate these isomers the crude sterols were dissolved in a large excess of acetic anhydride. On cooling this mixture, the acetates of α-spinasterol and β-spinasterol separated as flakey crystals and were filtered off while the δ-spinasteryl acetate remained for the most part in the acetic anhydride mother liquors. α-spinasterol and β-spinasterol were separated by saponifying the crude acetates and repeatedly recrystallizing the resultant sterols from 85% alcohol. β-Spinasterol is considerably more soluble than α-spinasterol in this solvent and was concentrated in the mother liquors.

α-Spinasterol was purified by extensive recrystallization from methanol and from chloroform-mehtanol, in an attempt to bring its rotation and melting point to the values reported by Kuwada and Yosiki[8,9] for bessisterol. This effort was not successful. α -spinasterol has previously been isolated from alfalfa seed oil[5]. Several investigators[6,10] have been able to reduce α -spinasterol to α -spinastenol which was subsequently identified as α -stigmastenol by Fernholz and Ruigh[11]. The α -spinasterol isolated from alfalfa seed oil gave α -stigmastenol on catalytic reduction.

The purification of β -spinasterol presented some difficulty since traces of α -spinasterol persisted. This α -spinasterol was removed by repeated recrystallization from 85% ethyl alcohol. β -Spinasterol has been previously observed by Heyl and Larsen[12] as a constituent of spinach fat. These authors noted that it absorbed one mole of hydrogen to give the same "spinastanol" as α -spinasterol. Their analysis indicated the formula was $C_{27}H_{46}O$. Since recent work has established the reduction product of α -spinasterol as α -stigmastenol[11], it is apparent that the β -spinasterol of spinach fat[12] has a basic structure containing twenty-nine carbon atoms.

The β -spinasterol isolated from Hardigan alfalfa seed oil has the formula $C_{29}H_{47}OH$. On catalytic reduction it gave α -stigmastenol ($C_{29}H_{49}OH$). The β -spinasterol of Hardigan alfalfa seed is therefore a doubly unsaturated sterol, isomeric with α -spinasterol, and identical with the β -spinasterol of spinach fat.

δ -Spinasterol was best purified by saponifying the crude acetates and recrystallizing from ethyl alcohol and from methanol. Analysis indicated the formula $C_{29}H_{47}OH$. On catalytic reduction it gave α -stigmastenol. δ -Spinasterol is therefore a doubly unsaturated sterol, isomeric with α -spinasterol.

Sobotka[1] tacitly identified the β -spinasterol of spinach fat[11] with the iso-spinasterol[13] produced when α -spinasterol was heated with chloroacetyl chloride. This assumption is not necessarily true since δ -spinasterol has essentially the same melting point as that reported for iso-spinasterol.

Summaries of the separation and of the relationships of the three isomeric spinasterols are presented in Fig. 1 and Fig. 2 .

Experimental

1. Separation of Crude Sterols from the Unsaponifiable Fraction.

One hundred and fifty grams of crude unsaponifiable material from Hardigan alfalfa seed oil,

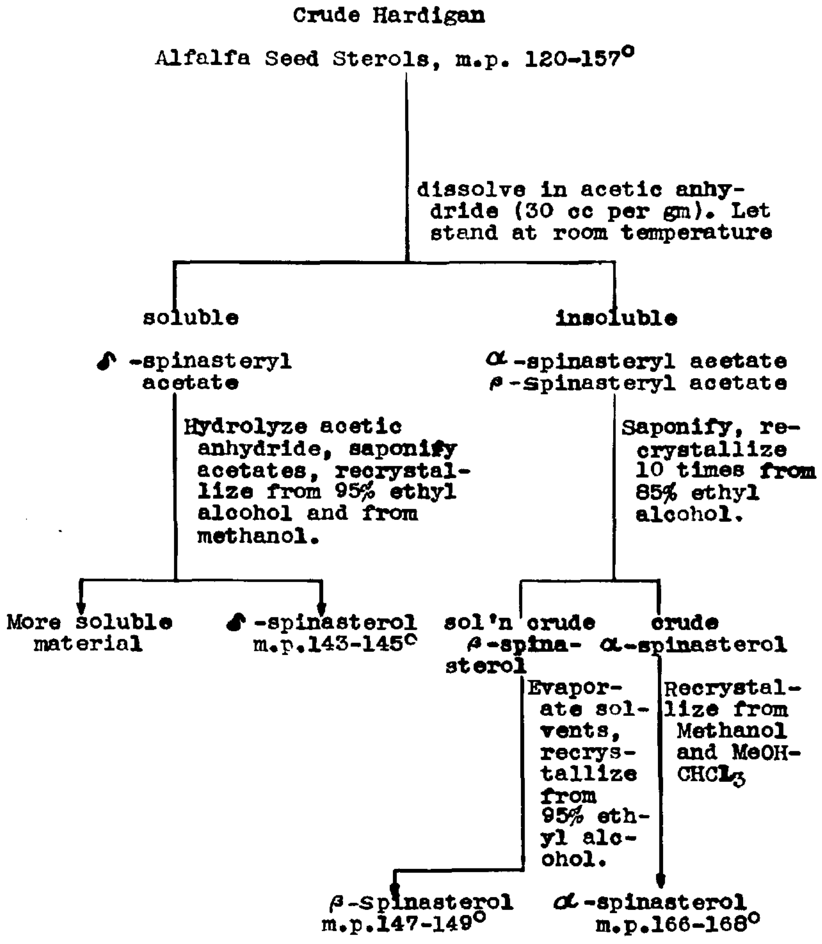


Fig. 1

Summary of the separation of α-spinasterol, β-spinasterol and δ-spinasterol

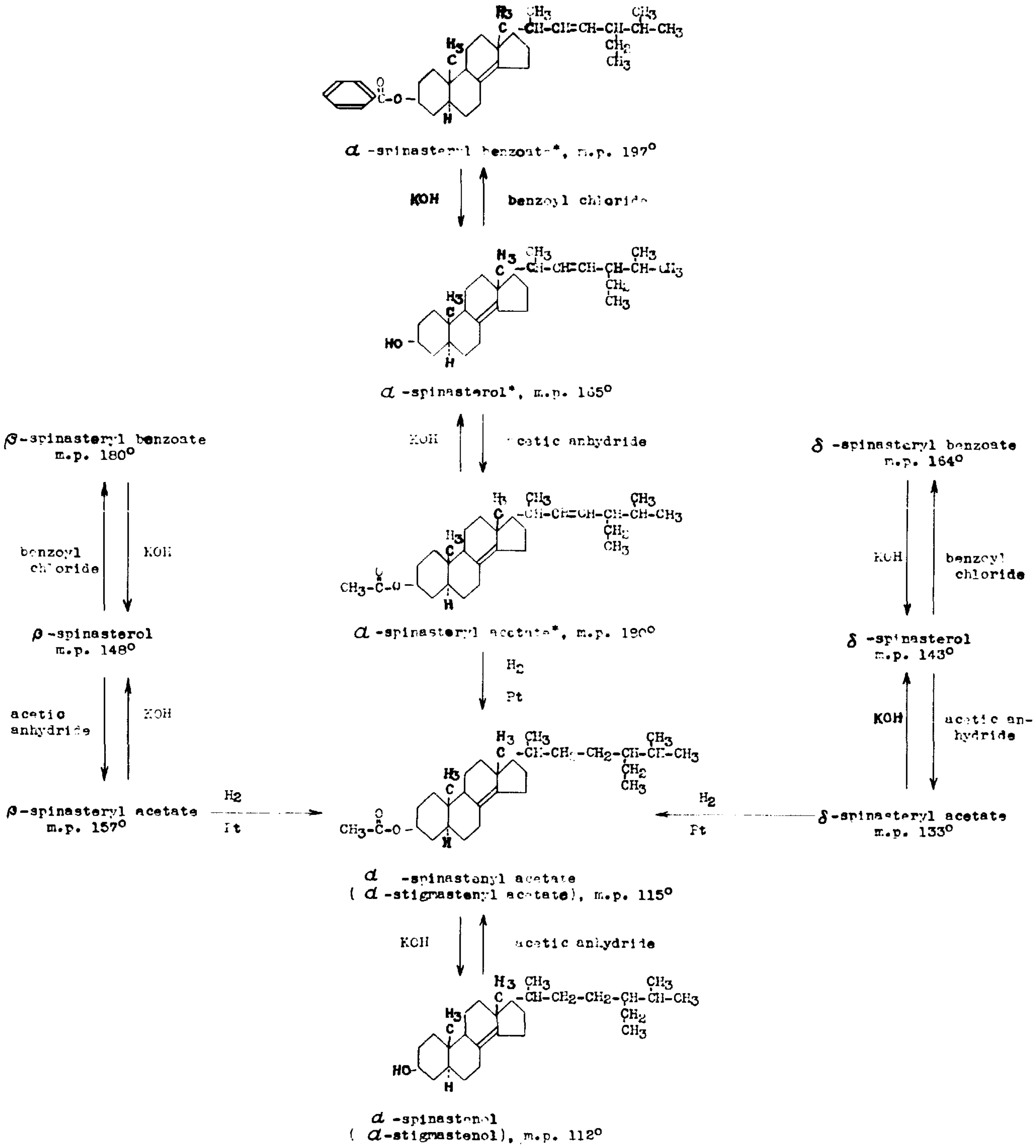


Fig. 2

Chemical reactions and relationships of α-spinasterol, β-spinasterol and δ-spinasterol

* Formula according to Fernholz and Ruigh

prepared as directed by King and Ball[5], was dissolved in enough ethyl ether to make about 500 cc. A stream of water vapor* was passed through the solution until a slight turbidity occurred and the mixture let stand over night at 5°. The crystalline mass which separated was filtered off and recrystallized from ethyl ether. The original mother liquors, and those from the subsequent recrystallizations were concentrated somewhat and the whole process repeated. In this way 26 fractions were obtained.

The 26 solid fractions weighing about 47 g. were nearly free of colored oily material. The first fraction isolated melted about 158°, subsequent fractions had lower and lower melting points, while the last fractions had very indefinite melting ranges in the vacinity of 60°. These crude fractions were combined and classified into two groups, those melting above 120° (Fraction A) and those melting below 120° (Fraction B). Fraction B will be considered in Section II of this thesis.

---

* A small amount of water greatly facilitates the separation of the sterols from the oily mixture. These sterols tend to crystallize with one-half mole of water, if water is available.

