A Preliminary Investigation of the Products of the Metabolism of Testosterone by Rat Liver *in vitro*

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A comprehensive summary of studies of the metabolism of androgens by animal tissues *in vitro* has been made by Samuels & West (1952). One of the points to which they draw attention, and which it is desired to emphasize here, is that, in order to permit the application of the orthodox methods of chemical manipulation, many workers choose grossly unphysiological ratios of substrate to tissue and work with suspensions or water-soluble derivatives rather than with true aqueous solutions of the steroids. This criticism is still applicable to much current work.

Thus the work of Kochakian and his associates (Clark & Kochakian, 1947; Clark, Kochakian & Lobotsky, 1947; Kochakian & Nall, 1953; Kochakian & Stidworthy, 1954) which indicates that dehydrogenases of liver or kidney act on the 3- and 17-positions and on the ethylenic linkage of testosterone, androst-4-ene-3:17-dione or 17 α -hydroxy-androst-4-ene-3-one (the so-called 'cis'-testosterone) was done entirely with suspensions of the substrates in serum-phosphate (1:1) buffer mixtures. The recently reported enzymic conversion of testosterone into androst-4-ene-3:17-dione by human serum in the presence of added diphosphopyridine nucleotide (DPN) (Richerich-van Baerle, Wotiz & Lemon, 1954) must be taken into consideration in evaluating these results. Wotiz and his associates (Wotiz & Lemon, 1954; Wotiz, Lemon & Voulgaropoulos, 1954) add the substrate to the incubation medium in solution in propylene glycol: using paper chromatography they have been able to demonstrate enzymic transformations of testosterone by a variety of tissues of which liver was the least active, contrary to the findings of Samuels, McCauley & Sellers (1947).

The development of methods for determining microgram quantities of testosterone and metabolites as Δ^4 -3-ketones and as 17-ketones in purified incubation extracts, and the recognition of the importance of cofactor activity (DPN in the presence of nicotinamide, citrate) are due to Samuels and his school, who obtained results which

are in many points at variance with those of other workers. The position of the intermediates in their metabolic scheme must remain uncertain, until individual metabolites have been characterized. In the papers cited above, Kochakian and his school report that androst-4-ene-3:17-dione accumulates. Samuels and his school find that, while the conversion of testosterone into androst-4-ene-3:17-dione can be brought about by a purified preparation of a DPN-linked liver dehydrogenase, this steroid does not accumulate if the hormone is incubated with liver mince.

The present paper reports a new attack on the problem. A number of micro procedures were applied, including tracer studies with [4-¹⁴C]testosterone, which was synthesized for the purpose of these experiments; conditions were used which favour 17-ketosteroid formation and ensure true aqueous solution of the substrate in the incubation medium.

EXPERIMENTAL

Melting points were observed on a microscope hot stage and are corrected.

Materials

Nicotinamide (Hopkin and Williams) was used without purification. DPN of 90% purity was kindly provided by Dr G. E. Glock (Courtauld Institute); material of 80% purity, purchased from C. F. Böhringer and Söhne, G.m.b.H., Mannheim, was used for the incubation of [4-¹⁴C]testosterone. DPN was measured by enzymic assay with yeast alcohol dehydrogenase (Horecker & Kornberg, 1948; Racker, 1950).

Testosterone was purified by two recrystallizations from acid-free ethyl acetate, followed by adsorption on and elution from a column of 20 times its weight of neutralized alumina (Peter Spence and Co., grade H) prepared in benzene (A.R.). Pooled fractions of the benzene eluate yielded a product of m.p. 156.5–157°.

All solvents were redistilled, unless stated otherwise. Acetone was dried over CaCl₂ and distilled from anhydrous K₂CO₃. CHCl₃ was dried over CaCl₂ and freshly distilled before use. For spectrographic and colorimetric purposes, the 95% ethanol used was 'Spiritus vini rectificatus' (James Burrough Ltd.). In 17-ketosteroid and thiosemicarbazone determinations, 95% ethanol consistently gave lower blank values than the absolute ethanol available in this laboratory.

