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Received September 1, 1964. P.S.E.B.M., 1964, v117.

Conversion of 4-Androstene-3β,17β-diol-4-C^{14} by Human Adrenals *in vitro*.* (29677)

JOHN C. COLLA,† MAJOR L. COHN† AND FRANK UNGAR
Department of Biochemistry, University of Minnesota Medical School, Minneapolis

Recent interest in the conversion of the Δ^4-3-hydroxyl to the Δ^4-3-ketone(1,2) has been generated by the isolation of Δ^4-3-hydroxyl compounds(3) or their artifacts from natural sources(4) and by demonstration of the conversion of the Δ^4-3-ketone to the Δ^4-3-hydroxyl under *in vitro*(5) and *in vivo*(3) conditions.

The conversion of Δ^4-androstene-3β,17β-diol and its 3α-isomer to testosterone had previously been shown to occur in both rat and chicken liver homogenates(1,2). The conversion of these isomers to testosterone and 4-androstene-3,17-dione occurred in both the supernatant fraction and thrice washed particulates from the 5000 $\times$ *g* fraction of rat and chicken liver acetone powders. The latter residue fraction, however contained only 30% of the activity of the supernatant. A stimulation by the pyridine nucleotides was also demonstrated.

In addition the conversion of the Δ^4-hydroxyl group to the Δ^4-3-ketone has been shown to occur in the testis tissue of the mouse(6). The present report demonstrates that human adrenal tissue also has the capacity to convert 4-androstene-3β,17β-diol to testosterone and 4-androstene-3,17-dione.

* Supported in part by Grants CA-5079 of Nat. Cancer Inst., and by Grant AM-02583 of Nat. Inst. for Arthritis and Metab. Dis.

† Postdoctoral Fellows of cancer training program in steroid biochemistry (CA-5079).

Methods. A total of 11 g of hyperplastic human female adrenals were homogenized with 80 ml of Krebs-Ringer bicarbonate buffer, pH 7.3, in a Potter-Elvehjem homogenizer. In a series of incubations, 2.5 ml of homogenate were placed in 50 ml Erlenmeyer flasks along with 5 μM ATP, 6 μM NADP, and 1 mg (73,000 cpm) of 4-androstene-3β,17β-diol-4-C^{14} (prepared by Dr. M. Gut, Worcester Foundation for Experimental Biology). The final volume was 2.8 ml. The flasks were incubated at 37° in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co.) under an atmosphere of 95% O_2 and 5% CO_2. The incubator was set at 100 oscillations per minute. Incubations were stopped with 10 volumes of acetone at zero time, 15, 30, 60, and 180 minutes.

The acetone extracts were filtered on a sintered glass filter funnel and the tissue residue was freed of residual steroids with successive washings of acetone, ethyl acetate and methanol. After removal of these solvents under vacuum the lipids were removed by partition between petroleum ether and 70% aqueous methanol. The methanol was removed and the steroids were extracted from the aqueous phase with ethyl acetate. Aliquots of the extracts were then analyzed by thin-layer and gas chromatography.

Chromatoplates were prepared for thin-layer chromatography by applying a slurry of silica gel GF (Merck 7730) in water (1:2)

TABLE I. Mobility of Steroids on Silica Gel. Thin-layer chromatography.*

Steroid	Movement (cm)
Testosterone	6.1
17α-Hydroxyprogesterone	7.5
4-Androstene-3,17-dione	9.3
Progesterone	10.9
Testosterone acetate	12.2

* Solvent system — benzene : ethyl acetate (4:3). Development time, 90-100 min. Solvent front, 15 cm.

to 20 × 20 cm plates at a layer thickness of 0.25 mm. The plates were dried at 100° for 2 hours and stored in a desiccator over calcium chloride. Plates were developed in a mixture of benzene : ethyl acetate (4:3) for 90 to 100 minutes at room temperature.

A ligroin-propylene glycol system(7) on Whatman No. 1 paper was used for separation of steroids by paper chromatography. Δ^4-3-Ketosteroids were located with ultraviolet light following a development time of 96 hours. Steroids were removed from thin-layer chromatoplates with a vacuum apparatus modified after Matthews *et al*(8) and from paper strips by successive extraction with methanol, methylene chloride and ethyl acetate using conventional techniques.

Radioactivity in solutions was measured with a Tri-Carb liquid scintillation Spectrometer (Model 314 EX, Packard Instruments) using a standard toluene scintillation solution. Radioacticity on paper strips was located with the aid of an automatic chromatogram scanner (Model 880, Vanguard).