2. Fractionation of the Crude Steryl Acetates from Cold Acetic Anhydride.

The crude sterol fractions melting above 120° (Fraction A) were combined and dissolved in acetic anhydride (30 cc. per gram). This mixture was heated one hour, let stand over night, and then filtered. The solid steryl acetates (Fraction A_1) so obtained consisted mostly of α -spinasteryl and β -spinasteryl acetates. Yield 24.5 g., m.p. 152-157°.

The acetic anhydride mother liquors from the above were hydrolyzed by heating with water. The solid steryl acetates precipitated by this treatment (Fraction A_2) were filtered off and recrystallized from 95% ethyl alcohol. Yield 2.9 g., m.p. 122-127°.

3. Separation and Identification of α -spinasterol and β -spinasterol.

α -Spinasterol— The crude acetates, m.p. 152-157°, (Fraction A_1) were hydrolyzed by boiling 1 hour with 5% alcoholic potassium hydroxide. The reaction mixture was poured into water and extracted with ethyl ether. The ether solution was washed with water and evaporated to dryness on the steam bath. The crude sterols obtained were dissolved in just sufficient boiling 85% ethyl alcohol to effect complete solution. This mixture was let stand over night and the crystalline maerial separated. The solid material was then redissolved in a minimum amount of boiling 85% ethyl alcohol and let stand again. This proceedure was repeated 10 times. The resultant crystalline material was then recrystallized from methyl alcohol, and from chloroform-methyl alcohol; m.p. 168.5-169°; $[\alpha]_D^{27} = -2.68^\circ$ (556 mg., 10 cc. chloroform, l = 2 dcm., $\alpha_D^{27} = -0.298^\circ$, <u>average</u> reading). All the attempts to bring the rotation of α-spinasterol to the value - 8.5° or - 13.5°, as reported by Kuwada and Yosiki[8,9] failed. α-Spinasterol isolated in this way forms a precipitate with digitonin.

α-Spinasteryl acetate— A quantity of α-spinasterol was dissolved in acetic anhydride and the mixture heated one hour. On standing the crystalline acetate separated. The product was filtered off and recrystallized from 95% ethyl alcohol; m.p. 180-182°; $[\alpha]_D^{27} = -6.35^\circ$ (52.9 mg., 2 cc. chloroform, l = 2 dcm., $\alpha_D^{27} = -0.336^\circ$, <u>average</u> reading). Attempts to bring the rotation to the value - 13.46° reported by Kuwada and Yosiki[9] were not successful.

α-Spinasteryl benzoate— Five hundred milligrams of α-spinasterol was dissolved in 1.5 cc. pyridine and 0.5 cc. benzoyl chloride added. The mixture was heated in a boiling water bath 2 hours, let stand over night, poured into ice cold 5% sulfuric acid and the product extracted with ethyl ether. The ether solution was washed first with 1% sodium carbonate, then with water, and finally evaporated to dryness on the steam bath. The product was recrystallized twice from 95% ethyl alcohol. Yield 350 mg.; m.p. 196-199°; $[\alpha]_D^{19} = 2.20°$ (51.6 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{19} = 0.114°$, <u>average</u> reading).

β-Spinasterol— The combined mother liquors from the isolation of α-spinasterol were evaporated to a small volume and water added. The precipitate was filtered off and taken up in the smallest volume of boiling 85% ethyl alcohol which would effect solution. After standing over night at room temperature the solid material was filtered off. From the mother liquors a fraction corresponding to the β-spinasterol of Heyl et al.[12], was isolated. On recrystallization from 95% ethyl alcohol it gave flakey transparent crystals; m.p. 148-150°. In melting they lost water of crystallization at 110-125°. $[\alpha]_D^{20} = 5.91°$ (52.7 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{20} = 0.3116°$, <u>average</u> reading). The substance formed an insoluble precipitate with digitonin.

Anal. 2.391 mg. gave 2.582 mg. HOH; 7.240 mg. CO_2
C, 82.56 ; H, 11.98
Calcd. for $C_{29}H_{47}OH \cdot \frac{1}{2}H_2O$ C, 82.58 : H, 11.72

Anhydrous β-spinasterol— β-Spinasterol as isolated above was heated at 50° in vacuo for 7 days. The product was free of water of crystallization; m.p. 148-150°.

Anal. 2.232 mg. gave 2.447 mg. HOH; 6.904 mg. CO_2
C, 84.35 ; H, 12.12
Calcd. for $C_{29}H_{47}OH$ C, 84.38 ; H, 11.73

This anhydrous product on recrystallization from 95% ethyl alcohol gave the original product $C_{29}H_{47}OH \cdot \frac{1}{2}H_2O$.

β-Spinasteryl acetate— One hundred milligrams of β-spinasterol was dissolved in acetic anhydride and the mixture heated 1 hour. On standing several hours at room temperature the crystalline acetate separated. The product was filtered off and recrystallized from 95% ethyl alcohol;

m.p. 153-155°; $[\alpha]_D^{19} = 5.10°$ (44.7 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{19} = 0.228°$, average reading).

Anal. 2.332 mg. gave 2.292 mg. HOH; 6.998 mg. CO_2
2.235 mg. gave 2.290 mg. HOH; 6.683 mg. CO_2
C, 81.83, 81.54 ; H, 10.92, 11.38
Calcd. for $C_{31}H_{50}O_2$ C, 81.88 ; H, 11.097

β-Spinasteryl benzoate— Five hundred milligrams of β-spinasterol in 1.5 cc. pyridine was treated with 0.5 cc. benzoyl chloride. The mixture was heated 2 hours on a boiling water bath, let stand over night, and then worked up as usual. The product was recrystallized from a mixture of ethyl alcohol and ethyl ether. Yield 450mg.; m.p. 181-183°; $[\alpha]_D^{19} = 7.51°$ (56.0 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{19} = 0.421°$, average reading).

Anal. 2.127 mg. gave 1.963 mg. HOH; 6.540 mg. CO_2
C, 83.81 ; H, 10.25
Calcd. for $C_{36}H_{52}O_2$ C, 83.65 ; H, 10.15

Hydrogenation of β-spinasteryl acetate— Nine hundred and fifty milligrams of β-spinasteryl acetate was dissolved in 25 cc. glacial acetic acid and shaken in an atmosphere of hydrogen for 2 hours in the presence of 100 mg Adam's catalyst[14]. An additional portion of catalyst was added and the reaction continued about 2 hours more. The reaction mixture was diluted with water and the product extracted with ethyl ether. The ether solution was washed with water, then with 1% sodium carbonate, and again with water. The solvent was then removed by evaporation on the steam bath. The product was recrystallized from 95% ethyl alcohol. Yield 625 mg.; m.p. 115-116°; $[\alpha]_D^{17}$ = 9.59° (53.1 mg., 2 cc. chloroform, ℓ = 2 dcm., α_D^{17} 0.510°, average reading).

Anal. 2.054 mg. gave 2.153 mg. HOH; 6.169 mg. CO_2
C, 81.90 ; H, 11.64
Calcd. for $C_{31}H_{52}O_2$ C, 81.50 ; H, 11.48

This compound gave no depression in melting point when mixed with an authentic specimen of α-stigmastenyl acetate (prepared from authentic α-spinasterol).

α-Spinastenol (α-stigmastenol)[11]— The acetate mentioned above was hydrolyzed with 5% alcoholic KOH. The reaction mixture was poured into water and the product extracted with ethyl ether. The ether solution was washed with water, the solvent evaporated, and the product was recrystallized from methanol; m.p. 111-112°; $[\alpha]_D^{15} = 21.17°$ (53.7 mg., 2 cc. chloroform, ℓ= 2 dcm., α_D^{15} = 1.137°, average reading).

Anal. 2.562 mg. gave 2.832 mg. HOH; 7.898 mg. CO_2
C, 84.05 ; H, 12.28
Calcd. for $C_{29}H_{49}OH$ C, 83.97 ; H, 12.13

There was no depression in melting point when a specimen of this substance was mixed with authentic α -stigmastenol.

4. Fractionation of the Material Soluble in Cold Acetic Anhydride.

The crude acetates (Fraction A_2), m.p. 122-127°, were hydrolyzed with 5% alcoholic potassium hydroxide. The reaction mixture was poured into water and the product extracted with ethyl ether. The ether solution was evaoprated to dryness and the residue fractionally crystallized from ethyl alcohol. The more soluble fractions yielded a crystalline substance; m.p. 122-125°; $[\alpha]_D^{20.5} = -4.80^\circ$ (48.7 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{20.5} = -0.234^\circ$, average reading). This material was not further studied.

δ -Spinasterol— The less soluble top fractions gave a product; m.p. 142-143°. This substance, after repeated crystallization from methanol, gave a substance which appeared to be a chemical individual; m.p. 143-145°; $[\alpha]_D^{19} = 6.15^\circ$ (49.6 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{19} = 0.305^\circ$, average reading).

Anal. 2.466 mg. gave 2.670 mg. HOH; 7.490 mg. CO_2
C, 82.80 ; H, 12.03
Calcd. for $C_{29}H_{47}OH\cdot\frac{1}{2}H_2O$ C, 82.58 ; H, 11.72

This substance forms an insoluble precipitate with digitonin and gives the common sterol color tests.

δ-Spinasteryl acetate— The sterol, m.p. 143-145°, was dissolved in a small amount of acetic anhydride and heated one hour. On standing at room temperature the product crystallized out. It was recrystallized from 95% ethyl alcohol; m.p. 132-133.5°; $[\alpha]_D^{16.5} = 0.82°$ (49.7 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{16.5} = 0.041°$, average reading).

Anal. 2.526 mg. gave 2.570 mg. HOH; 7.542 mg. CO_2
C, 81.42 ; H, 11.30
Calcd. for $C_{31}H_{50}O_2$ C, 81.88 ; H, 11.097

This acetate on hydrolysis gave the sterol; m.p. 143-145°. This acetate was also obtained from the mother liquors when crude iso-spinasteryl acetates were fractionated from 95% ethyl alcohol.

δ-Spinasteryl benzoate— Two hundred and ninety milligrams of the sterol was dissolved in 1 cc. pyridine and reacted with 0.5 cc. benzoyl chloride. The mixture was heated 2 hours on a boiling water bath and let stand over night. The product was recovered as usual, and recrystallized 4 times from ethyl alcohol; m.p. 165-168° (softening gradually to a viscous liquid) $[\alpha]_D^{19} = 11.17°$;

(50.4 mg., 2 cc. chloroform, ℓ = 2 dcm., α_D^{19} = 0.563°, average reading).

