[CONTRIBUTION FROM THE BIOCHEMICAL LABORATORY, NEW YORK AGRICULTURAL EXPERIMENT STATION]

THE SEPARATION OF UNSATURATED FROM SATURATED STEROLS

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Introduction

It has been shown in earlier papers from this Laboratory that the endosperm of corn[1] and wheat[2] contains a mixture of unsaturated and saturated sterols consisting of sitosterol and dihydrositosterol. A nearly complete separation of these sterols can be made by fractional crystallization from alcohol provided that a sufficient quantity of the crude unsaponifiable matter is available. The top fraction becomes richer in the saturated dihydrositosterol because it is less soluble than sitosterol. The complete removal of sitosterol has not been possible by this method because the last traces adhere to the dihydrositosterol with great tenacity or possibly the two sterols form mixed crystals in any proportion. Separation by recrystallization is therefore not only incomplete but it necessitates very tedious and laborious operations and requires large amounts of material.

We first attempted to remove the sitosterol after brominating the mixed acetyl derivatives. The dihydrositosterol does not absorb bromine and the dibromositosterol is quite soluble in alcohol. It was found to be practically impossible, however, by any reasonable number of recrystallizations to remove all of the bromine-containing compound. Bondzynski and Humnicki[3] were able to separate coprosterol and cholesterol after brominating a mixture of the two by digesting the resulting product in petroleum ether which dissolved only coprosterol. This method is useless in our case because dibromositosterol is perceptibly soluble in petroleum ether.

In studying the Liebermann-Burchard reaction[4] we became convinced that only the unsaturated sterols play any part in the formation of the color that is produced. In fact, a compound is formed between the sulfuric acid and the unsaturated sterol and when acetic anhydride is present it gives a deep blue or green color. This colored compound remains dissolved in the mixture of chloroform and acetic anhydride when the concentration is as low as it always is when this test is applied. When larger amounts of the unsaturated sterols are present, however, the entire quantity of the colored compound can be removed from the chloroformic solu-

[1] Anderson, THIS JOURNAL, **46**, 1450 (1924).

[2] Anderson and Nabenhauer, *ibid.*, **46**, 1717 (1924).

[3] Bondzynski and Humnicki, *Z. physiol. Chem.*, **22**, 396 (1896–7).

[4] Liebermann, *Ber.*, **18**, 1804 (1885). Burchard, *Chem. Centr.*, **1890**, 1, 25.

tion by the addition of a few drops of water. The mixture of acetic anhydride and sulfuric acid that settles to the bottom contains nearly all of the coloring matter in solution. In applying the Liebermann-Burchard reaction for the removal of unsaturated sterols, carbon tetrachloride may be substituted for chloroform and for this purpose we have found it to be a more suitable solvent.

Similar observations have been reported by Whitby[5] in his studies of sterol reactions. This author separated some of the colored compounds but applied the reaction for the detection and not for the separation of sterols.

We have not been able so far to isolate the reaction product between the unsaturated sterol and sulfuric acid in pure form. It forms an intensely dark green or bluish-green solution in the concd. sulfuric acid and on dilution with water it yields a clear grass-green solution. When this is neutralized or made alkaline with sodium hydroxide a clear yellow solution is produced.

The saturated sterol remains in the chloroformic or carbon tetrachloride solution. It exists in this solution partly as the acetyl derivative and probably in part as an ethereal sulfate. After saponification with alcoholic potassium hydroxide the free sterol is obtained.

The method may be of interest in separating unsaturated from saturated cholesterols if such mixtures should be found to occur in the animal body. It might also be used in separating cholesterol from coprosterol. Unfortunately we have not had any coprosterol at our disposal and have not been able to study this question.

Experimental Part

In carrying out the Liebermann-Burchard reaction on samples of the dextrorotatory sterol obtained from corn or wheat endosperm we observed several times, when the reaction mixture was allowed to stand until the chloroform had evaporated, that colorless crystals had separated on top of the dark green sulfuric acid layer. The fact that the intensity of coloration in the Liebermann-Burchard reaction decreased as the dextrorotatory sterol became more highly purified until only a faint blue color was produced led us to believe that the unsaturated sitosterol was responsible for the color reaction.

Preliminary experiments indicated that the unsaturated sitosterol could be easily removed from a mixture containing the dextrorotatory sterol. A mixture of the two sterols was dissolved in chloroform and the solution contained in a small separatory funnel was mixed with acetic anhydride. On the addition of concd. sulfuric acid a beautiful display of colors was observed; a momentary rose-red changed quickly into purple, deep blue and gradually into dark green. At the same time a considerable amount of sulfur dioxide was liberated. After the solution had been shaken for some

[5] Whitby, *Biochem. J.*, **17**, 5 (1923).

time a few drops of water were added; this caused the homogeneous liquid to separate, and on standing the mixture of acetic anhydride and sulfuric acid, containing the coloring matter in solution, settled to the bottom and was drawn off. The chloroformic solution retained some green color which was removed by repeating the treatment with acetic anhydride and sulfuric acid. The chloroform, after it had been washed with water, was distilled and the residue was saponified with alcoholic potassium hydroxide and finally recrystallized from alcohol. In the Liebermann-Burchard reaction the substance gave no coloration.

