THE ISOLATION OF 7(β)-HYDROXYCHOLESTEROL FROM
THE SERUM OF PREGNANT MARES*

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Little information is available as to the chemical nature of that portion of the unsaponifiable matter of blood serum which is not cholesterol. The only other sterol known to occur is β-cholestanol, which Schoenheimer (1) isolated in small amounts from a pathological human serum. The presence of other compounds related to cholesterol has been repeatedly suggested, mainly on the ground that the unsaponifiable matter after removal of the cholesterol with digitonin contains substances giving the Liebermann-Burchard reaction (2–5). Another unidentified entity is the digitonin-precipitable "oxycholesterol" of Lifschütz (6, 2), demonstrable by the color reaction bearing his name, as well as by the trichloroacetic acid reaction of Rosenheim.

In the hope that the isolation and identification of some of these constituents of the serum would add to our yet very scanty knowledge of sterol metabolism, we subjected the unsaponifiable matter of pregnant mare serum to systematic fractionation. This type of serum was chosen primarily because the lipid fraction, a by-product in the manufacture of gonad-stimulating hormone, was available to us in large quantities. It may safely be assumed that at least qualitatively similar results would be obtained with normal horse serum. From the alcoholic fraction a digitonin-precipitable sterol, 7(β)-hydroxycholesterol, has been isolated. This compound is not new; Barr, Heilbron, Parry, and Spring (7) prepared it in 1936 by oxidation of cholesterol acid phthalate

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with alkaline permanganate, and proved its structure by conversion to 7-dehydrocholesterol, but to our knowledge it has not previously been encountered in biological material. Interestingly enough, its dextrorotatory epimer, 7(\alpha)-hydroxycholesterol, has been shown by Haslewood (8) to occur in the unsaponifiable matter of ox liver. The identity of our isolated compound with the levorotatory isomer of Barr et al. was established by comparison with a sample prepared by the method of these workers. The melting point behavior of the natural as well as of the synthetic preparation showed some peculiarities, which we ascribe to the existence of solvated and solvent-free forms, which tend to give mixed crystals with each other. This obscured the mixed melting point determinations; however, crystal form, solubility properties, and specific rotations left no doubt as to the identity of the two preparations. Moreover, the properties of the respective dibenzoates showed full agreement.

The method of isolation of 7(\beta)-hydroxycholesterol is described in the experimental part. The sparing solubility of the diol in cold petroleum ether or pentane facilitated a partial removal of the cholesterol when the latter predominated in the mixture; for complete separation, adsorption of the acetates on aluminum oxide and fractional elution proved indispensable. The strong blue color given by the compound when dissolved in trichloroacetic acid (Rosenheim reaction) was employed as a guide in following the compound through the various fractionation steps.

Owing to the scattering of material unavoidable in an exploratory investigation and the undoubtedly heavy losses incurred in the fractionation, it is difficult to give an estimate of the amounts of 7(\beta)-hydroxycholesterol present in the unsaponifiable matter of the original serum. Starting from about 150 gm. of unsaponifiable matter we obtained from our best fraction 234 mg. of crude crystalline material, which yielded 84 mg. of the pure compound; an additional 70 mg. of somewhat less pure diol were obtained from the mother liquors and from other fractions. Taking into account the numerous side fractions, we may assume that the actual concentration in the unsaponifiable matter is probably several tenths of 1 per cent. On the other hand, we have evidence that not all of the chromogenic material in the final fractions is represented by 7(\beta)-hydroxycholesterol. In working up the
mother liquors of the diol we obtained a product crystallizable from absolute ether which exhibited a lower levorotation (−26°) than either 7(β)-hydroxycholesterol (−90°) or cholesterol (−38°). From methanol, in which it is much more soluble than 7(β)-hydroxycholesterol, this material deposited as a semicrystalline, gelatinous mass, reminiscent in appearance of the epimeric mixture of 7-hydroxycholesterols which is obtained by reduction of 7-ketocholesterol with aluminum isopropylate. The possibility exists that the dextrorotatory epimer, which is known to occur biologically (8), is likewise present in the diol fractions. The substance in question cannot be 7-dehydrocholesterol, because the latter is more strongly levorotatory than 7(β)-hydroxycholesterol.

