[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF HARVARD UNIVERSITY]

Cholesterol and Companions. V. Microdetermination of Δ^7-Stenols

BY KOJI NAKANISHI,[1] BIDYUT KAMAL BHATTACHARYYA[2] AND LOUIS F. FIESER

RECEIVED FEBRUARY 20, 1953

The Δ^7-stenol content of a sterol preparation is determinable with accuracy by a method based upon oxidation with selenium dioxide and spectrophotometric determination of iodine equivalent to the selenium formed. Cholesterol from typical human tissues contains 0.3–3% Δ^7-stenol.

The observation reported in paper III that lathosterol is oxidized rapidly at 0–25° by selenium dioxide in benzene–acetic acid, whereas cholesterol is not, seemed to offer promise of adaptation to an analytical method for determination of small amounts of the Δ^7-isomer in tissue sterol. The acetic acid and excess reagent can be removed by washing with water and bicarbonate solution and, in the range of concentrations involved, the selenium remains in colloidal solution in the benzene. Iodimetric and polarographic methods of determining the selenium were explored but seemed unsatisfactory. A simple but only approximate method reported in the experimental part is based on the darkening of silver foil due to formation of silver selenide. A more accurate method is based upon the spectrophotometric determination of iodine.

The selenium dioxide oxidation was carried out in the stopperless separatory funnel shown in Fig. 1. When clamped in an inverted position this funnel is leak-proof; in the horizontal position it can be shaken fairly vigorously. The funnel is particularly useful in extractions where a gas is evolved during shaking. For general purpose use it is best to have the side arm mounted at a slightly higher position; the principle can of course be adapted to funnels of other size and shape. In the present procedure the washed benzene solution was washed out into a Carius tube, the benzene evaporated, and the residue heated with nitric acid at 300° to convert the sterol to carbon dioxide and the selenium to the dioxide. Sulfamic acid was added to destroy nitrous oxides, followed by potassium iodide. The iodine was determined spectrophotometrically, small corrections applied for the reagents and for cholesterol (Fig. 2), and the Δ^7-stenol content read from a calibration curve (Fig. 3). Ergosterol also can be determined; the slope of the calibration curve (Fig. 4) for the diene is notably different from that for the stenol.

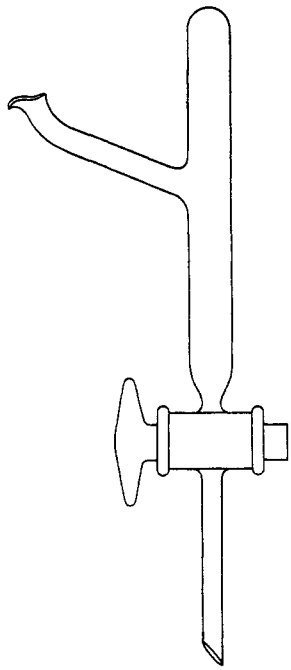

Fig. 1.—Stopperless separatory funnel (over-all length 18.5 cm., o.d. 12 mm., total volume about 8 cc.).

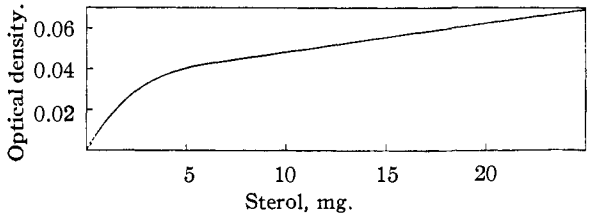

Fig. 2.—Correction factor due to reaction of purified cholesterol.

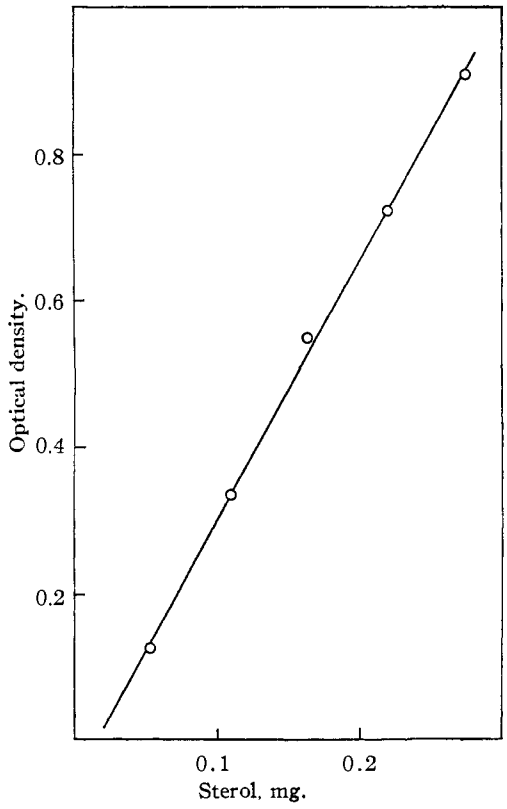

Fig. 3.—Calibration curve for lathosterol.