Anal. 2.391 mg. gave 2.232 mg. HOH; 7.294 mg. CO_2
C, 83.20 ; H, 10.37
Calcd. for $C_{36}H_{52}O_2$ C, 83.65 ; H, 10.15

Hydrolysis with 5% alcoholic potassium hydroxide gave the original sterol, m.p. 143-145°, and the sterol recovered from the benzoate was converted to the acetate; m.p. 131-134°.

Hydrogenation of δ-spinasteryl acetate— One hundred milligrams of δ-spinasteryl acetate was dissolved in 10 cc. of glacial acetic acid and reacted with an atmosphere of hydrogen in the presence of 50 mg. of Adam's catalyst. The product was recovered in the usual manner and recrystallized twice from 95% ethyl alcohol. Yield 40 mg.; m.p. 111-112°; $[\alpha]_D^{24}$ = 8.62° (44.5 mg., 2 cc. chloroform, ℓ = 2 dcm., α_D^{24} = 0.384°, average reading). This product when mixed with an authentic specimen of α-stigmastenyl acetate showed no depression in melting point.

Summary

1. From the unsaponifiable portion of Hardigan alfalfa seed oil three isomeric sterols of formula $C_{29}H_{48}O \cdot \frac{1}{2}H_2O$; namely, α-spinasterol,

β -spinasterol and δ -spinasterol have been isolated.

2. Several derivatives of each of the sterols have been prepared and the physical constants and analysis observed.

3. Each of the three isomers can be reduced to α -stigmastenol.

II. Isolation of an Hydrocarbon and of a Fourth Alcohol from the Unsaponifiable of Hardigan Alfalfa Seed Oil.

From the crude solid fractions of low melting point, obtained from the unsaponifiable fraction of Hardigan alfalfa seed oil (Fraction B), an hydrocarbon and a fourth alcohol were isolated.

The hydrocarbon was separated by means of its low solubility in acetone after removal of most of the sterols. It was not characterized.

The alcohol was separated as the acetate from acetone after removal of most of the hydrocarbon. Analysis indicates as a probable formula $C_{29}H_{47}OH$, isomeric with the three sterols mentioned in Section I. It is highly dextro rotatory. It reacts smoothly with halogens in such a way as to indicate one double bond. It did not precipitate with digitonin and could not be epimerized with sodium amylate to give a compound which did precipitate with digitonin. It gave a slight pink color to the sulfuric acid layer in the Lieberman-Burchard test.

A summary of the separation procedure used to obtain this hydrocarbon and this alcohol is given in Fig. 3 .

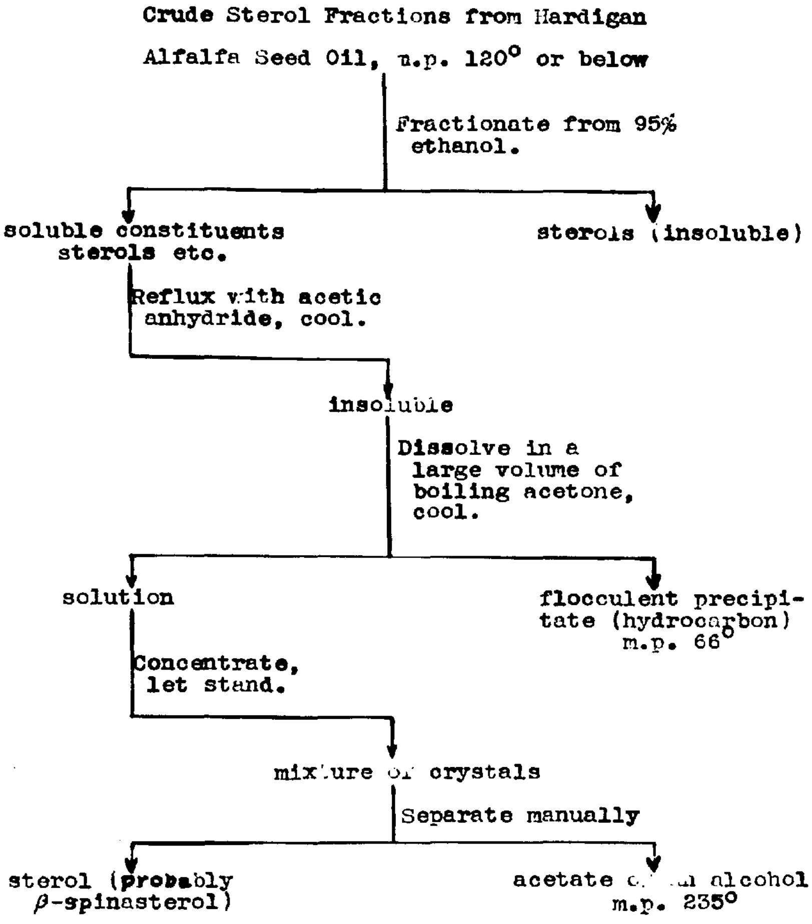


Fig. 3

Scheme for isolation of an hydrocarbon and a high melting alcohol from Hardigan alfalfa seed oil unsaponifiable

Experimental

Isolation of an Hydrocarbon— The crude sterol fractions (4 g.) melting below 120° (Fraction B, Section I), were combined and fractionated extensively from 95% ethyl alcohol. The first fractions melting above 120° appeared to consist of sterols identical with those already described and were set aside. The lower fractions had very indefinite melting points from 30-115°. These lower fractions were combined, refluxed with 500 cc. acetic anhydride and let stand over night. The solid material which separated was filtered off and dissolved in 200 cc. boiling acetone. A flocculent precipitate settled on cooling. It was filtered off and recrystallized from acetone; m.p. 64-66°. The substance had a waxy feel, was insoluble in cold concentrated H_2SO_4 and appeared to be an hydrocarbon. No further characterization was made.

Isolation of the acetate of an alcohol— The acetone mother liquors from which the hydrocarbon was isolated were concentrated to 100 cc. and let stand. Large rod-like crystals as well as large flakey crystals separated along with more of the hydrocarbon. The mixture was filtered in such a way as to retain only the very large rod-like and flakey crystals. These were separated manually. The flakey material consisted mostly of β -spinasteryl acetate. The rod-like crystals were recrystallized from acetone. Yield 0.5 g.; m.p. 238-239°; $[\alpha]_D^{19} = 79.8^\circ$ (50.6 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{19} = 4.040^\circ$, <u>average</u> reading).

Anal. 2.248 mg. gave 2.309 mg. HOH; 6.754 mg. CO_2
2.120 mg. gave 2.110 mg. HOH; 6.358 mg. CO_2
2.645 mg. gave 2.667 mg. HOH; 7.951 mg. CO_2
C, 81.83, 81.79, 81.97; H, 11.41, 11.05, 11.20
Calcd. for $C_{31}H_{50}O_2$ C, 81.88 ; H, 11.097

Reaction with Hanus iodine solution— To 5.018 mg. of the above acetate, dissolved in 0.2 cc. chloroform, was added 0.8 cc. Hanus iodine solution (equivalent to 1.039 cc. of 0.0988 M sodium thiosulfate). The reaction mixture was let stand thirty minutes. Halogen equivalent to 0.287 cc. of 0.0988 M sodium thiosulfate was taken up giving an iodine number of 72.6 . In a second experiment, 4.709 mg. took up halogen equivalent to 0.244 cc. of 0.0988 M sodium thiosulfate giving an iodine number of 65.7 .

Hydrolysis of the acetate— The above acetate was refluxed for 1 hour with 5% alcoholic KOH. The reaction mixture was diluted with water and

the product extracted with ether. The ether solution was washed with water and the solvent evaporated on the steam bath. The product was recrystallized from 95% ethyl alcohol; m.p. 194-
1crystallized from 95% ethyl alcohol; m.p. 194-
f196°; $[\alpha]_D^{18} = 86.5^\circ$ (24.8 mg., 2 cc. chloro-
rform, $l = 2$ dcm., α_D^{18} 2.149°, average

Anal. 2.070 mg. gave 2.127 mg. HOH; 6.418 mg. CO_2
2.310 mg. gave 2.464 mg. HOH; 7.160 mg. CO_2
C, 84.55, 84.52 ; H, 11.41, 11.85
Calcd. for $C_{29}H_{47}OH$ C, 84.38 ; H, 11.73

This alcohol on heating with acetic anhydride gave, in almost quantitative yield, a product identical with the original acetate; m.p. 234-238°. The regenerated acetate when hydrolyzed again gave the alcohol; m.p. 194-196°. This alcohol did not give a precipitate with digitonin. It colored the sulfuric acid layer cherry red but did not color the chloroform layer in the Lieberman-Burchard test.

Summary

From the unsaponifiable of alfalfa seed oil, a hydrocarbon and a fourth alcohol have been isolated.

III. Isolation and Composition of a Phytosterolin from Hardigan Alfalfa Seed Oil.

Sterol glycosides, called phytosterolins by Power and Salway[15], are known to occur generally in bark, leaves and seeds. Recent investigators[16, 17,18] have shown them to be prominent sterol containing constituents of soybean oil and of cotton seed oil. It is probable that phytosterolins occur much more commonly in seed oils than is commonly recognized.

From the cold concentrated ether extract of Hardigan alfalfa seed a phytosterolin was isolated. This same substance was also isolated almost pure as a precipitate in oil that had been standing in the refrigerator for several weeks. A gelatinous precipitate at the ether aqueous soap interphase of a saponification mixture of Hardigan alfalfa seed oil was also found to be a phytosterolin.

Because this alfalfa seed phytosterolin occurs at various points in the isolation scheme, and because it is occasionally contaminated by a nitrogenous substance, estimates of the yield are variable.

This phytosterolin was purified by recrystallizing from a large volume of amyl alcohol. It was characterized in general by a very low solubility, however, it formed an acetate which was very soluble in organic solvents. On acid hydrolysis the sterolin gave a mixture of sterols, from which α -spinasterol was isolated. Neither β -spinasterol nor δ -spinasterol reported in Section I could be observed. It seems probable that the original sterolin is a glycoside of α -spinasterol, and the sterol mixture is mostly due to breakdown products produced by the hydrolysis procedure.

A summary of the separation procedure and chemical relationship of the phytosterolin is given in Fig. 4 and 5 .

