A DERIVATIVE ISOTOPE DILUTION METHOD FOR DETERMINATION OF 5-α-CHOLESTAN-3β-OL:* ITS APPLICATION TO NORMAL AND TUMOROUS ADRENAL GLANDS

Masakiyo Kuroda

In a recent report (1) we described the identification of 5 α-cholestan-3β-ol in a crystalline sterol mixture isolated from adrenal glands of guinea pigs. It was subsequently shown that of the 23 % digitonin-precipitable, non-cholesterol sterols found in this crystalline sterol mixture, 16 % was 5-α-cholestan-3β ol (2). Because this stanol may be synthesized in the adrenal gland ( ) and also because of the possibility that it might be concerned there with the control of steroid synthesis ( ), we became interested in whether the high content of 5-α-cholestan-3β-ol foundin the adrenal of the guinea pig was peculiar to this animal or was of general occurrence.

Only two methods have been reported for the determination of this stanol. The procedure described by Schönheimer *et al.* ( ) is cumbersome and requires large amounts of tissue. The other, ased on two colorimetric determinations, is not specific ( ). The method described here, which is simple and specific, is based on derivative isotope dilution. It was adapted for the quantitation of the stanol content of normal and tumorous adrenal glands.

EXPERIMENTAL

Materials

Acetic anhydride-1-C^{14} (5.0 millicuries per μmole) was purchased from New England Nuclear Corporation. It was diluted with about 5 ml of unlabeled acetic anhydride (99 %) that had been distilled. All solvents were of reagent grade, delivered in glass bottles. 5-α-cholestan-3β-ol, a gift of Schering Corporation, was converted to the acetyl derivative ( ). Commercial cholesterol was purified by crystallization of the dibromide derivative ( ), and it was regenerated as described in ( ). Its acetyl derivative was also prepared. Acid-washed aluminum oxide suitable for chromatography was purchased from Merck, Sharp and Dohme. The adrenocorticotropic hormone was an Armour pruduct (ACTHAR Gel, 80 uuits per ml).

Extraction of Sterols

The whole adrenal glands of guinea pigs were weighed on a torsion balance and homogenized in a 3: 1 alcohol-ether mixture with a Potter-Elvehjem homogenizer. In the case of beef glands as much of the medulla as possible was removed by hand and the glands were homogenized in alcohol in a Waring blendor before the addition of ethyl ether.

The tissue was extracted twice by refluxing with a 3: 1 alcohol-ether solution, each time for one hour. The pooled extracts were diluted so as to yield a 50 % aqueous solution, which was then extracted three times with equal volumes of petroleum ether. The combined petroleum ether extracts

* Other names for this sterol: Dihgdrocholesterol

were washed with water and were dried over anhydrous sodium sulfate. After vacuum distillation of the petroleum ether, the lipid residue was chromatographed on silicic acid to separate free from the esterified sterols by the procedure described elsewhere ( ). The sterol esters were hydrolyzed with 10 % potassium hydroxide in alcohol by heating 1 hour on the steam bath. The cooled solution was diluted to 50 % with water and was extracted three times with petroleum ether. The combined petroleum ether extracts were washed and dried as des ribed above and the solvent was removed *in vacuo*. The dry residue obtained and the free sterols previously eluted from the silicic acid column were analyzed for 5-α-cholestan-3β-ol.

Cystalline digitonin-precipitable sterols were isolated from whole adrenal glands by the methods described in ( ).

Derivative Isotode Dilution Anatysis

Both the crystalliue and non-crystalline sterol fractions were transferred to 12 × 75 mm test tubes. To each tube 100 μl of acetic anhydride-1-C^{14} was added, and a small funnel containing a glass bead was placed in the mouth of the test tube. The tubes were heated in an oil bath maintained at 140° for one hour and were then coold. The contents of each tube were transferred quantitatively with chloroform to 100 ml-round bottom flasks containing 100.0 mg of 5-α-cholestan-3β-ol acetate, 100.0 mg of cholesteryl acetate and 5 ml of methanol. The methanol and chloroform were removed by vacuum distillation. Methanol was added to the residue dissolved in chloroform. After vacuum distillation of the solvents. the process was repeated twice more. The sterol residue was transferred to a small vial and dissolved in a chloroform solution containing about 0.4 g peroxybenzoic acid ( ). The solution was kept at room temperature 2 hours, and was then diluted with ether so as to yield a ratio of ether to chloroform of 4 : 1. The solution was extracted four times with 5 % sodium carbonate. After the ether-chloroform solution was washed with water and dried over anhydrous sodium sulfate, it was distilled to dryness. The residue, dissolved in petroleum ether, was transferred to a 10 g-column of aluminum oxide with the same solvent. The conumn was developed with 100 ml of petroleum ether containing 4 % benzene, and thé radioactive stanol acetate was eluted with 300 ml of petroleum ether containing 10 % benzene. The solvents were semoved by distillation *in vacuo* and the stanol acetate was recrystallized twice from an acetone-methanol (1 ; 1) mixture.