Chloroform is not an ideal solvent in the reaction outlined above. A permanent emulsion forms in washing when the acid-chloroform mixture is shaken with water. Even when the separatory funnel is gently rotated more or less emulsion forms which separates very slowly. We obtained, nevertheless, rather satisfactory results with this method when chloroform was used as solvent as is indicated in the examples given below. 1.6 G. of corn gluten sterols having $[\alpha] + 20.54°$ gave after the treatment described above 0.84 g. of substance that melted between 138° and 139° and had $[\alpha]$ +25.04°. Of another preparation from corn gluten, $[\alpha]_D^{20}$, +7.87°, 2.7 g. gave 1.28 g. of snow-white crystals that had $[\alpha]$ +23.74°.

Carbon tetrachloride was found to be a more suitable solvent in carrying out this reaction. The density of carbon tetrachloride is greater than that of chloroform and in consequence the sulfuric acid layer rises to the top of the solvent, thus permitting a cleaner separation of the two liquids.

The procedure outlined below was found to be quite satisfactory. Three g. of one of the middle fractions of sterols isolated from wheat bran, $[\alpha]_D^{20}$, +10.41°, was dissolved in 50 cc. of carbon tetrachloride. The solution was placed in a separatory funnel with 15 cc. of acetic anhydride and gradually 15 cc. of concd. sulfuric acid was added while the mixture was shaken and cooled in cold water. The color changed to rose-red and quickly into purplish-blue, while a considerable amount of sulfur dioxide was liberated. After the solution had stood for a few minutes a little water was added, a few drops at a time, while shaking and cooling in cold water were continued. The mixture was then allowed to stand until the sulfuric acid and acetic anhydride, containing the coloring matter in solution, had formed a thick, purplish layer on the carbon tetrachloride, while the latter solvent retained only a faint blue color. The carbon tetrachloride was drawn into another separatory funnel and was washed thrice with water. The first wash water retained all of the blue pigment, leaving the organic solvent nearly colorless. The carbon tetrachloride was distilled and the residue saponified with alcoholic potassium hydroxide, diluted with water and extracted with ether. The ether was distilled and the residue was dissolved in alcohol, decolorized with Norite, and finally recrystallized thrice from alcohol. The substance crystallized in colorless, large, hexagonal plates. The air-dried substance weighed 1.6 g. and melted[6] between 143° and 144°. On drying in a vacuum at 105° over phosphorus pentoxide it lost 4.99% in weight, corresponding to one molecule of water of crystallization. In chloroform solution it had a specific optical rotation of +22.60°. The dry substance was analyzed.

Anal. Subs., 0.1709: H_2O, 0.1866; CO_2, 0.5222. Calc. for $C_{27}H_{47}OH(388)$: C, 83.50; H, 12.37. Found: C, 83.34; H, 12.22.

The substance did not give any immediate coloration in the Liebermann-Burchard reaction but after the reaction mixture had stood for some time a faint green color developed. In chloroform solution there was no visible absorption of bromine. The specific optical rotation was somewhat low, probably due to incomplete removal of sitosterol. The experiment indicates, however, that it is possible in one operation by

[6] The melting points given in this paper are uncorrected.

this method to attain a degree of purity of the dihydrositosterol that it is absolutely impossible to secure by recrystallization of such a small amount of material.

The acetyl derivative was prepared and recrystallized from alcohol from which it separated in large, colorless plates free from water of crystallization; m. p., 139°.

Anal. Subs., 0.1573: H_2O, 0.1632; CO_2, 0.4651. Calc. for $C_{27}H_{47}O.CO.CH_3$ (430): C, 80.93; H, 11.62. Found: C, 80.64; H, 11.61.

In carrying out the reaction described above we have noticed sometimes when the acid-chloroform solution is washed with water that a solid white substance precipitates. While we have not obtained a sufficient quantity of this material for an examination, some tests indicate that it is dihydrositosterol sulfate. It is insoluble in water and is only slightly soluble in organic solvents. It contains sulfur and forms a potassium salt that crystallizes from alcohol in hexagonal plates.

The intensely bluish or purplish-green mixture of sulfuric acid and acetic anhydride that is obtained in the reaction described above contains the unsaturated sterol in a very stable chemical combination. When the acid mixture is diluted with water it forms a clear grass-green solution. When it is neutralized or made alkaline with sodium hydroxide the color changes to yellow. The green color can be extracted from the acid solution with amyl alcohol and on evaporation of the solvent a gummy brownish-green residue is obtained. It is freely soluble in water with a strongly acid reaction. When this aqueous solution is boiled with potassium hydroxide an aromatic terpene-like odor is given off. On extraction of the boiled alkaline solution with ether a substance is obtained that crystallizes from alcohol in aggregates of small, colorless plates, but this material has not been further examined.

Summary[7]

A method is described for the separation of unsaturated sterols from the saturated dihydrositosterol. The method is a slight modification of the Liebermann-Burchard reaction.

When a mixture of unsaturated and saturated sterols, dissolved in chloroform or carbon tetrachloride, is treated with acetic anhydride and concd. sulfuric acid the unsaturated sterol combines with the sulfuric acid forming a stable soluble compound of bluish or purplish-green color. The acids containing the coloring matter separate from the organic solvent on the addition of a few drops of water and can be removed.

The saturated sterol is obtained on evaporating the solvent, saponifying and crystallizing the residue from alcohol.

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[7] After the work described above had been completed and this paper had been prepared for publication we discovered that a similar procedure had been used by Windaus and Resau for the separation of unsaturated from saturated hydrocarbons belonging to the cholesterol series. Mention of this separation was made in a paper entitled, "Über die Oxidation des Cholesteryl Acetats mit Chromsäure," *Ber.*, **48**, 851 (1915).