Experimental

Isolation of phytosterolin— The ether extracts from 28 kg. of Hardigan alfalfa seed were concentrated to about 50% oil, whereupon a white insoluble powder separated out. This substance was filtered off, washed with ether and dissolved in a large volume of boiling n-amyl alcohol. The hot solution was filtered and cooled. A white powder-like precipitate settled out; yield 4.9 g. This substance turned brown at 240° and decomposed at 274-277°. A quantity of this sterolin also settled

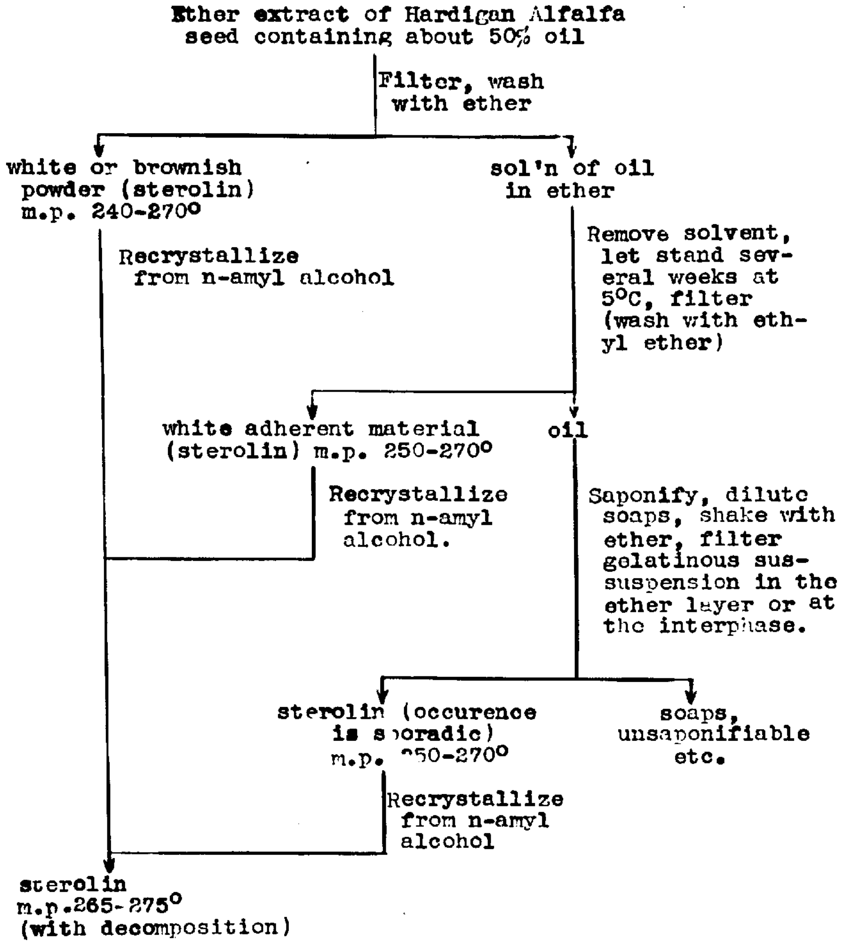


Fig. 4

Scheme for isolation of a phytosterolin from Hardigan alfalfa seed oil.

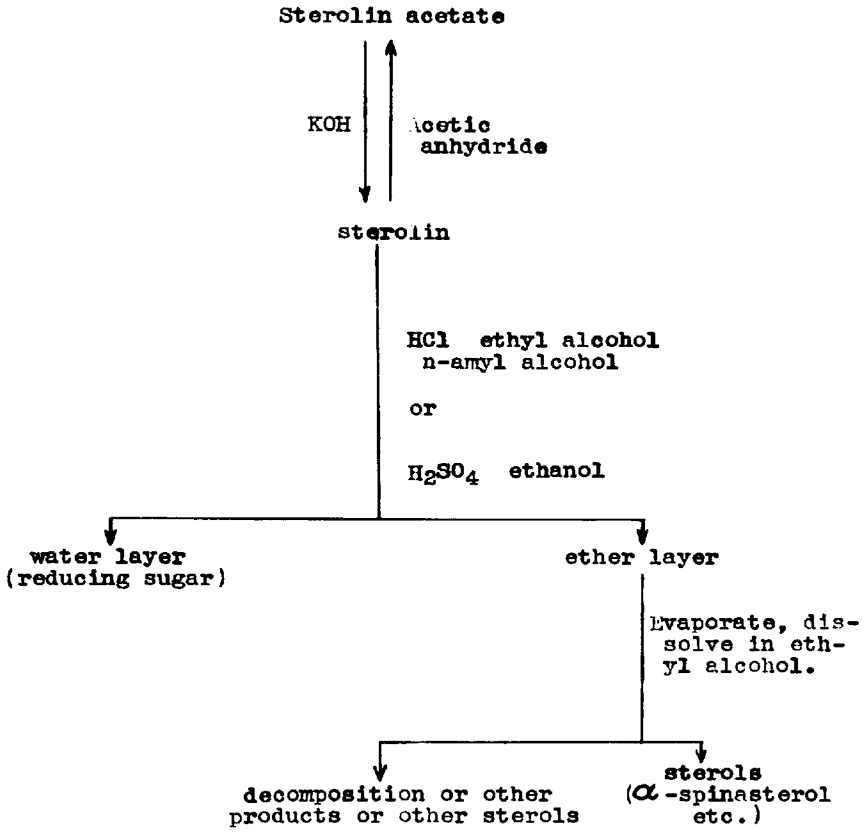


Fig. 5

Reactions of the phytosterolin from Hardigan alfalfa seed oil.

out of alfalfa seed oil when it had stood for several weeks in the refrigerator. A further quantity of this material was isolated as a gelatinous precipitate at the interphase of an ethyl ether extract of the alcoholic aqueous soap solution resulting from saponification of alfalfa seed oil.

This sterolin dissolved in acetyl chloride and acetic anhydride and formed in the process an acetate. It was soluble in cold concentrated sulfuric acid, and was slightly soluble in ether, n-amyl alcohol, and p-dioxane, but was nearly insoluble in most other common solvents. Qualitative analysis indicated the presence of carbon and hydrogen. This substance gave a positive Lieberman-Burchard test, and a positive Molisch test.

Acetylation of the sterolin— A small amount of the sterolin was dissolved in acetic anhydride. The mixture was decomposed with water, cooled and extracted with ether. The ether layer was washed with sodium carbonate then with water, and the solvent evaporated. The residue was dissolved in methyl alcohol and the hot solution filtered. After standing over night, the acetate was filtered off, and recrystallized twice from methanol; m.p. 160-162°

Hydrolysis of the sterolin acetate— About 0.1 g. of the above acetate was refluxed for ten minutes in 20 cc. ethyl alcohol containing 0.1 g. KOH. The precipitated sterolin was filtered off, washed with 95% ethyl alcohol and with water and finally dried. The product appeared to be identical with the original sterolin. It turned brown at 240° and decomposed at 260-270°.

Hydrolysis of the sterolin— Eight and six tenths grams of crude sterolin was boiled with 300 cc. absolute ethyl alcohol containing 2 cc. concentrated H_2SO_4 as described by Thornton, Kraybill and Mitchell[14]. After most of the solid had dissolved, the reaction mixture was diluted and the sterol fraction was extracted with ether. The ether solution was washed with sodium carbonate then with water and finally the solvent evaporated on the steam bath. The brownish residue was taken up in boiling 95% ethyl alcohol and the largest practical amount of crude colorless sterols fractionated from the solution; yield 2.6 g .

Isolation of α -spinasterol— The crude sterols obtained as above were recrystallized ten times from 85% ethyl alcohol and finally from methanol. Yield 250 mg.; m.p. 164-166°;

$[\alpha]_D^{23} = -0.83^\circ$ (48.4 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{23} = -0.040^\circ$, average reading). This substance gave no melting point depression when mixed with authentic -spinasterol.

α-Spinasteryl acetate— A quantity of the above sterol was reacted with acetic anhydride and the product recrystallized from 95% ethyl alcohol; m.p. 179-181°; $[\alpha]_D^{24} = -4.045^\circ$ (52.8 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{24} = -0.214^\circ$, average reading). This substance gave no melting point depression when mixed with authentic α-spinasteryl acetate.

α-Spinasteryl benzoate— Fifty milligrams of the above sterol was dissolved in pyridine and reacted with benzoyl chloride in the usual way. The product was recrystallized twice from ethyl alcohol; m.p. 194-196°; $[\alpha]_D^{22} = 0.46^\circ$ (23.5 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{22} = 0.011^\circ$, average reading). This product gave no depression in melting point when mixed with authentic α-spinasteryl benzoate.

Observation of a reducing substance— The aqueous layer from the above hydrolysis was heated for some time as recommended by Thornton et al.[17], it was then cooled, carefully neutralized with barium carbonate to a pH of 5 and the precipitate filtered off. The resulting solution was concentrated to dryness in vacuo and then purified by dissolving in water and adding methyl alcohol as suggested by Moore and Link[19]. The methyl alcohol was removed from a portion of this solution and the residue reacted with phenylhydrazine acetate. A greenish-yellow crystalline osazone was formed. A separate portion of the solution, after removal of methyl alcohol, reduced Fehlings solution.

Summary

1. A characteristically insoluble phytosterolin was isolated from Hardigan alfalfa seed oil.
2. This phytosterolin on hydrolysis gave a reducing substance and a sterol mixture containing α-spinasterol.

IV. The Sterols from the Unsaponifiable Fraction of Grim Alfalfa Seed Oil.

From the crude sterol fraction, m.p. 120-152°, obtained from the unsaponifiable fraction of Grim alfalfa seed oil in a manner identical with that described for Hardigan seed oil, Section I, three isomeric sterols, identical in each case with the corresponding products isolated from Hardigan alfalfa seed oil, have been isolated. The identity of these products with those obtained from Hardigan seed oil was established, in the case of α-spinasterol and β-spinasterol, by means of a comparison of the physical constants of derivatives. In the case of δ-spinasterol, correspondence was established by means of analysis of the sterol and its acetate as well as by comparison of physical constants (melting point, rotation, and mixed melting points) of the sterol and several derivatives. Interestingly enough, δ-spinasterol, isolated from Grim alfalfa seed oil on catalytic hydrogenation, gave α-stigmastenol.

Experimental

1. Separation of Crude Sterols from the Unsaponi-

fiable Fraction.

Ninety-two grams of crude unsaponifiable from Grim alfalfa seed oil, prepared as directed by King and Ball[5], was fractionated in a manner analogous to that used for Hardigan alfalfa seed oil, Section I . Four solid fractions weighing 33 g. and melting from 120-152°, were obtained.