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In adsorption chromatography of incubation extracts alumina (Savory and Moore; activity grade II-III, Brockmann & Schodder, 1941) was used throughout. In partition chromatography Hyflo-Supercel (Johns-Manville Co.) and dimethyldichlorosilane-treated Hyflo-Supercel were the supporting phases. The Hyflo-Supercel was treated with 10N-HCl for 24 hr. and washed with water till free from acid. The lightest particles were removed by decantation.

2:4-Dinitrophenylhydrazine was used without further purification. Thiosemicarbazide was decolorized by treatment with animal charcoal in boiling water and recrystallized to give white needles, m.p. 185-186° (decomp.).

Methods employed for the separation, identification and determination of C₁₉-ketosteroids

Unless stated otherwise, spectrophotometric measurements were made in a 1.0 cm. cell with either a Hilger Uvispek or a Unicam SP. 500 spectrophotometer. The infrared spectra were taken in CS₂ solution by means of a Perkin-Elmer double-beam recording infrared spectrometer (Model 21), using a microcell (3 mm. thickness).

The properties of testosterone, androst-4-ene-3:17-dione and their derivatives summarized in Table 1 were found to be useful for identification purposes.

αβ-Unsaturated ketonic structure. The amount of αβ-unsaturated ketone in a fixed volume of ethanol was determined by comparing the intensity of the ultraviolet absorption band at 240 mμ., against a solution of a suitable blank extract, with standard solutions of testosterone and androst-4-ene-3:17-dione. Optical densities were measured at 230, 240, 250 and 300 mμ.

Separation and identification of ketosteroids as 2:4-dinitrophenylhydrazones. Testosterone 2:4-dinitrophenylhydrazone and androst-4-ene-3:17-dione bis-2:4-dinitrophenylhydrazone were prepared as reference compounds (Table 1). Mixtures of the former (0.1-1.0 μmole) and the latter (0.1-0.5 μmole) could be separated quantitatively by adsorption chromatography on alumina (3 g.) prepared in *n*-hexane-benzene (1:1) (Tables 1 and 2).

Microgram quantities of ketosteroids were converted into their 2:4-dinitrophenylhydrazones by the procedure of Reich, Sanfilippo & Crane (1952); excess of reagent was destroyed by treatment with hot Benedict's solution as there described. Treatment with pyruvic acid was suggested

Table 1. *Properties of testosterone, androst-4-ene-3:17-dione and their derivatives*

Free steroids	Testosterone	Androst-4-ene-3:17-dione
Elution from alumina (micro procedure)	Eluted with 0.1-0.2% ethanol in benzene	Eluted with benzene
Elution from Hyflo-Supercel (ethylene glycol-cyclohexane) (for details see text)	Eluted with 55-72 ml.	Eluted with 8-16 ml.
Infrared maxima (CS ₂) (ketone region)	1675 cm. ⁻¹ (sharp peak, Δ ⁴ -3-ketone)	1675, 1740 cm. ⁻¹ (sharp peaks, Δ ⁴ -3-ketone and 17-ketone respectively)
Alkaline <i>m</i> -dinitrobenzene (solution)	λ _{max} ^{EtOH} : not determined	λ _{max} ^{EtOH} : 520 mμ.
Alkaline <i>m</i> -dinitrobenzene (paper)	Blue (fading rapidly)	Violet (stable colour)
2:4-Dinitrophenylhydrazones		Bis derivative
Melting points	206-208°	No change below 280°
Colour	Light red (cryst. from EtOH)	Deep red (cryst. from CHCl ₃ /EtOH)
Previously prepared by	Johnston (1947)	—
λ _{max} . (CHCl ₃)	390 mμ.	380 mμ.
ε _{max} . (CHCl ₃)	2.95 × 10 ⁴	5.05 × 10 ⁴
Found	N, 11.8%	C, 57.5; H, 5.1; N, 17.0%
Calc.	N, 11.95% (C ₂₅ H ₃₂ O ₅ N ₄)	C, 57.6; H, 5.3; N, 17.3% (C ₃₁ H ₃₄ O ₆ N ₈)
Adsorption chromatography on alumina	Eluted with benzene-CHCl ₃ (3:1) (sharp, light red band)	Eluted with benzene (yellow zone)
Thiosemicarbazones (only the testosterone derivative was prepared in milligram quantity)		
Melting points	152° (decomp.)	—
Colour	Very light yellow	—
Previously prepared by Ercoli, Koller & de Ruggieri (1951)		
λ _{max} ; ε _{max} . (MeOH)	247.5 mμ.; 1.11 × 10 ⁴	270 mμ.; 2.34 × 10 ⁴ *
λ _{min} ; ε _{min} . (MeOH)	265 mμ.; 7.84 × 10 ³	—
λ _{max} ; ε _{max} . (MeOH)	300-302 mμ.; 3.34 × 10 ⁴	300-302 mμ.; 3.48 × 10 ⁴ †
Elementary analysis	Results not reproducible	—
	MeOH	MeOH
* (Based on log (I ₀ /I) androst-4-ene-3:17-dione bis TSC - log (I ₀ /I) testosterone TSC).	270 mμ.	270 mμ.
	MeOH	MeOH
† (Based on log (I ₀ /I) androst-4-ene-3:17-dione bis TSC - log (I ₀ /I) androsterone TSC).	300-302 mμ.	300 mμ.
Androsterone TSC; λ _{max} . 270 mμ.; ε _{max} . (MeOH): 2.32 × 10 ⁴ .		