The results are reproducible to ±5% and hence the method appears to be more precise than the method of Moore and Baumann[3] based on the rapidity of response of Δ^7-stenols in the Liebermann–Burchard test. On the other hand, the latter method is much more rapid and convenient than ours, which requires meticulous attention to all details of the procedure.

(1) Research Fellow studying under the sponsorship of the Institute of International Education as participant in the Japanese Student Program of the Department of the Army and SCAP.

(2) On leave from the College of Engineering and Technology, Bengal (India).

(3) P. R. Moore and C. A. Baumann, *J. Biol. Chem.*, **195**, 615 (1953).

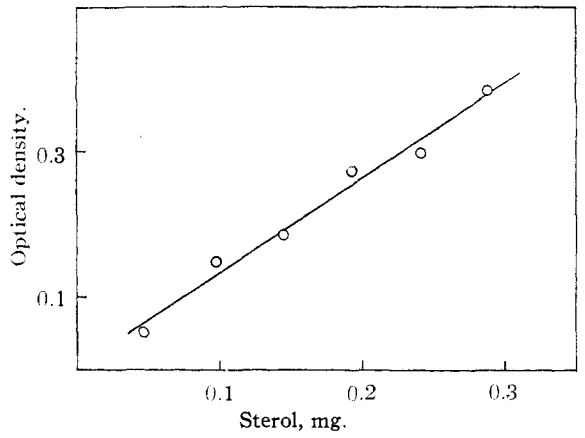

Fig. 4.—Calibration curve for ergosterol.

Results of exploratory analyses of cholesterol of various sources are listed in Table I. The skin sterols showed absorption at 280 mμ and hence consisted in part of 7-dehydrocholesterol; the high total Δ^7-stenol values confirm findings of Baumann and co-workers.[3,4] All other samples contain Δ^7-stenol in amounts ranging from 0.3 to 3%. Gallstones from individual patients contained the following amounts of Δ^7-stenol: 2.59, 2.72, 2.76, 2.45, 3.11, 2.19, 2.63, 2.62%. Samples of sterol from atherosclerotic plasma kindly supplied by Dr. David Kritchevsky were analyzed with the following results: Gofman Sf 10–20 lipoprotein sterol, 0.10, 0.10%; Sf 4–8 lipoprotein sterol, 0.29%; other fractions combined, 0.24% Δ^7-stenol.

TABLE I

Δ^7-STENOL CONTENT OF CHOLESTEROL SAMPLES

Source	Δ^7-Stenol, %
Spinal cord and brain of cattle (Wilson Co.)	0.60
Human brain	1.43
Human serum, normal	0.60
Gallstones (average of 8)	2.63
Egg yolk	0.39
Beef adrenal[a]	.65
Hog liver[b]	.35
Wool fat	2.97
Cancer tissue[c]	0.29
Blood plasma, nephrotic syndrome[d]	2.49
By glandular perfusion[b]	2.00
Rat skin	18.9
Dog skin	9.1

[a] From Parke, Davis Co. [b] Dr. Erwin Schwenk. [c] From Drs. Jacob Fine and Arnold M. Seligman, Beth Israel Hospital. [d] From Dr. David Rittenberg.

A number of sterol samples (free or as acetate) kindly supplied by Dr. Werner Bergmann were first assayed roughly by micro selenium dioxide and Liebermann–Burchard tests (paper III) and two that were found rich in Δ^7-stenol were analyzed with the following results: sterol acetate from *Terpios fugax* (Bermuda sponge), 10.8%; sterol acetate from *Spongia sp.* (Bahamas bath sponge), 34.3%. Sterols from the following animals were estimated to contain less than 1% of Δ^7-stenol (also the original spongosteryl acetate mixture): *Arenicola* (California), *Limulus* (New England), *Ascidia prunum* (Massachusetts Bay), *Plexaura sp.* and *Dysidea crashayi* (Bermuda), *Gorgonia flabellum*, *Geodia gibberosa*, and *Verongia fulva* (Florida); *Aplysia sp.* (Adriatic), *Ophiura sarsi* (Bay of Fundy), *Gorgonocephalus sp.* (Carolinas), *Crinoid spec.* (Cuba), *Geodia zetlandica* (Shetland Islands).

Acknowledgment.—For able help in the processing of some of the tissues, we are indebted to a group of students of Chemistry 20.