2. Fractionation of Crude Steryl Acetates from Cold Acetic Anhydride.

This operation was carried out in a manner similar to that used for Hardigan alfalfa seed sterols, Section I . This procedure gave 16 g. of insoluble acetates, m.p. 150-157°, and 1.5 g. of soluble acetates, m.p. 120-127°.

3. Separation and Identification of α -Spinasterol and β—spinasterol.

α-Spinasterol— The crude α -spinasterol fraction, 5.8 g., obtained by repeated recrystallization from 85% ethyl alcohol, was purified by recrystallization from methyl alcohol, from chloroform-methyl alcohol, and finally from 95% ethyl alcohol; m.p. 164-166°; $[\alpha]_D^{24} = -2.54°$ (51.5 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{24} = -0.131°$, <u>average</u> reading). A mixed melting point of this sterol and the corresponding substance from Hardigan alfalfa seed oil gave no depression.

α-Spinasteryl acetate— A quantity of the above sterol was dissolved in acetic anhydride and the mixture heated. The acetate was recovered in the usual way and recrystallized from 95% ethyl alcohol; m.p. 180-182°; $[\alpha]_D^{21} = -4.19°$ (51.0 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{21} = -0.214°$, average reading). A mixed melting point of this acetate and the corresponding substance from Hardigan seed oil gave no depression.

β-Spinasterol— The crude β-spinasterol fraction, 6.3 g., obtained as in Section I , was recrystallized from 95% ethyl alcohol; m.p. 147-149°; $[\alpha]_D^{21} = 4.21°$ (54.5 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{21} = 0.230°$, average reading). A mixed melting point of this sterol and the corresponding substance from Hardigan seed oil gave no depression.

β-Spinasteryl acetate— One gram of the above β-spinasterol was heated with acetic anhydride. The acetate was recovered as usual, and recrystallized from 95% ethyl alcohol. Yield 1 g.;

m.p. 155-159°; $[\alpha]_D^{21} = 4.081°$ (58.0 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{21} = 0.237°$, average reading). A mixed melting point of this acetate and the corresponding substance from Hardigan alfalfa seed oil gave no depression.

β-Spinasteryl benzoate— To 0.7 g. of the above β-spinasterol dissolved in 2 cc. pyridine, 1 cc. of benzoyl chloride was added. The reaction mixture was heated several hours on the steam bath. The benzoate was recovered in the usual way and recrystallized from 95% ethyl alcohol; m.p. 179-182°; $[\alpha]_D^{21} = 5.87°$ (50.5 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{21} = 0.297°$, average reading).

4. Fractionation of the Material Soluble in Cold Acetic Anhydride.

δ-Spinasterol— The crude acetates, 1.5 g., m.p. 120-127°, were saponified and the sterol recrystallized repeatedly from methanol; m.p. 143-145°; $[\alpha]_D^{19} = 5.97°$ (51.1 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{19} = 0.305°$, average reading).

Anal. 2.712 mg. gave 8.187 mg. CO_2; 2.917 mg. HOH
C, 82.33 ; H, 11.94
Calcd. for $C_{29}H_{47}OH \cdot \frac{1}{2}H_2O$ C, 82.58; H, 11.72

δ-Spinasteryl acetate— A quantity of the above sterol was dissolved in acetic anhydride and the mixture heated. The acetate was recovered in the usual way and recrystallized from 95% ethyl alcohol; m.p. 131-131.5°; $[\alpha]_D^{14} = 0.85^\circ$ (50.7 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{14} = 0.044^\circ$, <u>average</u> reading).

Anal. 2.169 mg. gave 2.254 mg. HOH; 6.502 mg. CO_2
C, 81.74 ; H, 11.54
Calcd. for $C_{31}H_{50}O_2$ C, 81.88 ; H, 11.097

Reduction of δ-spinasteryl acetate— Two hundred milligrams of the acetate was reduced with an atmosphere of hydrogen in the presence of Adam's catalyst. The product was recovered as previously described and recrystallized repeatedly from ethyl alcohol. Yield 50 mg.; m.p. 111-112°; $[\alpha]_D^{14} = 8.74^\circ$ (43.8 mg., 2 cc. chloroform, $\ell = 2$ dcm., α_D^{14} 0.383°, <u>average</u> reading). This acetate gave no depression in melting point when mixed with authentic α-stigmastenyl acetate.

Summary

Three isomeric sterols, α-spinasterol, β-spinasterol and δ-spinasterol, have been isolated from the unsaponifiable of Grim alfalfa seed oil.

V. Isolation and Composition of a Phytosterolin from Grim Alfalfa Seed Oil.

From Grim alfalfa seed oil a phytosterolin identical in all properties with the corresponding compound found in Hardigan alfalfa seed oil has been obtained. The acetate of this sterolin prepared with acetic anhydride and pyridine has a saponification equivalent of about 200 . From this value, after making allowance for the sterol residue, and for the minimum requirements of the possible polyhydroxy alcohols, it appears that the molecular weight of the acetate is approximately 800 . The sterolin is, therefore, a tetrahydroxy alcohol. This observation, when considered in connection with the other properties noted, indicates that the sterolin is a glycoside of a hexose sugar.

Experimental

The concentrated ether extracts of 28 kg. of Grim alfalfa seed gave 4.8 g. of crude phytosterolin. On recrystallization from n-amyl alcohol this substance gave a product which turned brown at 240° and decomposed at 260-270°. The product was slightly soluble in p-dioxane, ethyl ether, and n-amyl alcohol and easily soluble in hot acetic anhydride or acetyl chloride. It was nearly insoluble in most other common solvents.

Acetylation of the sterolin— To two grams of crude sterolin was added 30 cc. acetic anhydride and 15 cc. pyridine. The mixture was let stand 24 hours at room temperature and then filtered. The filtrate was decomposed with water and the precipitated acetate filtered off. This residue was washed thoroughly with water and recrystalllized from methyl alcohol. Yield 2 g.; m.p. 162-163°; $[\alpha]_D^{21} = -7.92^\circ$ (45 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{21} = 0.357^\circ$, <u>average</u> reading).

Saponification of the sterolin acetate— To 106 mg. of the above sterolin acetate weighed quantitatively into a 50 cc. Erlenmeyer flask, 20 cc. of ethyl alcohol containing about 100 mg. of potassium hydroxide was added. The mixture was refluxed 20 minutes and the excess potassium hydroxide titrated with standard acid to a phenolphthalein end point. An amount of potassium hydroxide equivalent to 5.5 cc. of 0.0952 N hydrochloric acid was used up in the reaction. The saponification equivalent calculated from this data was 202, the percentage acetyl (H_3C-CO-) was 21.2 .

The insoluble sterolin precipitated by the above saponification was collected quantitatively, washed with water, and with ethyl alcohol, and dried; yield 83.5 mg. The saponification equivalent calculated from this data was 197 . While the percentage acetyl (H_3C-CO-) was 21.7 . This latter value was calculated by means of the formula

$$\% \text{ acetyl} = \frac{106 - 83.5}{106} \times \frac{43}{42} \times 100$$

The recovered sterolin turned brown at 240° and decomposed at 260-270°.

The required values for the saponification number and percentage acetyl (H_3C-CO-) in the tetraacetate are 186 and 23.1% respectively, assuming the formula of the sterol hexoside tetraacetate to be $C_{43}H_{66}O_{10}$.

Hydrolysis of the sterolin— A mixture of 3.9 g. of this sterolin dissolved in 1.5 liters of boiling n-amyl alcohol and 200 cc. of 15% HCl solution[15], was refluxed 1 hour. The amyl alcohol was then removed by steam distillation, and the suspension extracted with ether. The ether extract was washed with sodium carbonate, then with water and the solvent evaporated. The brownish residue was taken up in 95% ethyl alcohol and the crude sterols allowed to crystallize.

α-Spinasterol— The crude sterols from hydrolysis of the sterolin were repeatedly recrystallized from 85% ethyl alcohol and twice from methanol. Yield 243 mg.; m.p. 164-165.5°; $[\alpha]_D^{26} = -2.56°$ (48.0 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{26} = -0.123°$, average reading).

α-Spinasteryl acetate— Seventy milligrams of the above sterol was reacted with acetic anhydride. The product was worked up as usual, and recrystallized from 95% ethyl alcohol. Yield 46.5 mg.; m.p. 177-181°; $[\alpha]_D^{27} = -4.09°$(46.5 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{27} = -0.190°$, average reading).

Observation of reducing substance— The water layer obtained from the above hydrolysis was neutralized with silver carbonate to a pH of 5 . The insoluble silver chloride was filtered off and the solution concentrated. The remaining aqueous solution reduced Benedict's solution. A portion of the solution was treated with phenylhydrazine, acetic acid and the mixture heated. A greenish-yellow crystalline osazone separated; m.p. about 197°.

Summary

1. A phytosterolin identical with the corresponding substance in Hardigan alfalfa seed oil, has been isolated from Grim alfalfa seed oil.
2. This substance had the properties of a tetrahydroxy alcohol and on acid hydrolysis gave a reducing substance and a sterol mixture containing α-spinasterol.

VI. The Sterols from the Unsaponifiable Fraction of Dutch White Clover Seed Oil.

Several authors[20,21,22,23,24] have made observations on the oil obtained from clover flowers and plants. Rogerson[25] mentioned the isolation of a phytosterol from Trifolium incarnatum while Finnemore et al.[26] observed a similar substance in Trifolium repens.

Fink and Richter[27] extracted the oil from Trifolium incarnatum seeds and determined its constants. As far as can be determined there is no other account of an investigation of clover seed oil.

About 8% of the weight of Dutch White clover seed may be extracted with ethyl ether as a clear yellow green oil and about 3.8% of the oil is unsaponifiable. The unsaponifiable portion consists of about 33% of crude sterols and of about 67% of reddish oily liquid.

A portion of the sterol fraction obtained from the unsaponifiable of Dutch White clover seed oil after recrystallization from 85% ethyl alcohol was acetylated and brominated. About 10% of the weight of crude sterols precipitated on standing as an insoluble tetrabromide. This substance on debromination and saponification gave stigmasterol.

Experimental

Fourteen kilograms of Dutch White clover seed was finely ground and extracted seven times with ethyl ether. The solvent was evaporated, giving 1073 g., or 7.6% of a yellow green oil. The fat constants of this oil determined by the usual methods[32] were as follows: Iodine number 133.2 , saponification number 186.6 , unsaponifiable 3.83%.

