

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Sterols of Rye Germ Oil

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Early investigators found indications of a marked similarity in the sterols which they isolated from wheat and rye germ oils. Interest in the unsaponifiable matter of the latter, however, has not kept pace with that aroused by the former for the sterols of wheat germ oil have been extensively investigated since the turn of the century. For this reason it was deemed desirable to re-investigate the nature of the sterols of rye germ oil; particularly in the light of the newer knowledge² that the principal phytosterol, sitosterol, is indeed a mixture of several sterols. These are now designated, in the order of the insolubility in the common non-aqueous solvents, as γ -, β -, and α -sitosterol.

γ -Sitosterol, which is the least soluble of this group, was isolated by Anderson and co-workers² from corn and wheat germ oils. Wallis and Chakravorty³ found that the β -form is the major component of the sterol fraction of cottonseed oil and Ichiba⁴ isolated it from wheat germ oil. Wallis and Fernholz⁵ succeeded in separating from the most soluble sterol fraction of wheat germ oil two components which they designated as α_1 - and α_2 -sitosterol, respectively. We have succeeded in isolating from rye germ oil not only those sterols which are known to be characteristic of wheat germ oil, or α_1 -, β -, and γ -sitosterol, but also a hitherto unreported compound which we tentatively designated as α_3 -sitosterol.

α_1 -Sitosterol was isolated from the most soluble sterol fraction by taking advantage of the fact, as did Wallis and Fernholz,⁵ that the corresponding *m*-dinitrobenzoates differ greatly in solubility, the α_1 -sitosterol derivative having the lesser solubility. They isolated α_2 -sitosteryl *m*-dinitrobenzoate from the mother liquor. We found no evidence for the presence of this sterol in rye germ oil. In its place there was isolated a sterol-like compound which we have named α_3 -sitosterol. Combustion data on the dinitro-

benzoate indicate the empirical formula $C_{36}H_{50}N_2O_6$ which corresponds to $C_{29}H_{48}O$ for the free sterol. Perbenzoic acid titrations indicate the presence of two double bonds, thereby making α_3 -sitosterol an isomer of stigmasterol and also of α_1 -sitosterol.

α_1 - and α_3 -sitosterol give the Liebermann-Burchard color reaction, the final color being a dark purple. The Salkowski reaction for both is similar to that of ergosterol in that the sulfuric acid layer becomes colored while the chloroform layer remains colorless. The Rosenheim color reaction with chloroacetic acid is negative for both. α_1 - and α_3 -sitosterol are precipitated by digitonin.

Dihydrositosterol, which has been reported in as much as from 1–1.5% yields in wheat germ oil, has not been isolated from rye germ oil in this study. Although the specific rotation could be reduced from -31.53 to -8.41 by recrystallization, nevertheless the amounts present were too small to isolate by either the Schöenheimer⁶ or the Anderson-Nabenhauer⁷ methods.

Experimental

Approximately 12 kg. of rye oil was obtained by extracting thoroughly sifted, cleaned rye germ stock⁸ with ethyl ether, first in the cold and then in a continuous extractor. An ether extraction of the saponified oil yielded some 800 g. of orange-colored unsaponifiable matter.

The sterol fraction was separated from the non-crystalline fraction with ice-cold petroleum ether yielding 474 g. of crude sterol, which is equivalent to 58% of the unsaponifiable matter. The crude sterol was submitted to a systematic triangle scheme of crystallization from alcohol and benzene in which, during the course of 115 recrystallizations, 12 fractions were obtained.

Isolation of γ -Sitosteryl Acetate.—The third and fourth top fractions (m. p. 141–142°, $[\alpha]^{27D} -31.26$; m. p. 140–141°, $[\alpha]^{27D} -30.68$) were acetylated and then brominated.⁹ A small amount of material precipitated. It was debrominated by refluxing its alcohol solution with zinc and acetic acid, after which it was recrystallized from alcohol. The melting point, 138–139°, $[\alpha]^{24D} -41.51$, showed that it was not stigmasteryl acetate.

The dibromide from the filtrate of the original brominated solution was precipitated, by the addition of water,

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(2) R. J. Anderson, R. L. Shriner and G. O. Burr, *THIS JOURNAL*, **48**, 2897 (1926); see also Anderson and Shriner, *ibid.*, **48**, 2976.