An inquiry whether the isolated compound bears some relationship to the “oxycholesterol” which Lifschütz (6, 2) detected, by means of his color reaction, in the unsaponifiable matter of blood and of various organs, seems hardly profitable. Chemical and physical data, other than spectroscopic, on this entity are entirely lacking. Lifschütz was able to prepare from cholesterol by oxidation and other chemical means resinous products which possessed identical chromogenic properties and the composition C_{27}H_{48}O_2 required for a hydroxycholesterol (9), and the fact that such a preparation, made from cholesterol dibromide by treatment with sodium acetate, was shown by Rosenheim and Starling (10) to contain a diol later identified as Δ^4-cholestenediol-3,6 (11) has little bearing on the problem. The Lifschütz test is certainly not specific, as it is given not only by Rosenheim’s diol but also by α- and β-7-hydroxycholesterol, and probably by all the numerous sterols that yield a blue color in the Rosenheim test.

Of greater interest are the numerous reports that “oxycholesterol” is formed from cholesterol by the influence of light, heat, and oxygen (12–16), and by aeration of aqueous colloidal solutions in the presence of soaps (15). Indeed, Bischoff (16) could not obtain a positive Lifschütz reaction on the unsaponifiable matter of blood, brain, and egg yolk when oxygen was excluded in the operations requiring heating, and he therefore doubted the existence of preformed “oxycholesterol.” The question may then be asked whether the compound isolated by us is of true biological origin. Conditions, especially during the large scale operations, may have approximated those in the experiments quoted. To the
work done in our laboratory, beginning with the saponification of the lipid fraction, this applies only to a limited degree, since all procedures requiring heat or prolonged standing, as well as the vacuum distillations, were carried out in an atmosphere of nitrogen, and all solutions at rest were kept in the dark refrigerator. Nevertheless the mother liquors of the first crude cholesterol fraction gave a distinct Rosenheim reaction. A similar result, moreover, was obtained in two small scale experiments with 1.3 liters of pregnant mare serum and 3.7 liters of normal horse serum, which were collected from individual animals. In these all operations, from the drawing of the blood on, were conducted with the least possible delay and all practicable precautions against oxidation by air and light were taken. The succinates prepared from the cholesterol mother liquors gave the color reaction, which also points to the existence of preformed chromogenic products in serum. However, in working with the large quantities necessary in the search for new compounds it is virtually impossible to exclude all factors which might favor the formation of artificial "oxycholesterol," so the question of the source of any isolated compound giving the Lifschütz reaction is unanswerable. It is clear that the approach to this problem must be made by trying to establish the nature of the "oxycholesterol" formed from cholesterol. The yield of pure 7(β)-hydroxycholesterol represented a substantial portion (about 30 per cent) of the cholesterol-free, Rosenheim-positive fractions. On purely chemical grounds the possibility that 7(β)-hydroxycholesterol may have been formed by such accidental oxidation seems remote. If, on the other hand, in vitro oxidation to a well defined substance actually takes place with such facility, it can well be argued that an identical reaction may occur also under biological conditions; a line of thought, incidentally, which also underlies the work on the artificial preparation of "oxycholesterol" (Lifschütz, Rosenheim).

Our finding bears suggestively on the question of the biological origin of provitamin D₃, 7-dehydrocholesterol, which has been shown by Windaus (17) to occur in a variety of mammalian tissues. Both α- and β-hydroxycholesterol can be dehydrated by way of the dibenzoates to the provitamin, and may well also figure as intermediates in its biological formation.
EXPERIMENTAL

The starting material1 was the lipid fraction (about 750 gm.) from some 150 liters of pregnant mare serum, obtained by precipitation of the serum proteins with acetone, which probably eliminated most of the phospholipids. The extract was saponified with 10 per cent potassium hydroxide in 90 per cent methanol by allowing the mixture to stand at room temperature for 7 days in an atmosphere of nitrogen. It was then exhaustively extracted with purified, low boiling petroleum ether in a continuous extractor. The material extracted by the first charge of petroleum ether was subjected to a second saponification for 12 days. The subsequent charges on concentration in vacuo deposited a clean looking, crystalline precipitate, which was separated by filtration. The complete removal of soaps from the rest of the petroleum ether-soluble products proved somewhat troublesome. Only after most of the soaps and other disturbing substances had been eliminated by extraction of the dry residue with acetone, in which they remained undissolved, could the remainder be effectively washed out of the ether or petroleum ether solutions with aqueous potassium carbonate. The crystallizable portion of the neutral fraction was then subjected to an extensive fractionation, in which methanol, petroleum ether, and acetone were employed as solvents. 50 gm. of almost pure cholesterol (m.p. 146–147°, $[\alpha]_D = -38.8^\circ$ in chloroform), which gave no trace of blue color with trichloroacetic acid, were thus isolated. The rest of the material was largely contained in two fractions, 26.3 gm. of methanol-soluble products (Fraction A), and 70.8 gm. of a reddish brown oil, which was almost insoluble in methanol (Fraction B).2

1 We wish to express our sincere thanks to Dr. E. Schwenk of the Schering Corporation, Bloomfield, New Jersey, for making this material available to us.