Experimental

Analytical Procedure (K.N.).—The terminal determination of the optical density of a solution of liberated iodine can be done most satisfactorily with a 5–10 mg. sample of sterol containing 1% Δ^7-stenol. Hence, prior to an analysis, the Δ^7-stenol content of the sample was estimated roughly by colorimetric tests conducted in melting point capillaries as described in paper III and a sample estimated to contain about 50 γ of Δ^7-stenol was weighed into a weighing stick. The stick was inverted into the side-arm of the separatory funnel shown in Fig. 1 and the sample was rinsed in with 2 cc. of pure benzene and 2 cc. of a 0.1 *M* solution of selenous acid made by dissolving 1.29 g. of selenous acid in 2 cc. of water and diluting to 100 cc. with acetic acid. The funnel was secured in an inverted position (to prevent leakage through the stopcock) in a Castaloy double buret clamp holder (Fisher Scientific Co.) and let stand for 2 hr. in a cold room at 5–8°. The resulting solution of colloidal selenium was then washed with two 2-cc. portions of water, two 2-cc. portions of 5% sodium bicarbonate solution, and two further 2-cc. portions of water. The residual benzene solution was then poured into a Pyrex Carius tube (300 mm. long × 9 mm. i.d.) and the funnel rinsed with 1 cc. of benzene. By heating the tube on the steam-bath and blowing in a stream of compressed air, the benzene was completely evaporated in less than 5 min. After addition of 0.5 cc. of C.P. concentrated nitric acid the tube was sealed and heated at 300° for 5 hr. The resulting aqueous solution was washed into a 10-cc. beaker with approximately 1 cc. of water, excess sulfamic acid was added to destroy nitrous acid, and the solution was heated on the steam-bath for 20 min. and made slightly alkaline (about 0.8 cc. of 8.5 *N* sodium hydroxide solution). The solution is conveniently stirred with a micro glass hook about 4 cm. long. A yellow-red coloration sometimes appeared, but the color disappeared later on acidification and did not interfere with the determination. The alkaline solution was treated with three drops of a freshly prepared solution of 3 g. of potassium iodide in 25 cc. of distilled water, diluted to a volume of 3 cc. with distilled water, and acidified with 0.5 cc. of 36% hydrochloric acid. After the solution had stood for 5 min., the optical density was measured in a 1-cm. cell (4 cc.) with a Beckman spectrophotometer set at 425 mμ. When the Δ^7-stenol content was greater than the optimum amount specified, fairly satisfactory determination of the optical density could be made by diluting the solution with a solution of acidified potassium iodide solution prepared exactly as in the procedure outlined and let stand for 5 min. before use.

The directly read optical density was corrected by subtracting two correction factors determined as follows.

Correction 1. Reagent Factor.—Three drops of the same fresh potassium iodide solution were added to 3 cc. of distilled water, 0.4 cc. of 36% hydrochloric acid was added, and the optical density measured after 5 min. Observed correction factors were in the range 0.050 to 0.075.

Correction 2. Cholesterol Factor.—Cholesterol purified through the dibromide or by 22 crystallizations from acetic acid (paper III) gave a slight positive response for which correction was made by reference to the calibration curve of Fig. 2, determined under standard conditions with purified cholesterol.

Calculation of Δ^7-Stenol Content.—A calibration curve based upon the results of oxidative analyses of pure lathosterol at five different concentrations is recorded in Fig. 3; each reading of optical density was corrected by subtracting the value found for the reagent factor. The relationship between optical density and concentration is linear. In an

(4) D. R. Idler and C. A. Baumann, *J. Biol. Chem.*, **195**, 623 (1952).

analysis of an unknown sample the observed optical density of the iodine solution was corrected by subtracting the small densities due to factors 1 and 2 and the weight of Δ^7-stenol corresponding to the corrected optical density was read from the calibration curve of Fig. 3. The results were reproducible to within ±5%.

Typical analyses illustrating the size of sample and the magnitude of the correction factors are shown in Table II.

TABLE II

Sample, mg.	Optical density				Δ^7-Stenol	
	Reading	Cor. 1	Cor 2	Cor. reading	Mg.	%
9.8	0.431	0.063	0.046	0.322	0.108	1.10
14.8	.243	.063	.052	.128	.056	0.38
5.6	.618	.063	.042	.513	.157	2.49
9.8	.265	.063	.047	.155	.063	0.64

Determination of Ergosterol.—Ergosterol is determinable by the same procedure by reference to the calibration curve of Fig. 4.

Approximate Silver Foil Assay.—Test pieces were made from a sheet of silver foil, the surface of which was first roughened with fine emery paper and then cleaned thoroughly with ethanol. Pieces approximately 1 × 4 mm. were cut as uniformly as possible and stored in the dark.