(3) E. S. Wallis and P. N. Chakravorty, *J. Org. Chem.*, **2**, 335 (1937).

(4) A. Ichiba, *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **28**, 112 (1935).

(5) E. S. Wallis and E. Fernholz, *THIS JOURNAL*, **58**, 2446 (1936).

(6) R. Schöenheimer, *Z. physiol. Chem.*, **192**, 81 (1930).

(7) R. J. Anderson and F. P. Nabenhauer, *THIS JOURNAL*, **46**, 1957 (1924).

(8) Acknowledgment is made to the Messrs. Frank H. Blodgett, Inc., of Janesville, Wisconsin, who contributed some 700 pounds of rye germ stock for this investigation.

(9) A. Windaus and A. Hauth, *Ber.*, **39**, 4378 (1906).

debrominated, and recrystallized sixteen times from alcohol. The remaining material, which melted at 143–143.5°, $[\alpha]^{25D} -45.02$, corresponded to pure γ -sitosteryl acetate (26.24 mg. in 2 cc. of chloroform in a 2-dm. tube gave $\alpha^{25D} -1.094$).

Anal. Calcd. for $C_{31}H_{52}O_2$: C, 81.51; H, 11.48. Found: C, 81.62; H, 11.45.

γ -Sitosterol.—The acetate was hydrolyzed with a 2% alcoholic solution of potassium hydroxide, precipitated with water and recrystallized twice from alcohol, yielding the free sterol. It melted at 146°, $[\alpha]^{24D} -42.02$ (21.29 mg. in 2 cc. of chloroform).

Anal. Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.16. Found: C, 83.96, 84.03; H, 12.33, 12.47.

Isolation of β -Sitosteryl Benzoate.—The three bottom or most soluble fractions, weighing 63 g. (m. p. 139.5–140°, $[\alpha]^{27D} -17.90$; m. p. 139.5–140°, $[\alpha]^{27D} -17.00$; m. p. 139.5–140°, $[\alpha]^{24D} -17.66$), were dissolved in 260 cc. of pyridine. To this solution there was added benzoyl chloride (55 cc.), after which the mixture was heated on a boiling water-bath for one hour. After working up the product in the usual manner, the benzoate was four times recrystallized. It melted at 146–147°; $[\alpha]^{24D} -13.75$ (30.54 mg. in 2 cc. chloroform in a 2-dm. tube gave $\alpha^{24D} -0.389$).

Anal. Calcd. for $C_{30}H_{50}O_2$: C, 83.33; H, 10.50. Found: C, 83.28; H, 10.61.

β -Sitosterol.—The benzoate was hydrolyzed and the liberated sterol precipitated as before. On recrystallization from alcohol it melted at 137–137.5°; $[\alpha]^{24D} -36.66$ (23.82 mg. in 2 cc. of chloroform).

Anal. Calcd. for $C_{29}H_{50}O$: C, 83.98; H, 12.16. Found: C, 84.03; H, 12.26.

Isolation of α_1 -Sitosteryl *m*-Dinitrobenzoate.—In the mother liquor from the first crystallization of β -sitosteryl benzoate there remained the most soluble corresponding α -derivative. These benzoates were saponified with alcoholic potassium hydroxide solution. Conversion to the *m*-dinitrobenzoates was effected by dissolving the free sterol in 110 cc. of pyridine and adding 15 g. of *m*-dinitrobenzoyl chloride. This mixture was kept on a boiling water-bath for two hours, after which it was worked up in the usual manner. The crude dinitrobenzoate was recrystallized four times from ethyl acetate, yielding 0.8 g. of very insoluble, tiny needles of m. p. 225°; $[\alpha]^{25D} +37.05$ (19.16 mg. in 2 cc. of chloroform).

Anal. Calcd. for $C_{30}H_{50}N_2O_4$: C, 71.24; H, 8.31; N, 4.62. Found: C, 71.12; H, 8.32; N, 4.67.

α_1 -Sitosterol.—The dinitrobenzoate was hydrolyzed and the liberated sterol precipitated as before. On recrystallization from alcohol it melted at 163°; $[\alpha]^{25D} -1.84$ (26.42 mg. in 2 cc. of chloroform).

Anal. Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73. Found: C, 83.93¹⁰; H, 11.68.