2 The solubility properties of Fraction B suggested that it consisted at least in part of hydrocarbons. Small amounts of similar material were obtained from Fraction A after removal of the alcohols as acid succinates and of the ketones with Girard's reagent. These were subjected to further fractionation with methanol, and finally to fractional distillation in a high vacuum. No crystalline products could be isolated. Analysis of one of the distilled fractions showed that it consisted entirely of hydrocarbons. Since it appeared doubtful that all of the methanol-insoluble material was of biological origin, we prepared the unsaponifiable matter from 1.3
Fraction A was separated into alcoholic and non-alcoholic fractions by means of succinic anhydride and pyridine (18). The crude acid succinates were thoroughly extracted with petroleum ether at room temperature; the undissolved residue (10 gm.) consisted mainly of cholesterol acid succinate. The oily, petroleum ether-soluble material was dissolved in methanol and treated with an excess of lead acetate dissolved in the same solvent, in the hope that differences in the solubility of the resulting lead salts in organic solvents might effect a separation of the residual alcohols. A dark brown oil deposited, which was extracted with several portions of ether at room temperature, leaving a granular residue. The me thanol supernatant was diluted with water and extracted with ether. Three lead salt fractions were thus obtained, (1) one soluble in methanol, (2) one soluble in methanol, but insoluble in ether, and (3) one insoluble in both these solvents. To remove the lead, each of these fractions was distributed between dilute hydrochloric acid and ether; the ether residues were saponified at room temperature (4 days) with 5 per cent methyl alcoholic potassium hydroxide. Not all of the saponified material could be extracted with petroleum ether after the addition of water. The aqueous phase was therefore concentrated in vacuo, and the extraction repeated with ethyl ether. The distribution of material in the resulting six fractions (petroleum ether residues, Fractions 1a, 2a, 3a, and ether residues, Fractions 1b, 2b, 3b) liters of pregnant mare serum and 3.7 liters of normal horse serum, and fractionated it in a similar manner. Strict precautions against accidental contamination with foreign matter were taken. The total lipid fractions were obtained by treatment of the serums with 5 volumes of ether-alcohol (1:1). The phospholipids were removed by precipitation with acetone; ethyl ether instead of petroleum ether was used for the extraction of the unsaponifiable matter. The yields of products free from alcohols and ketones indicated that no more than 5.6 and 3.7 per cent of the unsaponifiable material (from pregnancy serum and normal serum respectively) behaved like hydrocarbons. We conclude from these results that, though small amounts of hydrocarbons are probably present in serum, the corresponding fraction in our large batch must have been derived largely from contaminations introduced during the factory operations. The normal horse serum for the above experiments was made available to us by Dr. W. G. Malcolm of the Lederle Laboratories, Inc., Pearl River, New York, and the pregnant mare serum by Mr. W. O. Osborn of the Ben Venue Laboratories, Inc., of Cleveland, Ohio; we wish to extend to them our sincere thanks for these gifts.
was as follows: Fractions 1a 0.738 gm., Fraction 1b 0.247 gm., Fraction 2a 1.20 gm., Fraction 2b 0.364 gm., Fraction 3a 1.416 gm., and Fraction 3b 0.558 gm. All six fractions gave a strong Rosenheim reaction, but subsequent work showed that the largest amount of the diol was present in Fraction 3a. This fraction was extracted in a 50 cc. centrifuge tube, with 20 cc. of pentane, by stirring the suspension for a few minutes after short initial boiling. The insoluble material, a gelatinous mass, was centrifuged and the extraction repeated twice with the same amount of pentane, and then with two 10 cc. volumes of petroleum ether (b.p. 40-60°). The insoluble residue (433 mg.) was acetylated with acetic anhydride and pyridine at room temperature. The acetylated produce (514 mg.) was dissolved in 25 cc. of pentane and passed through a column of Brockmann's aluminum oxide (250 X 12 mm.). The column was washed in succession with 75 cc. of pentane, 100 cc. portions of pentane-benzene (9:1, 8:2, 6:4, 4:6), benzene, and finally acetone. The filtrate was cut for every ingoing 25 cc. The 8:2 pentane-benzene washings left on evaporation a colorless, mostly crystalline residue, which on purification yielded cholesterol acetate. The Rosenheim-positive products, colorless oils, were mostly contained in the 6:4 and 4:6 pentane-benzene washings (274 mg.). To assure the complete removal of cholesterol the absorption procedure was repeated on this fraction. The Rosenheim-positive material was distributed as before; the 6:4 and 4:6 pentane-benzene fractions were saponified separately with cold methyl alcoholic potassium hydroxide. Both solutions deposited needle-shaped crystals, which were centrifuged and washed with 90 per cent methanol (31 and 92 mg.). Since both preparations possessed the same melting point, namely 179-184°, no marked fractionation of the chromogenic material had apparently been effected. The remainder of the fraction was recovered by ether extraction of the mother liquors (111 mg.).