One-cc. portions of benzene solutions of unknowns, or of standard solutions containing from 0.01 to 0.10 mg. of lathosterol per cc., were measured into separatory funnels of the type shown in Fig. 1, 1 cc. of 0.1 *M* selenous acid solution was introduced to each funnel, and the funnels were left in an inverted position at room temperature (26°) for 2 hr. Precipitation of red selenium during this period indicated that the test solution was too concentrated. Each benzene solution was washed with 1-cc. portions of water (twice), 5% sodium bicarbonate solution (twice), and water (twice). The benzene layer was then poured into a small test-tube and the funnel rinsed with 0.5 cc. of benzene. The combined benzene solution was treated with 1 cc. of 95% ethanol and two drops of 36% hydrochloric acid, a strip of silver foil was added, and the solution heated at 60° for 20 min. The silver strip from an unknown was washed with alcohol, placed against a light background, and the surface darkening compared with that of a series of strips prepared from the standard solutions. If the sample contains more than 0.1 mg. of Δ^7-stenol per cc. the surface darkening is too intense for satisfactory comparison.

Preparation of Cholesterol Samples (B.K.B.; see also paper VI). (a) **Liver.**—A lipid concentrate (15 g.) from liver kindly supplied by Dr. Erwin Schwenk was dissolved in 50 cc. of absolute ethanol and 30 cc. of benzene and added to a solution prepared from 7 g. of sodium and 128 cc. of absolute ethanol. The mixture was warmed on the water-bath at 50–60° for 30 hr., cooled, and 10 cc. of water was added. Most of the alcohol was removed by distillation and the residue was diluted with water and extracted with ether. The ether layer was washed, dried and evaporated, and the dark semisolid residue crystallized once from acetic acid, then from methanol, then from 95% ethanol (Norit). The yield of colorless sterol, m.p. 148–149°, was 2.6 g.; a second crop of 2 g. melted at 144–146°.

(b) **Beef Adrenals.**—A sterol fraction concentrate (80 g.) kindly supplied by Parke Davis and Co. was saponified as above and the sterol crystallized from 95% ethanol (Norit); yield 2.8 g., m.p. 148–149.5°.

(c) **Hog Testes.**—A sterol preparation kindly supplied by the Upjohn Co. on crystallization from methanol gave a little solid, m.p. 152–155°, which was discarded. The mother liquors afforded material that after two crystallizations from methanol melted at 145–147°.

(d) **Cancer Tissue.**—A carcinoma of the stomach (100 g.) was cooled in Dry Ice and broken up in a mortar into small pieces. The powdered material was then refluxed with 1.5 l. of a 3:1 mixture of alcohol and ether for 1.5 hr., the solution was filtered, and the residue refluxed again with 1.5 l. of the same solvent mixture. The filtrates were combined and evaporated under nitrogen to a dark gum. This was digested with two 50-cc. portions of petroleum ether, and the combined extracts cooled and filtered. Evaporation of the petroleum ether solution gave 5 g. of residue, and this was refluxed with methanolic (50 cc.) potassium hydroxide (5 g.) for 5 hr. After dilution with water, ether extraction afforded a yellow sterol fraction that on two crystallizations from methanol gave 143 mg. of colorless sterol, m.p. 145.5–147°; a second crop of 70 mg. melted at 144–146°. Chromatography of the material in the mother liquors afforded 160 mg. more sterol, m.p. 143–145°.

The residual tissue was further extracted in a Soxhlet apparatus with acetone (100 cc.) for 6 hr. and then with chloroform (100 cc.) for 8 hr. and the extracts were combined and concentrated to a volume of about 3 cc. A small crop of crystals separated, m.p. 225–235° dec. Recrystallization from ether–petroleum ether gave 2.9 mg. of product, m.p. 232–238° dec.

(e) **Egg Yolk.**—The analysis previously reported[5] was later found to be in error because the sample contained a contaminating oily substance eliminated by further crystallization from methanol.

(f) **Dog Skins.**—Fresh dog skins were depilated with alkali, scraped, superficially dried, soaked in acetone, cut into pieces, and 100 g. of this material was extracted with 350 cc. of acetone and 5 cc. of ethanol in a Soxhlet extractor for 48 hr. A little saturated magnesium chloride was added and the extract filtered from slime. The residue left on evaporation was saponified with alcoholic alkali for 12 hr. and the neutral material chromatographed; yield 80 mg. of sterol, m.p. 141.5–142°.

(g) **Rat Skins.**—Rats were skinned very conveniently after first blowing compressed air through a needle inserted under the skin of the small part of the leg. Sterol was extracted by the method cited above and crystallized from methanol. The yield from 100 rats was 300 mg., m.p. 127–133°; recrystallized, 130–136°.

CAMBRIDGE, MASS.