Preparation of C^{14}-Acetate Derivative of 5-α-Cholestan-3β ol as a Staudard

One hundred mg of unlabeled 5-α-cholestan-3β-ol was acetylated, chromatographed and recrystallized as described above. No sterol carrier was used.

Determination of C^{14}-Radioactivity

All samples were cenuted in 15 ml of toluene contaiuing 45 mg of 2, 5-diphenyl-oxazole. Its C^{14} was assayed by liquid scintillation spectrometry in the automatic Packard liquid scintillation spectrometer, model 314 E. The stanol acetate standard was weighed, diluted with toluene, and an aliquot was counted.

Calculations

The amount in milligrams of 5-α-cholestan-3β-ol in the sample assayed was calcuated from the following eqnation :

Specific activity of 5-α-cholestan-3β-ol acetate × 90.2 *Specific activity of standard*

where ; specific activity is disintegsations per minute per mg

$$90.2 = \frac{100 \times \text{Mol. wgt. 5-}\alpha\text{-cholestan-3}\beta\text{-ol}}{\text{Mol. wgt. of 5-}\alpha\text{-cholestan-3}\beta\text{-ol acetate}}$$

RESULTS

In order to evaluate the possible interference of cholesterol, the major sterol component in adrenal glands, in the analysis for 5-α-cholestan-3β-ol, several mxitures containing varying amounts of 5-α-cholestan-3β-ol and cholesterol were assayed for the stanol. These data are presented in Table I. The error range for single determinations of about 100 μg of the stanol was from −7.3 to 10.0 %. However, if the average values of duplicate determinations was used to calculste the errors of the ataly ses, a considerably smaller range was encountered, from −4.8 to + 4.3 %. Provided the average of triplicate anaysas was used for the computation, the analytical error was less than 0.5 %. The average error of duplicate analyses of 50 μg of the stanol was 3.3 %, while that of a triplicate analysis was 2.8 %.

The data in Table II shows that when added to crystalline tissue sterols, 20 and 100 μg of 5-α cholestan-3β ol can be recovered in amounts ranfing from 80 to 98 %.

Table I Analysis of 5-α-Cholestan-3β-OL in Mixtures Containing Known Amounts of Stanol and Cholesterol

Composition of prepared mixtures		Amount of 5-α-cholestan-3β-ol found	% error of		
5-α-Cholestan-3β-ol	Cholesterol		a single analyses	average of any 2 analyses	average of 3 analyses
μg	μh				
98.8	200	96.6	− 2.3	− 4.8	
98.8	200	91.6	− 7.3	+ 4.3	−0.41
98.8	200	109.4	+10.7	+ 1.7	
99.4	200	105.7	+ 6.3	+ 3.2	
99.4	200	99.4	0	+ 3.4	+0.22
99.4	200	99.8	+ 0.4	− 0.2	
49.7	400	46.3	− 6.8	+ 1.6	
49.7	400	54.7	+10.0	− 0.8	+ 2.8
49.7	400	52.2	+ 5.0	+ 7.6	
25.2	400	27.0	+ 7.1	+10.4	
25.2	400	28.6	+13.6	+12.8	+13.0
25.2	400	29.8	+18.4	+16.0	

Table II Recovery of 5-α-Cholestan-3β-OL Added to Crystalline Sterol Mixture Isolated from Adrenal Tissues

Adrenal sterol mixture		5-α-cholestan-3β-ol		Total stanol found*	Recovery of added stanol	% Recovery of added stanol
Obtained from	Free or ester	Found in crystalline tissue sterols*	Added to crystalline sterol			
		μg	μg	μg	μg	
Beef	Free	7.2	103.4	107.9	100.7	98
Beef	Free	9.8	20.2	25.8	16.0	79
Guinea pig	Ester	167.9	103.1	269.4	101.5	98

* Average or triplicate analysis.

Values for the 5-α-cholestan-3β-ol content of crystalline sterols isolated from normal and tumor bearing adrenal glands of several mammalian species are given in Table III. In general, the percentages of the free and esterified stanol in the crystalline sterols from normal tissue parallel each other.