Gas chromatography was performed on a 6 foot glass column using a β^- ionization detector (Model 600 series, Research Scientific Co.). Areas under peaks were determined with a planimeter (Gelman Instrument Co., Ann Arbor, Mich.).

Results. The movement of standard steroids on thin-layer chromatography is shown in Table I.

The developed thin-layer chromatoplates of the extracts were sectioned off into zones using standards and ultraviolet absorbing properties as references. Although the extracts visually demonstrated increasing ultraviolet absorbing properties with time of incubation for the testosterone and 4-androstene-3,17-dione zones, the radioactivity in the testosterone zones was rather constant. It became apparent that the testosterone zone was contaminated with a radioactive material which did not absorb ultraviolet light and which was subsequently shown to be 4-androstene-3β,17β-diol. The origin zone and testosterone zone from thin-layer chromatography were then rechromatographed for 96 hours in the ligroin system on paper. The developed chromatogram revealed an origin zone, 4-androstene-3β,17β-diol zone and testosterone zone which were then eluted from these strips. The radioactivity eluted from the 4 zones, containing the origin, 4-androstene-3β,17β-diol, testosterone and 4-androstene-3,17-dione are shown in Table II. The zone containing radioactivity at the origin which presumably represented more highly oxygenated metabolites of the starting material was not further analyzed at this time.

A portion of the testosterone zone eluted from the paper strips was converted to the acetate and after addition of 30 mg of carrier testosterone acetate, was recrystallized to constant specific activity. The movement of radioactivity of the testosterone acetate, before dilution with carrier, was identical with that of standard testosterone acetate on both thin-layer and paper chromatography. The results of recrystallization are shown in Table III.

TABLE II. Radioactivity of Zones Isolated Following Thin-Layer and Paper Chromatography.

Incubation time (min)	Origin zone	4-Androstene-3β, 17β-diol zone	Testosterone zone	4-Androstene-3, 17-dione zone	% Recovery*
0	5,300	53,800	4,800	1,850	86
15	5,800	45,050	18,200	1,350	96
30	6,300	31,900	22,000	1,750	86
60	7,200	20,900	28,700	3,550	88
180	9,550	13,900	33,300	4,000	84

* Per cent of C^{14} extracted from tissues present in the 4 isolated zones.

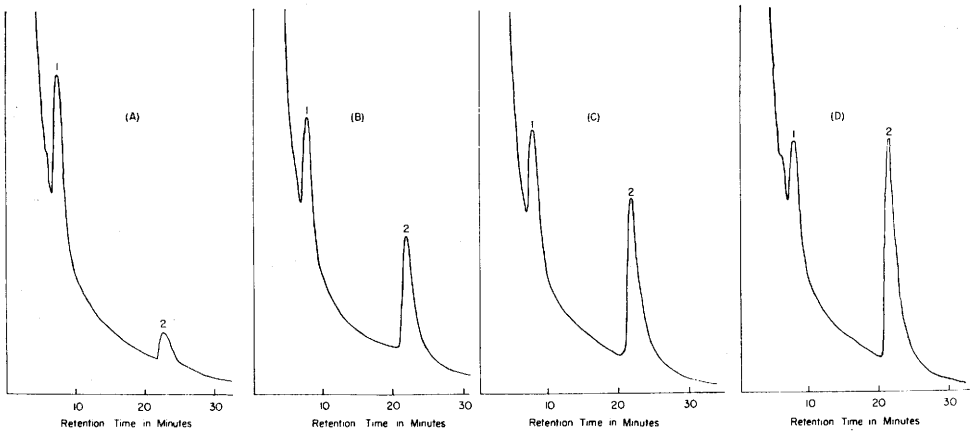


FIG. 1. Tracings of gas chromatographic analyses of extracts obtained from adrenal incubations with 4-androstene-3β,17β-diol-4-C^{14}. Gas chromatographic unit employed was Research Specialties, Model 600 series with β-ionization detector; glass column—6-ft length by ¼ inch I.D.; coating, 3% QF-1 on ABS, 100/110 mesh (Analabs); high voltage—1200, gas pressure—30 psi, attenuation —2, temperatures °C: column 240, vaporizer 330, detector 242, output 247. One microliter of extract sample injected. Incubation times for A, B, C and D are 0, 15, 30 and 60 min respectively. 1) 4-androstene-3β,17β-diol; 2) testosterone.