Preparation of the unsaponifiable fraction— Fifteen hundred grams of Dutch White clover seed oil was saponified in 200 gram lots by dissolving in 1500 cc. ethyl ether and slowly adding to the vigorously stirred mixture, a solution of freshly prepared sodium ethylate (made by dissolving 40 g. of sodium in about 500 cc. 95% ethyl alcohol). When the reaction was complete the ether was filtered from the soaps in a closed vessel. The solid soaps were extracted several times with ether and finally dissolved in 2-3 liters of water and extracted repeatedly with ether. The combined extracts were washed free of alkali and the solvent distilled off. Yield 58 g. of a reddish oil.

Separation of sterols— Fifty-eight grams of the above unsaponifiable fraction was dissolved in 600 cc. of ethyl ether and 5 crude sterol fractions separated out in a manner similar to that described for the separation of Hardigan alfalfa seed sterols. Yield 19 g.; m.p. 120-130°. The crude sterols were fractionated from 85% ethyl alcohol. A top fraction weighing 15 g., m.p. 136-137°, was obtained.

Bromination of the sterols— Ten grams of the sterol fraction, m.p. 136-137°, was heated with acetic anhydride. The acetates obtained were dissolved in 100 cc. ethyl ether and treated with 7.0 g. of bromine in glacial acetic acid (5 g. bromine per 100 cc. glacial acetic acid) as recommended by Windaus[28]. After standing over night in the refrigerator, the insoluble tetrabromides were filtered off and washed with cold ether. Yield 1.07 g.; m.p. 192-196°, with browning.

Stigmasteryl acetate— To 0.986 g. of the above tetrabromide, dissolved in 10 cc. glacial acetic acid, was added 1 g. of zinc dust, the mixture was refluxed 1 hour. The reaction mixture was filtered while hot, diluted with water and extracted with ethyl ether. The ether layer was washed with sodium carbonate and then with water and the solvent evaporated. The residue was recrystallized from 95% ethyl alcohol. Yield 342 mg.; m.p. 137-139°; $[\alpha]_D^{18} = -58.4^\circ$ (26.2 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{18} = -1.53^\circ$, average reading). A mixed melting point of this substance with authentic stigmasteryl acetate gave no depression.

Stigmasterol— A portion of the above acetate was saponified with alcoholic potassium hydroxide. The product was recrystallized from 95% ethyl alcohol and from methyl alcohol; m.p. 162-164°; $[\alpha]_D^{25} = -50.76^\circ$ (47.5 mg., 2 cc. chloroform, $l = 2$ dcm., $a_D^{25} = -2.411^\circ$, average reading).

Summary

1. The oil has been extracted from Dutch White clover seed and some of its common constants determined.
2. From the unsaponifiable fraction of this oil stigmasterol has been isolated.

VII. Isolation and Composition of a Phytosterolin from Dutch White Clover Seed Oil.

In 1910 Power and Salway[29] and later Rogerson[25] isolated a high melting alcohol from clover. They named the substance Trifolianol and characterized it as a dihydroxy alcohol. In 1913 Power and Salway[15] recognized Trifolianol as a sterol glycoside and suggested the term phytosterolin for alcohols of this type.

From the concentrated ether extracts of Dutch White clover seed a phytosterolin was isolated. This substance has properties which agree well with those given by Power and Salway[29] for Trifolianol. On acid hydrolysis this substance gave a sterol mixture from which stigmasterol was obtained.

Experimental

Twelve kilograms of Dutch White clover were extensively extracted with ethyl ether and the extracts concentrated to about 75% oil. On standing, 6 g. of a slightly colored material separated. It was filtered off, dissolved in n-amyl alcohol and the hot solution filtered. On standing, a white gelatinous precipitate settled out.
It was filtered off and the excess n-amyl alcohol

was removed by heating in vacuo; yield 4.82 g. It turned brown at 260° and decomposed at 280-290°. This compound gave tests for carbon and hydrogen only. It gave positive Lieberman-Burchard and positive Molisch tests.

Acetylation of the sterolin— About 1 g. of the above sterolin was heated with 50 cc. of acetic anhydride until the solid had all gone into solution. The product was recovered as usual, and recrystallized from methyl alcohol. Yield 1 g.; m.p. 161-163°; $[\alpha]_D^{21} = -26.3°$ (51.0 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{21} = -1.343°$, average reading).

Hydrolysis of the sterolin— Three grams of sterolin from Dutch White clover was suspended in 150 cc. ethanol containing 1 cc. of concentrated sulfuric acid and the mixture refluxed until complete solution was effected. The reaction mixture was worked up in the usual way; yield 2 g. crude sterols.

Bromination of sterols— The crude sterols weighing 2 g. were converted to the acetates. The acetates were dissolved in 40 cc. ethyl ether and treated with 2 g. of bromine in 40 cc. glacial acetic acid. The reaction mixture was placed in the refrigerator over night and the crude tetrabromides filtered off. Yield 390 mg.; m.p. 180-185°. On recrystallization from chloroform-ethyl alcohol the melting point improved to 190-195°.

Stigmasteryl acetate— A portion of the above tetrabromide fraction was debrominated in the usual way and the product recrystallized from ethyl alcohol; m.p. 136-138°; $[\alpha]_D^{24} = -54.6^\circ$ (44 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{24} = -2.406^\circ$, <u>average</u> reading).

Stigmasterol— A portion of the above acetate was saponified with alcoholic potassium hydroxide and the product recrystallized from ethyl alcohol; m.p. 159-161°; $[\alpha]_D^{21} = -49.5^\circ$ (34.5 mg., 2 cc. chloroform, $\ell = 2$ dcm., $\alpha_D^{21} = -1.711^\circ$, <u>average</u> reading).

Detection of a reducing substance— The aqueous layer from the above hydrolysis was heated as recommended by Thornton et al.[17]. A portion of this solution when neutralized and heated with Fehling's solution caused reduction of the copper solution.

Summary

1. A phytosterolin probably identical with Trifolianol[27] has been isolated from Dutch White clover seed oil.

2. This phytosterolin on acid hydrolysis gave a reducing substance and a sterol mixture from which stigmasterol was isolated.

VIII. The Sterols from the Unsaponifiable Fraction of Medium Red Clover Seed Oil.

Medium Red clover seed oil is very similar in properties to that obtained from Dutch White clover seed.

From the unsaponifiable fraction of Medium Red clover seed oil, a quantity of crude sterols was separated. A fraction, m.p. 135-136°, obtained from these sterols by recrystallizing from 85% ethyl alcohol, was acetylated and brominated. About 10% of the weight of crude sterols separated on standing as an insoluble tetra bromide. This substance on debromination and saponification gave stigmasterol.

Experimental

Extraction of oil— Thirteen and eight tenths kilograms of Medium Red clover seed was finely ground and extracted eight times with ethyl ether. The solvent was evaporated; yield 1250 g. of a greenish-yellow oil. The common fat constants of this oil were as follows; unsaponifiable 3.41%, iodine number 129.4, saponification number 187.6 .

Preparation of the Unsaponifiable fraction— Two kilograms of Medium Red clover seed oil was saponified in 200 g. lots in a manner similar to that described in Section VI; yield 55 g. of a reddish oil.

Separation of sterols— Sixty-five grams of the above unsaponifiable fraction was dissolved in 500 cc. of ethyl ether and 4 crude sterol fractions separated out in a manner similar to that described for the separation of Hardigan alfalfa seed oil sterols, Section I . Yield 28 g.; m.p. 130-136°. The crude sterols on fractionation from 85% ethyl alcohol gave a fraction weighing 10 g.; m.p. 135-136°.

Bromination of the sterols— One gram of the sterol fraction was acetylated using acetic anhydride; yield 0.9565 mg. The acetates obtained were dissolved in 20 cc. of ethyl ether and treated with 0.7 g. bromine in 15 cc. glacial acetic acid. The mixture was let stand over night in the refrigerator, and the insoluble tetrabromides filtered off and washed with ether; yield 96 mg. On recrystallization from chloroform-alcohol the product melted at 195° with browning.

Stigmasteryl acetate— The tetrabromide above was debrominated and the product recovered as in the case of the corresponding compound, Section VI. The product was recrystallized from 95% ethyl alcohol; m.p. 138-140°; $[\alpha]_D^{24} = -58.6^\circ$ (37.0 mg., 2 cc. chloroform, l = 2 dcm., $\alpha_D^{24} = -2.170^\circ$, average reading). This product gave no depression in melting point when mixed with an authentic specimen of stigmasteryl acetate.

Stigmasterol— The above acetate was hydrolyzed with alcoholic potassium hydroxide. The product was recovered in the usual way and recrystallized from ethyl alcohol; m.p. 160-162°; $[\alpha]_D^{24} = -49.2^\circ$ (38.5 mg., 2 cc. chloroform, l = 2 dcm., $\alpha_D^{24} = -1.904^\circ$, average reading). This substance gave no depression in melting point when mixed with authentic stigmasterol.

Summary

1. The common fat constants of Medium Red clover seed oil have been determined.
2. From the unsaponifiable fraction of Medium Red clover seed oil, stigmasterol has been isolated.

IX. Isolation and Composition of a Phytosterolin from Medium Red Clover Seed Oil.

From the concentrated ether extracts of Medium Red clover seed oil a phytosterolin has been isolated. This substance appears to be identical with Trifolianol[27] and with the corresponding compound isolated from Dutch White clover seed oil.

Experimental

Twenty-seven kilograms of Medium Red clover seed was repeatedly extracted with ethyl ether and the extracts concentrated to about 75% oil. On standing 3.5 g. of an isoluble compound separated. This was filtered off and recrystallized from n-amyl alcohol. Yield 2.8 g.; m.p. 285-295°, with decomposition. This substance gives a positive Lieberman-Burchard and a positive Molisch test.

Acetylation of the sterolin- About 1 g. of the above sterolin was acetylated at room temperature using acetic anhydride and pyridine. The product was recovered as usual and recrystallized from methanol; m.p. 162-163°; $[\alpha]_D^{28} = -28.5^\circ$ (50.5 mg., 2 cc. chloroform, $l = 2$ dcm., $\alpha_D^{28} = 1.444^\circ$, average reading).

Hydrolysis of the sterolin— About 1 g. of the Medium Red clover seed sterolin was suspended in 100 cc. of ethanol containing 1 cc. of concentrated sulfuric acid. The mixture was refluxed until complete solution was effected. The mixture was then diluted and the sterol fraction extracted with ether; yield 0.6 g.