Table III Percentages of Free and Esterified 5-α-Cholestan-3β-OL in the Crystalline Digitonin-Precipitable Sterols Isolated from Normal and Tumorous Mammalian Adrenal Glands

Animal	Tissue	% of 5-α-cholestan-3β-ol found in sterol mixture in	
		Free form	Esterified form
Rat	Normal adrenal	0.43	0.56
	Adrenal tumor (494H)	0.91	0.53
	Adrenal tumor (464)	0.72	0.13
Human	Normal adrenal	1.26	1.13
	Adrenal tumor (Cushing syndrome)	0.39	0.44
Beef	Normal adrenal	0.71	1.32
Chicken	Normal adrenal	5.9	6.7
Guinea pig	Normal adrenal	10.0	12.1
Add ACTH values			

* Aldosterone secreting tumor one abrenal. Other abrenal on which analyses carried out was normal.

‡ Intraperitoneal injection of 20 units ACTH (pooled Samples).

The stanol content of normal guinea pig and chicken adrenal glands were high when compared to the values found in normal rat, beef, and human adrenal glancs.

In the crystallinc sterols isolated from the two rat adrenal tumors the percenage of esterified stanol is lower than that of the free stanol. Also, the percentage of free stanol is elevated when compared to the value given for the normal control rat. There is a marked depression of the percentage of esterified stanol in the crystalline sterol isolated from rat adrenal tumor 494 while the value is similar to that of the cotnrol rat in tumor 494-H.

The percentage of free and esterified 5-α-cholestan-3β-ol found in the crystalline sterols isolated from the human adrenal tumor (Cushing's syndrome) were lower than that found in a histologically normal adrenal gland taken from a man with an aldosterone secreting tumor.

Stimulation of guinea pig adrenal glands by an intraperitoneal injection of adrenocorticotropic hormone results in an elevation of the percantage of free and esterified 5-α-cholestan-3β-ol in the crystalline adrenal sterols. Thus the percentage of esterified stanol has risen from a control value of 12.1 % to 16.8 % while the percentage of free stanol is increased from a control value of 10.0 % to 17.4 % 6 hours after adrenocorticotropic ahministration.

Table IV presents the results of the 5-α-cholestan-3β-ol analysis of the total lipid material that was extracted from rat adrenal glands and separated by silicic acid chromatography into free and esterified fractions. There is about 7 times more esterified than free stanol in the whole rat adrenal gland.

In Table V are given the results of the 5-α-cholestan-3β-ol analysis on the total lipid extract of normal rat adrenal glands and of those bearing tumors. Although the content of the free stanol is not effected by tumor growth, the tumor tissue shows a seven-to eight-fold drop in the content of

Table IV Free and Esterified 5-α-Cholestan-3β-OL* Content in Micrograms Per 100 Milligram of "Wet" Male rat Adreual Glanbs

Groub	Free ‡	Ester ‡
I	2.58	12.1
II	3.41	15.8
III	3.54	19.4
IV	1.44	25.4
V	2.06	19.8
	Av. 2.61	18.5

State

Each group consists of 8 adrenal gland

* Separated by silicic acid chromatography.

‡ Each analysis was performed on the lipid extract which was obtained from four animals.

Add total weight of adrenal glands.

Table V Content of Free and Esterified 5-α-Cholestan-3β-OL in Rat Normal and Tumor Adrenal Glands

Adrenal tissue	5-α-Cholestan-3β-ol per 100 mg "wet" adrenal glands	
	Free	Ester
	μg	μg
Normal	4.2	48.6
Tumor 494	3.8	5.6
Tumor 494H	5.8	7.0

esterified stanol.

DISCUSSION

Since the work of Schoenheimer and his collaborators in 1930, only sporadic attempts have been made to elucidate the origin, metabolism and function of 5-α-cholestan-3β-oi in animal organs. At least a part of the paucity of research with this stanol may have stemmed from the absnece of a facile and accurate method for its analysis in tissues. The method of Schoenheimer *et al.* ( ) is laborious, requires large amounts of tissue and cannot be applied directly to the lipid extracts. The procedure for 5-α-cholestan-3β-ol analysis described in this report, although somewhat time consuming, nevertheless obviates the other difficulties encountered with the older method ( ).