Fractions 1a, 1b, 2a, and the pentane-soluble part of Fraction 3a were all treated separately with digitonin in 80 per cent alcohol. An excess of the reagent and prolonged standing were necessary to make the precipitation complete. The sterols regenerated from the digitonides (209, 37.5, 550, and 288 mg. respectively) contained, as further fractionation and chromatographic analysis
showed, varying amounts of cholesterol and Rosenheim-positive products. Altogether about 200 mg. of the latter, free from cholesterol, were obtained from these fractions, but only 30 mg. of this amount could be recovered in form of the crystalline diol.

Undoubtedly the procedure of isolation as described could be simplified in several respects, with probable advantage to the yield. The fractionation of the succinates as lead salts, which resulted only in a scattering of the diol without effectively separating it from the cholesterol still present, seems unnecessary. The soluble succinates could preferably be saponified directly, and the remaining steps applied to the digitonin-precipitable products only. We plan to repeat the isolation from a new batch of starting material in this manner.

\(\gamma(\beta)\)-Hydroxycholesterol—The combined crystalline fractions (123 mg.) yielded on two recrystallizations from methanol beautiful long needles melting at 183–186°. The whole preparation was dried for 3 hours at 110° and 12 mm. of Hg for the analysis and the determination of the specific rotation. The weight loss on drying was 7.3 per cent; calculated for 1 mole of methanol of crystallization, 7.37 per cent.

\[
\text{Analysis} - C_{37}H_{48}O_2. \quad \text{Calculated. } C \ 80.52, \ H \ 11.52
\]
\[
\text{Found. } C \ 80.36, \ H \ 11.25
\]
\[
\left[\alpha\right]_D^0 = -96.8^\circ \ (0.095\% \ \text{in chloroform})
\]

A \(3 \times 10^{-3}\) M solution of the dried preparation in alcohol showed no specific absorption in the ultraviolet region above 220 m\(\mu\).

With trichloroacetic acid the compound gives a deep blue color. The green pigment formed with Lifschütz’s reagent, prepared as described by Blix and Löwenhielm (15), exhibited the typical band at about 630 m\(\mu\).

The digitonide crystallizes from 80 per cent alcohol in beautiful small needles. Precipitation takes place more slowly than from cholesterol solutions of comparable strength.

The melting point of the original preparation (186°) was slightly depressed (183°) after drying at 110°. On recrystallization from methanol crystals indistinguishable in appearance from those of the undried sample were obtained, but their melting point was considerably lower (170–173°) and could not be raised by further crystallization. Still more puzzling was the behavior of the
material recovered from the chloroform solution used in the rotation experiment. It proved to be much more soluble in methanol than the original crystals; seeding with the sample melting at 183° was of no avail. Large rods melting at 154-157°, quite different in appearance from the long, fine needles of the higher melting samples, were finally obtained from a small volume of methanol on prolonged standing in the refrigerator. This preparation lost no weight on drying at 110° and 12 mm. of Hg for 1 hour. As was ascertained on another sample, prolonged heating at that temperature leads to decomposition. The same is true of the higher melting preparations.

**Analysis**—C_{17}H_{36}O_{2}. Calculated. C 80.52, H 11.52

  Found. “ 80.42, “ 11.47

44.5 mg. of the diol, melting at 167-175°, were recovered from the combined mother liquors of the original crystals. The melting points on three subsequent recrystallizations from methanol were 175-181°, 175-182°, and 176-179°. The last crop was analyzed after drying *in vacuo* at room temperature.