Bromination of sterols— The crude sterols weighing 0.6 g. were converted to the acetates. The crude acetates were dissolved in ether and treated with 0.6 g. bromine in 10 cc. of glacial acetic acid. The crude tetrabromides settled out on standing over night. Yield 50 mg.; m.p. 185-190° . The crude product after recrystallization from chloroform-ethyl alcohol melted at 194-198°. This product gave no depression in melting point when mixed with crude stigmasteryl acetate tetrabromide, prepared from soybean sterols.

Detection of a reducing substance— The aqueous layer from the above hydrolysis was heated as recommended by Thornton et al.[17]. A portion of the resultant solution reduced Fehling's solution.

Summary

1. A phytosterolin probably identical with Trifolianol[27] has been isolated from Medium Red clover seed oil.
2. This phytosterolin on acid hydrolysis gave a reducing substance and a sterol mixture.
3. Bromination of the sterol mixture gave a product that appears to be stigmasteryl acetate tetrabromide.

X. Observations on the Reactions of Sterols of the Spinasterol Type with Halogens.

Several attempts have been made to prepare halogen derivatives of α-spinasterol[3,5,10], all of them resulted in oily decomposition products or in unreacted starting substances.

A preliminary study of the action of halogens on α-spinasterol, α-stigmastenol, β—spinasterol and δ-spinasterol indicated that steroid compounds which have double bonds in ring "C" near or at positions 8 and 14 are extensively decomposed by reaction with halogens. This decomposition was so consistant that a green color, which developed, was used to detect sterols of the spinasterol type. It is probable that the irregular results obtained by Eck[30] in his attempts to determine the number of double bonds in hydrocarbons related to cholestane, are in part due to this anomalous reaction of halogens with unsaturation in positions 8 and 14.

In a preliminary study it has also been observed that compounds of the spinasterol type react with perbenzoic acid in such a way that the amount of perbenzoic acid used depends on the excess reagent. It is possible that the anomalous results of Fernholz and Moore[6] for α-spinasterol and α-stigmastenol can be explained on this basis.

Experimental

Reaction of α-spinasteryl acetate with bromine— To 0.1 g. of α-spinasteryl acetate in 2 cc. ether was added 0.1 g. bromine in 2 cc. of glacial acetic acid and the mixture let stand at room temperature. After about 5 minutes the mixture began to darken rapidly. The same experiment was repeated except that the reaction was carried out with cold reagents and let stand in the ice box. After about 20 minutes a rapid darkening occurred. The same phenomena occurred if α-stigmastenyl acetate, β—spinasterol or δ-spinasterol were used.

Reaction of α-spinasteryl acetate with Hanus iodine solution— About 5 mg. of α-spinasteryl acetate was weighed quantitatively into a 10 cc. iodine flask, 0.2 cc. of chloroform, and a measured excess of Hanus iodine solution were added. After standing about 1 minute the mixture was observed to darken rapidly. It was let stand 30 minutes, treated with 2 cc. of saturated potassium iodide, diluted, and titrated with 0.0988 M sodium thiosulfate to a starch end point. This reaction was repeated several times with variation in the excess of Hanus iodine solution present during the reaction. The chloroform layer of the above titration mixture had a characteristic dark green color. β—Spinasteryl acetate, α -stigmastenyl acetate, δ -spinasteryl acetate and the free sterols as well react with Hanus iodine solution in the same manner.

Data

Data obtained for α -spinasteryl acetate and α -stigmastenyl acetate by means of the above prodecure are listed in Tables I and II .

For purposes of illustration the data given in Table I is recalculated in Table III so that the milliequivalents for halogen added for each gram of sample may be compared with the milliequivalents of halogen used by each gram of sample, and with the percentage excess halogen left at the end of the reaction. These values are plotted in Fig. 6 .

Summary

1. From preliminary investigation it appears that sterols which are unsaturated in position 8 and 14

react with halogens in such a way that the extent of the reaction is dependent on the excess of halogen present.

2. A characteristic green color remaining in the chloroform layer after addition of Hanus iodine solution and discharging the excess iodine color, was used as a test for sterols of the spinasterol type.

Table I

Data for the Reaction of α-Spinasteryl Acetate with various Excess Amounts of Hanus Iodine Solution

Weight of Sample mg.	cc. Hanus solution added	Titration cc. of 0.0988 M $Na_2S_2O_3$	I No. Calculated
4.14	.750	.997	163.0
0.0	.750	1.53	
4.176	.900	1.198	202.0
0.0	.900	1.862	
5.0782	.500	.665	95.8
0.0	.500	1.050	
5.148	.500	.660	95.1
0.0	.500	1.046	
4.413	.500	.687	103.5
0.0	.500		
5.5105	.500	.652	91.0
0.0	.500		

Table II

Data for the Reaction of α-Stigmastenyl Acetate with Various Excess Amounts of Hanus Iodine Solution.

Weight of Sample mg.	cc. Hanus solution added	Titration cc. of 0.0988 M $Na_2S_2O_3$	I No. Calculated
5.058	.3	.370	65.2
0.0	.3	.630	
4.805	.5	.620	105.0
0.0	.5	1.037	
5.070	.5	.650	97.6
0.0	.5	1.040	
5.026	.5	.637	101.8
5.0693	.3	.373	65.1
0.0	.3	.636	
5.0305	.3	.372	65.8
0.0	.3	.636	

Table III

Values for α-Spinasteryl Acetate, Calculated from Table I

These data are Plotted in Fig. 6

Weight of Sample mg.	Milli-equivalents of Halogen added per g. sample	Milli-equivalents of Halogen used per g. sample	% Halogen used	% Excess Halogen left
4.14	.036	.012	33%	200%
4.176	.044	.015	34%	193%
5.078	.0206	.0074	36%	179%
5.148	.0183	.0074	40%	147%
4.413	.0236	.0081	34%	192%
5.51	.0189	.0071	37%	166%

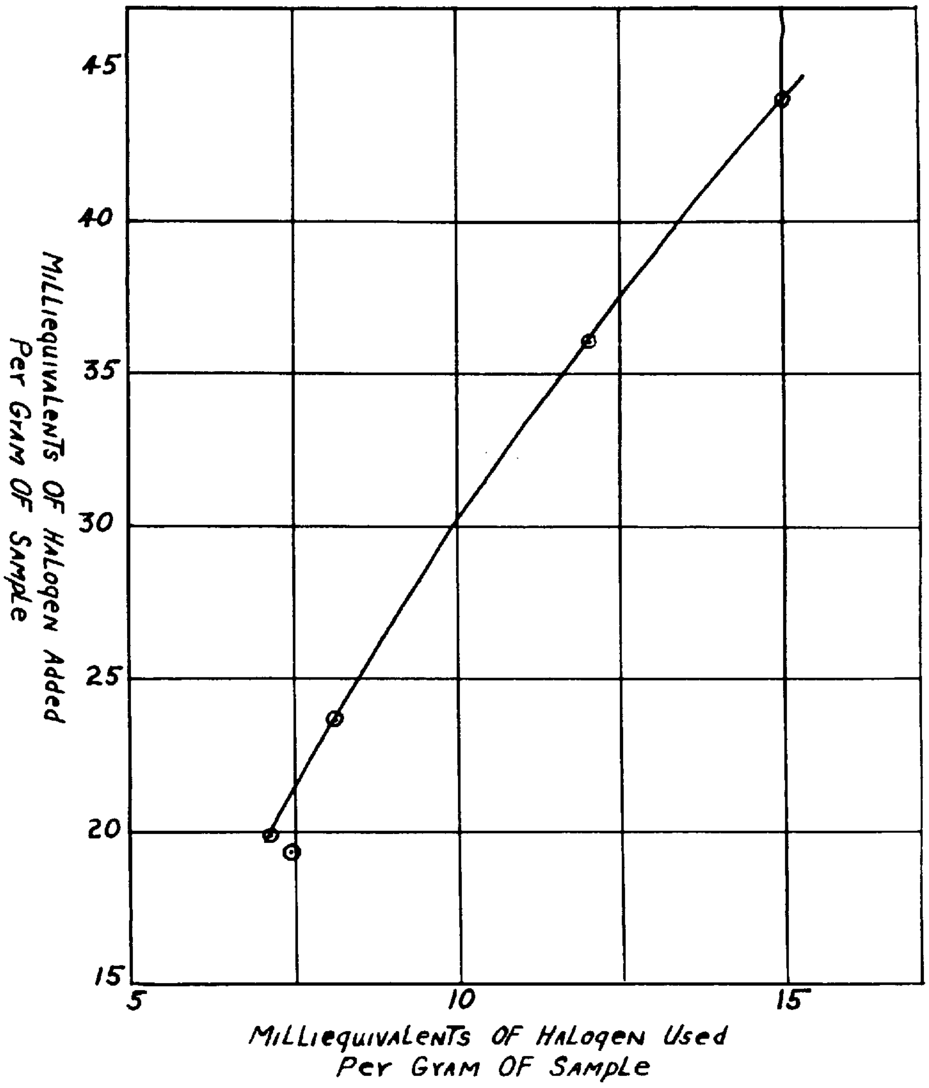

45
40
35
30
25
20
15
5
10
15
MilliequivalenTs OF Haloqen Added Per Gram OF Sample
MilliequivalenTs OF Haloqen Used Per Gram OF Sample

Fig. 6

XI. Discussion

1. A comparison of the Sterol Composition of Hardigan Alfalfa Seed Oil, Grim Alfalfa Seed Oil, Dutch White Clover Seed Oil and Medium Red Clover Seed Oil.

The seed used in this work was purchased from the Michigan State Farm Bureau and under their guarantee had the characterstics indicated in Table IV.

Certain fat constants of the oil obtained from each of these seeds by ethyl ether extraction are listed in Table V.

A comparison of Sections I, III, IV and V shows clearly that Grim alfalfa seed and Hardigan alfalfa seed are identical in steroid composition. To facilitate the comparison, the yields of the various fractions are tabulated in Tables VI and VII . This complete correspondence in steroid composition is the basis of the introductory statement, that the steroid composition is the same for different varieties within a single species. A comparison of Sections VI, VII, VIII and IX indicates that Medium Red clover seed and Dutch White clover seed are identical in steroid composition. The yields of the various fractions are tabulated in Tables VII and VIII. The correspondence in steroid composition is complete as far as the work has progressed and forms the basis of the introductory statement, that the sterol composition is the same for different species within a single genus.