Aliquots of the ethyl acetate extracts were analyzed by gas chromatography. These results are shown in Fig. 1. Testosterone standards in concentrations ranging from 0.125 to 1.0 μg in one lambda of chloroform were injected under identical conditions on the same day during the period of analyses of the extracts. The areas under the standard testosterone peaks when plotted against concentration gave a straight line. The area of the testosterone peaks from the incubates were then determined and the total amount of testosterone in the incubates was calculated using the standard curve. These values were compared with the amounts of testosterone calculated from the radioactivity measurements. Both sets of data are plotted in Fig. 2.

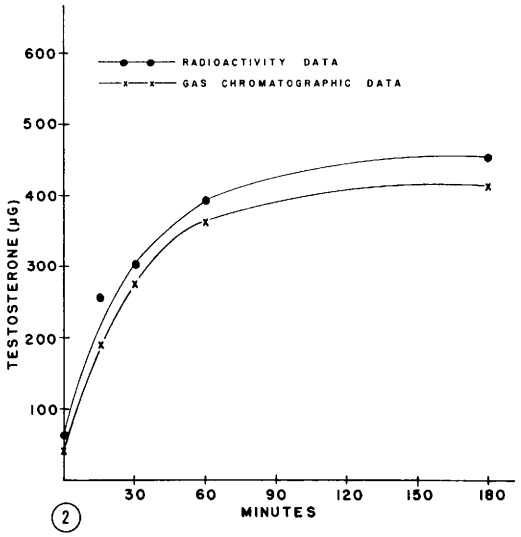
TABLE III. Recrystallization of Testosterone Acetate to Constant Specific Activity.

Sample	No. of recrystallization	mg*	Counts/min/mg
A	1	24.8	90
	2	14.9	83
	3	13.1	82
	4	9.7	78
B	1	26.1	96
	2	20.5	88
	3	18.3	88
	4	12.5	85

* 30 mg testosterone-acetate added as carrier before 1st recrystallization to an aliquot of the zone taken from the 60 min incubation (Sample A); and that taken from the 180 min incubation (Sample B).

Discussion. 4-Androstene-3β,17β-diol was readily converted to testosterone and 4-androstene-3-17-dione (Fig. 3) in a homogenate prepared from adrenal tissue obtained from a human female with adrenal hyperplasia. This conversion was similar to that demonstrated in mouse testis tissue with respect to the formation of large amounts of testosterone and relatively minor amounts of 4-androstene-3,17-dione. This adrenal preparation like the mouse testis mince had a relatively weak 17β-hydroxy dehydrogenase activity. By contrast, in the liver, the 4-androstene-3,17-dione increased with time as the newly formed testosterone was oxidized to the 17-ketone. The Δ^4-3β-hydroxy dehydrogenase activity of the adrenal appears to be similar to that observed in testis and liver. Whether or not the 3β-hydroxy group on ring A saturated and Δ^4-steroids are oxidized by the same enzyme systems in the 3 tissues examined, remains to be proven.

The identity of the testosterone formed was verified by paper, thin-layer and gas chromatography. The analyses using gas chromatography procedures warrant further comment. As seen in Fig. 2 the amount of



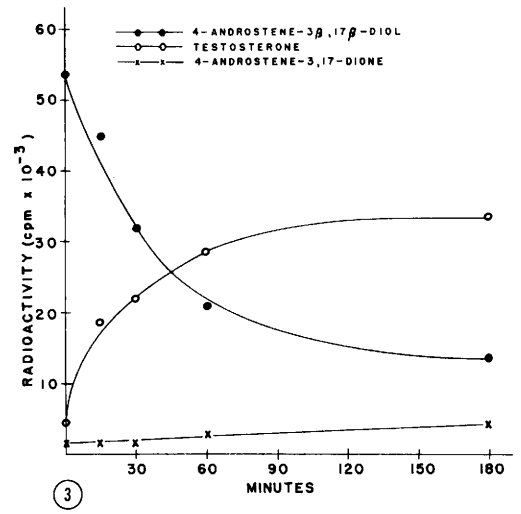


FIG. 2. Comparison of testosterone formation as measured by radioactivity recovered in testosterone zone and by the testosterone peak area observed following gas chromatography.

FIG. 3. Radioactivity recovered from zones representing testosterone and 4-androstene-3,17-dione following chromatography of extracts of adrenal tissue incubated with 4-androstene-3β,17β-diol-4-C^{14} for periods ranging from 0 to 180 min.

testosterone calculated from gas chromatographic data agrees well with the values obtained for testosterone calculated from the radioactivity measurements. The area under the peaks for the unknowns were compared with those obtained from a series of standard testosterone solutions during the period in which the unknowns were being analyzed. A straight line relationship was obtained for the standards at the concentrations employed and reasonable values for the unknowns could be obtained. It should be emphasized that the amounts of testosterone formed from the precursor were relatively large, permitting injections of small (1λ) samples of extracts. The measurement of endogenous testosterone which would normally be present in small concentrations in adrenal tissue containing relatively high concentrations of background material and other steroid components probably would not be feasible without further extensive modifications of the procedures and conditions employed.