α_1 -Sitosteryl Benzoate.—The free sterol was converted to the benzoate in pyridine solution in the usual manner. On recrystallization from alcohol it had a melting point of 167–168°; $[\alpha]^{26D} +40.77$ (22.69 mg. in 2 cc. of chloroform).

(10) These sterols apparently retain a small amount of solvent of crystallization, thereby lowering the value for the percentage of carbon.

Anal. Calcd. for $C_{30}H_{52}O_2$: C, 83.68; H, 10.15. Found: C, 83.62; H, 10.42.

Titration with Perbenzoic Acid.—To 19.56 and 26.14 mg. of α_1 -sitosteryl *m*-dinitrobenzoate there was added an excess of a chloroform solution of perbenzoic acid. After standing in the refrigerator at -7° for fifty-six hours there was added 5 cc. of a 10% potassium iodide solution, and the liberated iodine titrated with 0.02007 *N* sodium thiosulfate solution. The compound consumed 0.98 and 1.32 mg. of oxygen, respectively. The theoretical amount for 2 atoms of oxygen is 1.03 and 1.37 mg., respectively. These results correspond to 1.91 atoms of oxygen consumed, thus indicating the presence of two double bonds.

Isolation of α_3 -Sitosteryl *m*-Dinitrobenzoate.—To the concentrated mother liquor from the first crystallization of α_1 -sitosteryl *m*-dinitrobenzoate alcohol was added, while the solution was being boiled, until needles began to crystallize out. The material thus obtained (m. p. 183–184°) was recrystallized five times from ethyl acetate and alcohol, and twice from acetone, after which the melting point and the specific rotation remained unchanged. The yield of pure α_3 -sitosteryl *m*-dinitrobenzoate was increased by concentrating and recrystallizing the material in the mother liquors from α_1 -sitosteryl *m*-dinitrobenzoate, and by giving similar treatment to the material from the mother liquors obtained in recrystallizing the crude α_3 -dinitrobenzoate. The total yield was 3 g. The product had a melting point of 202.5–203°; $[\alpha]^{27D} +15.35$ (31.69 mg. in 2 cc. of chloroform).

Anal. Calcd. for $C_{30}H_{50}N_2O_4$: C, 71.24; H, 8.31; N, 4.62. Found: C, 71.23, 71.04; H, 8.32, 8.55; N, 4.72.

α_3 -Sitosterol.—The dinitrobenzoate was hydrolyzed and the liberated sterol precipitated as before. On recrystallization from alcohol it melted at 142°; $[\alpha]^{25D} +1.65$ (25.72 mg. in 2 cc. of chloroform).

Anal. Calcd. for $C_{29}H_{48}O$: C, 84.38; H, 11.73. Found: C, 83.65, 83.81¹⁰; H, 11.55, 11.64.

α_3 -Sitosteryl Benzoate.—The free sterol was converted to the benzoate in pyridine solution in the usual manner. On recrystallization from alcohol it melted at 167.5–168°; $[\alpha]^{28D} +14.85$ (22.20 mg. in 2 cc. of chloroform).

Anal. Calcd. for $C_{30}H_{52}O_2$: C, 83.68; H, 10.15. Found: C, 83.65; H, 9.89.

Titration with Perbenzoic Acid.—To 17.42 and 28.16 mg. of α_3 -sitosteryl *m*-dinitrobenzoate was added an excess of a chloroform solution of perbenzoic acid. After standing in the refrigerator at -7° for a week there was added 5 cc. of a 10% potassium iodide solution, and the liberated iodine titrated with 0.02007 *N* sodium thiosulfate solution. The compound consumed 0.96 and 1.24 mg. of oxygen, respectively. The theoretical amount for two atoms of oxygen is 0.92 and 1.47 mg., respectively. These results correspond to 2.10 and 1.70 atoms of oxygen consumed, giving an average of 1.90 atoms of oxygen. These results, although not as good as those obtained with α_1 -sitosteryl *m*-dinitrobenzoate, indicate the presence of two double bonds in this sterol.

Attempted Isolation of Dihydrositosterol.—The two top fractions were investigated for the presence of dihydrositosterol and stigmasterol. These fractions, $[\alpha]^{27D}$

-28.62, and $[\alpha]^{27D}$ -28.06, respectively, were brominated by the Schönheimer⁶ method. To the reaction mixture was added a one per cent. solution of digitonin. A small amount of digitonide precipitated, which upon decomposition with pyridine¹¹ yielded a sterol of somewhat lower rotation, $[\alpha]^{24D}$ -21.30. This value seemed to indicate that dihydrositosterol, if present, would be found only in very small amount.