The sterol composition of the seed of the genus Medicago (alfalfa) is obviously quite different from that of the genus Trifolium (clover). The former probably consists entirely of sterols of the spinasterol type, while the latter contains stigmasterol.

Table IV

	Hardigan Alfalfa	Grim Alfalfa	Medium Clover	Dutch White Clover
Lot No.	C9622	C9732	C9600	C9422
Purity	99.5%	90.02%	99.40%	99.15%
Crop Seed	.2%	.78%	.34%	.66%
Inert	.2%	.10%	.12%	.15%
Weed Seed	.1%	.10%	.14%	.04%
Where grown	Michigan	Michigan	Michigan	Wisconsin
When grown	1939	1939	1939	1939

Table V

	Hardigan Alfalfa	Grim Alfalfa	Medium Clover	Dutch White Clover
% Ether Extract	10.6	10.2	8.9	7.7
Iodine Number	172.15	172.0	129.4	133.2
Saponification Number	187.9	187.2	187.6	186.6
Unsaponifiable	4.32%	3.86%	3.41%	3.83%

Table VI

A Summary of Yields of the Various Fractions and Compounds Isolated from 3.75 kg. of Hardigan Alfalfa Seed Oil and from 2.67 kg. of Grim Alfalfa Seed Oil.

Fraction or Compound	Yields	
	Hardigan Alfalfa Seed Oil	Grim Alfalfa Seed Oil
Total unsaponifiable	150 g.	92 g.
Total crude sterols	47 g.	35 g.
α-Spinasterol isolated	11.1 g.	5.8 g.
β-Spinasterol isolated	18.7 g.	9.4 g.
δ-Spinasterol isolated	2.9 g.	1.5 g.
Yield of unsaponifiable material	4.0%	3.44%
Yield of crude sterols on the basis of oil saponified	1.2%	1.2%
on the basis of total unsaponifiable	31.0%	35.0%
Yield of α-spinasterol on the basis of oil saponified	0.29%	0.2%
on the basis of total unsaponifiable	7.4%	6.3%
on the basis of total crude sterols	23.0%	17.0%

Table VI (cont'd)

Fraction or Compound	Yields Hardigan Alfalfa Seed Oil	 Grim Alfalfa Seed Oil
Yield of β-spinasterol on the basis of oil saponified	0.5%	0.35%
on the basis of total unsaponifiable	12.0%	10.0%
on the basis of total crude sterols	39.0%	28.0%
Yield of δ -spinasterol on the basis of oil saponified	0.07%	0.05%
on the basis of total unsaponifiable	1.8%	1.6%
on the basis of total crude sterols	6.1%	4.5%

Table VII

Summary of the Yield of Phytosterolin from the Various Seeds

	Hardigan Alfalfa	Grim Alfalfa	Medium Red Clover	Dutch White Clover
Amount of seed extracted	28 kg.	28 kg.	27 kg.	14 kg.
Amount of phytosterolin obtained	4.9g.	5.8 g.	2.79 g.	4.82 g.
Yield of phytosterolin on the basis of seed	0.018%	0.02%	0.01%	0.034%
Yield of phytosterolin on the basis of oil.	0.17%	0.19%	0.112%	0.44%

Table VIII

A Summary of the Various Fractions and Compounds Isolated from 1500 g. of Dutch White Clover Seed Oil, and from 2000 g. of Medium Red Clover Seed Oil.

Fraction or Compound	Yields	
	Dutch White Clover Seed Oil	Medium Red Clover Seed Oil
Total unsaponifiable	58 g.	65 g.
Total crude sterols	19 g.	28 g.
Sterols brominated	10 g.	1.0 g.
Total crude tetrabromides	1.07 g.	0.096 g.
Yield of unsaponifiable	3.86%	3.25%
Yield of crude sterols based on oil saponified	1.2%	1.4%
based on total unsaponifiable	33.0%	43.0%
Yield of crude stigmasteryl acetate tetrabromide based on sterols brominated	10.7%	9.09%

2. A Consideration of the Structure and Homogeneity of Sterols of the Spinasterol Type.

In this work the isolation of a new sterol which was called δ-spinasterol was described. It should be noted that the ordinary criteria of purity, such as constancy of melting point, recovery of original products from derivatives and so forth, are not completely reliable in the steroid field. The tendency of these compounds to form mixed crystals of constant properties makes the recognition of a chemical individual very uncertain.

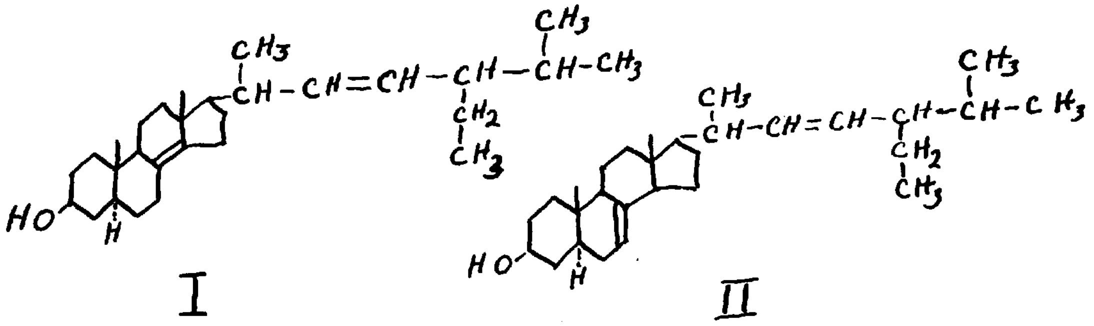
Recently Bernstein, Kauzmann and Wallis[31] have formulated a working method for calculating the specific rotation of steroids from the structures assigned. The extension and revision of the idea will probably provide an excellent criterion for the establishment of the structure and "einheitlichkeit" of members of the steroid group. In connection with this, it is of interest to note that the formula for α-spinasterol I, proposed by Fernholz and Ruigh[11] cannot be correlated with the specific rotation calculated according to Bernstein et al.[31]. The calculated rotation using the constants of these authors is:

$$\boxed{\alpha}_D = \frac{[M]_D}{\text{mol. weight}} = \frac{C + Nt D_{8:14} + \text{stig } D_{22:23}}{\text{mol. weight}}$$

or

$$\boxed{\alpha}_D = \frac{9160 + (-1260) + (-4530)}{412} = 9.2^\circ$$

whereas the observed value is - 2.7°.


I

II

On the other hand the specific rotations of β-spinasterol, 5.91°, and of δ-spinasterol, 6.15°, are in fair agreement with the value calculated for I .

The bessisterol isolated by Kuwada and Yosiki[8] agrees closely in properties[7] with α-spinasterol except that it has a specific rotation of - 13°.

Now the calculated rotation of the structure II is:

$$[\alpha]_D = \frac{[M]_D}{\text{mol. weight}} = \frac{C + Nt\,D_{7:8} + Stig\,D_{22:23}}{\text{mol. weight}}$$

or

$$[\alpha]_D = \frac{9160 + (-9160) + (-4530)}{412} = -10.9^{\circ}$$

This value is in fair agreement with the observed value for bessisterol.

It seems possible that bessisterol in II and either β spinasterol or δ -spinasterol is I , and that α -spinasterol as ordinarily isolated is a mixture of I and II . However all attempts in this laboratory to separate α -spinasterol into two such components failed.

Bibliography

1. Sobotka, Chemistry of the Sterids, page 86, Willians and Wilkins, Baltimore (1938).

2. Heyl, Wise and Speer, J. Biol. Chem. 82, 111 (1929).

3. Simpson, J. Chem. Soc. 730 (1937).

4. Dam, Geiger, Glavind, Karrer, Karrer, Rothschild and Solomon, Helv. Chim. Acta. 22, 310 (1939).

5. King and Ball, J. Am. Chem. Soc. 61, 2910 (1939).

6. Fernholz and Moore, J. Am. Chem. Soc. 61, 2467 (1939).

7. Kuwada and Yosiki, J. Pharm. Soc. (Japan) 60, 161 (1940).

8. Kuwada and Yosiki, J. Pharm. Soc. (Japan) 57, 155 (1937).

9. Kuwada and Yosiki, J. Pharm. Soc. (Japan) 59, 282 (1939).

10. Larsen and Heyl, J. Am. Chem. Soc. 56, 2663 (1934).

11. Fernholz and Ruigh, J. Am. Chem. Soc. 62, 2341 (1940).

12. Heyl and Larsen, J. Pharm. Assoc. 22, 510 (1933).

13. Hart and Heyl, J. Biol. Chem. 95, 311 (1932).

14. Gilman and Blatt, Organic Synthesis, Collective Volume I . 2nd ed., page 463, John Wiley and Sons, New York.

15. Power and Salway, J. Chem. Soc. 103, 399 (1913).

16. Jantzen and Gohdes, Biochem. Z. 272, 166 (1934).

Bibliography (cont'd)

17. Thornton, Kraybill and Mitchell, J. Am. Chem. Soc. 62, 2006 (1940).

18. Thornton, Kraybill and Broome, J. Am. Chem. Soc. 63, 2079 (1941).

19. Moore and Link, J. Biol. Chem. 133, 293 (1940).

20. Mitumaru Tuzimoto, J. Soc. Chem. Ind., (Japan) 43, suppl. binding 208 (1940). C. A. 34, 7974 (1940).

21. Guido Colombo, Boll. ufficiale staz. sper. seta 4, 57 (1934). C. A. 29, 1849 (1935).

22. Kirk, J. Am. Soc. Agron. 18, 385 (1926).

23. Dunbar and Wells, J. Oil and Fat Ind. 382 (1926).

24. Stoeckert, Oel und Fettind (1920) 61. C. A. 15, 2991 (1921).

25. Rogerson, J. Chem. Soc. 97, 1004 (1910).

26. Finnemore, Cooper, Stanley, Cobcroft and Harris, J. Soc. Chem. Ind. 57, 162 (1938).

27. Fink and Richter, Časopis Českoslov Lékarnictva 17, 262 (1937). C. A. 32, 1501 (1938).

28. Windaus and Hauth, Ber. 39, 4378 (1906).

29. Power and Salway, J. Chem. Soc. 97, 231 (1910).

30. Eck and Hollingsworth, J. Am. Chem. Soc. 64, 140 (1942).

31. Bernstein, Kauzmann and Wallis, J. Organic Chem. 6, 319 (1941).

32. Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists. 4th ed., 1935. Association of Official Agricultural Chemists. Washington D.C.