**Analysis**—C_{17}H_{36}O_{3}·CH_{3}OH. Calculated. C 77.35, H 11.60

  Found. “ 77.08, “ 11.51

  C_{17}H_{36}O_{2}·H_{2}O. “ 77.30, “ 11.04

The product was then recrystallized once more from methanol. The melting point was now 167-171°. The optical rotation was determined on the desiccator-dry material. \([\alpha]_D^{27} = -91.2°\) (0.704 per cent in chloroform).

*Synthetic 7(β)-hydroxycholesterol*, prepared by the method of Barr et al. (7), showed the same irregularities in melting point behavior. In fact we never succeeded in raising the melting point to that of the diol isolated from serum, namely 186°, which corresponds to the value given by the English workers (185°). The highest melting point attained was 179°, but on subsequent recrystallization it fell to 169°. As with some preparations of the natural compound, irregular rises and drops were observed in the intermediate crystallizations. On account of these difficulties the mixed melting point determination could be carried out only with products differing by 8° in their melting points (171° and 179°). The mixed sample melted at 176°. The preparation
lost 4.24 per cent of solvent on drying at 110° and 12 mm. of Hg. The weight became constant after 1 hour.

Analysis—C₂₇H₄₄O₂. Calculated. C 80.52, H 11.52

Found. " 80.60, " 11.62

The specific rotation ([α]₂⁰ = −89.9°) of the (desiccator-dry) synthetic product, m.p. 169°, compared well with the value, −91.2°, obtained with a natural preparation melting at 171°. Furthermore, a weighed sample of the same material was dried to constant weight at 110° at 12 mm. pressure and its rotation determined. [α]₂⁰ on the basis of the original weight was again −89.9°, and −94.5° on the basis of the dry weight. It is probable that the value given by Barr et al., [α]₂⁰ = −86.4°, was obtained with a preparation containing solvent. When the synthetic sample recovered from the chloroform solution was recrystallized from methanol, it exhibited the same peculiarities as the natural material, and a highly concentrated solution finally yielded rods mixed with a small amount of needles.

The weight loss on drying in the above experiment was only 5.50 per cent; the natural compound, m.p. 186°, under identical conditions, lost 7.3 per cent, which would account for 1 mole of methanol of crystallization. Taking into account all the observed facts, we are inclined to believe that the diol exists in two modifications, a high melting form, m.p. 186°, crystallizing in needles which contain 1 mole of methanol, and a low melting form, m.p. 154–157°, crystallizing in rods without solvent of crystallization. The products with intermediate melting points and solvent content are obviously mixed crystals of the two forms. If once the low melting form, which seems to be the more stable one, is produced, for instance, by drying, or the use of chloroform as a solvent, it is apparently not possible to secure again the fully solvated form by crystallization from methanol.

7(β)-Hydroxycholesterol Dibenzoate—The dibenzoate was prepared from 40 mg. of a preparation of the natural diol melting at 173° by allowing its solution in 1 cc. of pure pyridine and 0.5 cc. of benzyol chloride to stand at room temperature for 24 hours. The reaction mixture was worked up in the usual way. The crystals obtained from the ether residue were washed with cold methanol, and then recrystallized once from 1:1 methanol-acetone,
and twice from absolute alcohol. 33 mg. of fine needles, m.p. 155–156.5°, were obtained.

Analysis—C41H64O4. Calculated. C 80.60, H 8.92

Found. " 80.41, " 9.15

[α]D = −112.5° (0.68 % in chloroform)

A synthetic preparation (m. p. 169°) yielded on benzylation needles of identical appearance and melting point (155–157°); this is somewhat higher than the melting point reported by Barr et al. for their dibenzoate (150–151°). The melting point of a sample mixed with the dibenzoate of the isolated compound showed no depression. [α]D = −110.4° (0.79 per cent in chloroform); Barr et al. found [α]D = −105.3°.

Our attempts to prepare the crystalline diacetate (m. p. 122°) described by Barr et al. were unsuccessful. Both preparations, from the natural and from the synthetic diol, failed to crystallize from dilute alcohol as well as from pentane. Distillation in a high vacuum at 120–140° likewise yielded only an oily product.

SUMMARY

A sterol not hitherto encountered in biological material, 7(β)-hydroxycholesterol, has been isolated from the unsaponifiable matter of pregnant mare serum. Its identity was established by comparison with the synthetic compound which Barr, Heilbron, Parry, and Spring prepared by oxidation of cholesterol acid phthalate with permanganate.

The microanalyses reported in this paper were carried out by Mr. William Saschek.

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