The dibromide still in solution was precipitated with water, debrominated, and then acetylated. The acetyl derivative was recrystallized ten times in the hope of concentrating the dihydrositosterol, if present, in the top fraction. It was then brominated,⁹ but since no precipitate of insoluble stigmasterol tetrabromide was formed, the presence of this sterol was eliminated. The dibromide was precipitated with water and debrominated according to the method of Anderson and Nabenhauer.⁷ The regenerated acetyl derivative was dissolved in carbon tetrachloride and treated twenty times with portions of acetic anhydride and sulfuric acid. There was only a slight color reaction after these treatments. The carbon tetrachloride was evaporated but only a small amount of brown resinous mass remained from which no crystals of dihydrositosterol could be obtained.

A separate sample of 33 g. of crude sterol (m. p. 138-139°; $[\alpha]^{25D}$ -31.53) was recrystallized twenty-three

(11) R. Schönheimer and H. Dam, *Z. physiol. Chem.*, **215**, 59 (1933).

times, after which it had a melting point of 137°; $[\alpha]^{24D}$ -8.41. This sample was now dissolved in alcohol, brominated by the Schönheimer method⁶ and an excess of digitonin solution added. However, no insoluble digitonide was formed.

The fact that the specific rotation could be lowered to -8.41 and that the rotation of the two top fractions was lowered by treatment with digitonin would indicate the possibility that small amounts of dihydrositosterol were present. However, these amounts were too small for isolation.

Summary

A new doubly unsaturated sterol which is an isomer of stigmasterol has been isolated in a pure state from the most soluble sterol fraction of rye germ oil.

The presence of α_1 -, β -, and γ -sitosterol has been demonstrated.

The absence of α_2 -sitosterol and stigmasterol has been shown.

An analysis for dihydrositosterol failed to yield the pure compound by either the Schönheimer⁶ or Anderson-Nabenhauer⁷ method.

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RECEIVED JUNE 12, 1939

[CONTRIBUTION FROM THE FRICK CHEMICAL LABORATORY, PRINCETON UNIVERSITY]

Studies in the Sitosterol Complex. The Isolation of α_3 -Sitosterol

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That sitosterol is in reality a mixture of several sterols was first shown by Anderson and his co-workers.² These investigators showed that sitosterol obtained from various sources contains dihydrositosterol and at least three other sterols which they named α -, β - and γ -sitosterol.

In 1936 Wallis and Fernholz³ showed that the most soluble fraction of the sitosterol complex from wheat germ oil named by Anderson and his co-workers α -sitosterol was in reality a mixture of at least two new sterols α_1 - and α_2 -sitosterol. A detailed method of their separation and isolation was given, and certain properties of these sterols and their derivatives were listed.

In this paper we wish to report the results of certain experiments which show that a third sterol, α_3 -sitosterol, is also present in the sterol complex from wheat germ oil. Our work in this

connection was prompted by the fact that for continuance of our studies on the structure of α_1 -sitosterol, the results of which will be reported shortly in another paper, it was necessary to obtain further quantities of this interesting material. Following the procedure described by Wallis and Fernholz,³ we obtained from recrystallization of 1627 g. of a sitosterol from wheat germ oil, 6.5 g. of α_1 -sitosteryl *m*-dinitrobenzoate and 6.0 g. of the more soluble fraction which in melting point corresponded to α_2 -sitosteryl *m*-dinitrobenzoate. Hydrolysis of this latter fraction and conversion into its acetate gave a product from which not only α_2 -sitosteryl acetate (m. p. 126°, $[\alpha]_D +17$) was isolated but also there was obtained in a pure condition 1.0 g. of a new acetate, α_3 -sitosteryl acetate (m. p. 152-153° $[\alpha]^{20D} +6.1$).

Several derivatives of this new sterol were prepared and their properties together with those of α_1 - and α_2 -sitosterol are given in Table I.

(1) Research Assistant on Special Funds from the Rockefeller Foundation.

(2) Anderson, Shriner and Burr, *THIS JOURNAL*, **48**, 2987 (1926). See also Anderson, *ibid.*, **46**, 1450 (1924).

(3) Wallis and Fernholz, *ibid.*, **58**, 2446 (1936).