The conversion of the 3-hydroxyl-Δ^4 group to the Δ^4-3-ketone has now been demonstrated to occur in human adrenal tissue as well as mouse testis(6) and rat liver(1,2). In contrast, this enzyme system may be deficient or absent in ovarian tissue as evidenced by our failure to obtain a conversion of the same precursor to testosterone or 4-androstene-3,17-dione in the one instance attempted using tissue obtained from a human with polycystic ovaries.‡ Our results with ovarian tissue as well as those with adrenal tissue were obtained using human subjects in abnormal states. The activity of normal tissues obtained from the human has not been evaluated.

The presence of the Δ^4-3-hydroxyl form of steroids has been demonstrated in the human by the isolation from urine of 11β-hydroxy-3,5-androstadiene-17-one(3,4) and of 3β,11β-dihydroxy-4-androstene-17-one(3) following the administration of 11β-hydroxy-4-androstene 3,17-dione. The enzymatic conversion of a Δ^4-3-ketosteroid containing a 6α-fluoro group to the corresponding Δ^4-3-hydroxyl derivative (5) could be shown, using rat liver preparations, whereas the reaction was not observed with 4-androstene-3,17-dione(5,1). However, the conversion of 4-androstene-3,17-dione-4-C^{14} to 3β-hydroxy-4-androstene-17-one-C^{14} by a supernatant fraction of rabbit skeletal muscle(9) and the conversion of 17α-hydroxyprogesterone to 3α,17α-dihydroxy-4-pregnene-20-one(10) by perfusion of bovine adrenals

‡ Unpublished data.

have been reported recently. The role of a nuclear substituant on the Δ^4-steroid molecule for reduction of the ketone at carbon 3 to occur as discussed by Ringold *et al*(5), if it is in fact necessary, may have various possible *in vivo* counterparts in terms of protein-steroid interactions. The forward and reverse reactions involving the 3-hydroxyl-Δ^4 group merit further investigation to establish their significance in the biosynthetic or catabolic pathways of steroid metabolism.

ADDENDUM ADDED IN PROOF: The authors wish to point out an error in nomenclature in the article by Rosner, Horita and Forsham, *Endocrinology,* 1964, v75, 299. The compound called androstenediol should be designated as Δ^5 not Δ^{4-}. They demonstrate a conversion of the Δ^5-3β-hydroxyl group to the Δ^4-3 ketone. Our investigations are concerned with the allyl structure, Δ^4-3β-hydroxyl group.

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Received September 2, 1964. P.S.E.B.M., 1964, v117.

Alteration in Citrate Metabolism in Parathyroid Extract-Treated Calvaria.* (29678)

CHRISTYNA E. MECCA,† G. R. MARTIN, E. SCHIFFMANN AND P. GOLDHABER‡
(Introduced by I. Zipkin)
National Institute of Dental Research, Bethesda, Md. and Harvard School of Dental Medicine, Boston Mass,

Mouse calvaria, when grown in tissue culture under appropriate conditions, resorb when parathyroid extract (PTE)§ is added to the medium(1). During resorption, citrate levels are increased in the medium(2). Although bone contains large quantities of citrate relative to other tissues, the release of citrate bound to mineral can account for less than 1/40 of the total. The major proportion of the citrate is newly synthesized. Since citrate is a potent demineralizing agent and may be one of the acids concerned with mineral resorption, we have attempted to determine if the oxidative metabolism of citrate is altered during PTE-induced resorption.

Methods. In these studies the tissue culture methods developed by Goldhaber were employed(1,2). In general, calvaria weighing approximately 6 mg were obtained from 5-day-old mice and were attached to either a coverslip or a piece of 0.45 μ Millipore® fllter (HA) with a plasma clot. These preparations were placed in glass tubes containing 2 ml of a medium composed of 20% Gey's salt solution, 80% heated horse serum and 100 units/ml each of penicillin and streptomycin. PTE was added to the medium of

* This work was supported in part by USPHS Research Grant D-1298.

† Portions of this paper are excerpts from the thesis submitted to the Zoology Department of the George Washington University in partial fulfillment of the requirements for the degree of Master of Science.

‡ U.S.P.H.S. Research Career Development Awardee.

§ Lilly Parathormone.