We have assumed that 5-α-cholestan-3β-ol is the only stanol that is determined in tissuse by the new meshod. Since other stanols as coprostanol have never been reported in animal tissues, the assumption is indoubtedly valid. The assumption is also supported by our finding that a minimum of 75 % of the β-digitonin-precipitable stanol synthesized from cholest-4-ene-3one by guinea pig adrenal gland homogenates is 5-α-cholestna-3β-ol. Any unsaturated sterols that may be preaent in tissues, for example, lanosterol, zymosterol, 7-dehydrochtlesterol, lathosterol, desmosterol and eight others that are cited in a report by Irie *et al.* ( ), are converted by the peroxybenzoic acid to epoxide derivatives ( ) more polar than 5-α-cholestan-3β-ol acetate, and they are thereby eliminated during the chromatographic step in the procedure. Cholestane 3β-5α-6β-triol if present in the tissues being analyzed, would be converted to a triacetate that would be eluted prior

to 5-α-cholestan-3β-ol acetate in the chromatographic step. We were unable to evaluate the possible intesference of cholestan-3β-ol 6-one, which has been found in some tissues ( ), because this sterol was not available to us.

The date in Table III show considerable variation of the 5-α-cholestan-3β-ol content of the crystalline adrenal sterols isolated from various species. The guinea pig and chicken may be unique in having a rather high percentage of stanol, above 5 %, in both the free and esterified crystalline sterols isolated from this endocrine organ.

The adrenal glands of man and the rat have low 5-α-cholestan-3β-ol content compared to that of the guinea pig even though the adrenal glands of the three species are known by histological examination ( ) to be rich in lipids.

In the case of the guinea pig, the elevated adrenal stanol content may be related to the presence of a 27-carbon 5-α-reductase and 3-β-dehydrogenase which we found in this organ. Recently, Forchielli ( ) described the presence of 19 and 21 carbon atom 5-β-reductases in the microsomal fraction of guinea pig adrenal glands. The stereospecificity of a similar reductase found in the supernatant fraction by these workers was not determined.

Our data in a previous publication suggest that the guinea pig adrenal stanol is derived both from the blood by transport and from endogenous precursors through biosynthesis. The presence of enzymes in the adrenal glaud which can readily convert cholest-4-ene-3-one to 5.α-cholestan-3β-ol make the unsaturated ketone a most like naturally occurring precursor for the stanol.

Since most of the digitonin-precipitable sterols in the adrenal glauds of the rat ( ), man ( ), guinea pig ( ), and chicken ( ), exist in the esterified form, it is obvious from the data in Table III, that 5-α-cholestan-3β-ol is present principally in the esterified state in these species. The reverse is true for the bovine adrenal glands, * the stanol being found principally as the free sterol.

Adrenocorticopic is known to diminish the total cholesterol content of both guinea pig and rat adrenal glands ( ), In the case of the rat the reduction of cholesterol is principally in the esterified fraction, Since the cholesterol ester constitutes the major sterol fraction in both rat and guinea pig adrenal glands it is reasonable to assume that adrenocorticotropin also reduces only the sterol ester fraction in the guinea pig adrenal gland. Thus at least a part of the rise in the 5-α-cholestan-3β-ol content of the crystalline sterols 6 hours after adrenocorticotropic administration may be explained by the declinc in the cholesterol ester content. The elevation in the perceutage of free cholestan-3β-ol in the crystalline sterols may reflect either 1) altered adrenal metabolism of free 5-α-cholestan-3β-ol, the glend possessing either an increased synthetic capacity or a decreased catabolic activity or 2) a concomitant decline of free cholesterol after adrenocorticotropin in contrast to the findings in the rat.

On the basis of Hechter's data ( ) for the cholesterol concentration in rat adrenal glands, the esterified 5-α-cholestan-3β-ol (Table IV) represents about 0.7 % of the esterified cholesterol content while the free stanol content (Table IV) is 0.3 % of the free cholesterol value.

It is of interest that the rat tumors studied were found to have a decreased estesified stanol content and the content of stanol in the crystalline esterified sterols isolated from the adrenal tumor of the patient with Cushing's syndrome was also lower than that found in normal human adrenal tissue. In this connection Kellner ( ) isolated only 40 mg of crystalline cholesterol from an adrenal

* Slaughterhouse material.

tumor weighing 180 g. Although his methods were not quantitative, this is considerably below the 6 to 8 g of cholesterol that have been isolated from normal human adrenal glands. Of three adrenal tumors removed from patients with Cushing's syndrome, 2 were found to have cholesterol contents lower than normal while one was elevated ( ). These data reflect an altered sterol metabolism in adrenal tumors in comparison with that found in normal glands. In this connection it is of interest to note that ascites tumor cells are unable to utilize acetate for sterol synthesis.

本論文は *Dr. I. L. Chaikoff 及び *Dr. H. Werbin との共同研究によるものであり，原著は近く J. Biol Chem に発表予定である．したがって文献は書かなかった．尚研究費の大部分は N. I. H. からこれを得た．ここに深謝の意を表す．

* Dept. of Physiology, Univ. of Calif. Berkeley, Calif. U. S. A.