Table 2. Separation of the 2:4-dinitrophenylhydrazones of testosterone and androst-4-ene-3:17-dione

After elution from alumina the derivatives were dissolved in CHCl_3 (10 ml.). Three results for each separation.

Steroid	μmoles	Benzene- CHCl_3 (3:1)		Benzene eluate	
		$E_{\text{CHCl}_3}^{390 \text{ m}\mu}$	Recovery	$E_{\text{CHCl}_3}^{380 \text{ m}\mu}$	Recovery (%)
Testosterone 2:4-dinitrophenylhydrazone	0.2	0.565	96 $\pm$ 0.9	—	—
Androstenedione bis-2:4-dinitrophenylhydrazone (authentic specimens)	0.1	—	—	0.495	98 $\pm$ 0.9
Testosterone*	0.2	0.555	94 $\pm$ 2.3	—	—
Androstenedione*	0.1	—	—	0.490	97 $\pm$ 2.5
Control*†	0.2	0.530	90 $\pm$ 3.3	—	—

* 2:4-Dinitrophenylhydrazones prepared by micro procedure, see text.

† Recovery of testosterone from boiled medium containing inactivated tissue.

(Reich, Crane & Sanfilippo, 1953) as an alternative method, but proved unsatisfactory here, even when freshly distilled acid or purified sodium pyruvate was used, owing to the persistence of neutral ketonic impurities (derivatives, $\lambda_{\text{max}}^{\text{CHCl}_3}$ 360 $\text{m}\mu$; eluted from alumina with benzene).

Determination and identification of ketosteroids as their thiosemicarbazones. Testosterone thiosemicarbazone was prepared as a reference compound (Table 1). For the preparation of the thiosemicarbazones from microgram quantities of ketosteroids, the reagent mixture of Bush (1953) was used (5 mg. recrystallized thiosemicarbazide/ml. 5% acetic acid in methanol). Methanol was distilled from 2:4-dinitrophenylhydrazine, acetic acid from CrO_3 and then from 2:4-dinitrophenylhydrazine. The extract containing the ketosteroid (5–50 $\mu\text{g.}$) in 95% ethanol (0.1 ml.) and the reagent (0.1 ml.) were heated for 20 min. at 95° in loosely stoppered tubes. CHCl_3 (15 ml.) and 0.1N-HCl (5 ml.) were then added immediately, the stoppered tube was shaken vigorously and the HCl layer removed with a pipette. The CHCl_3 solution of the thiosemicarbazone was further washed with 0.1N-HCl (5 ml.) and with water (2 $\times$ 5 ml.). It was then evaporated to dryness under reduced pressure and the residue taken up in sufficient methanol to record, at the absorption maximum, an optical density reading in the most sensitive range of the spectrophotometer. Blank determinations as a check on the 95% ethanol and the reagent were included in each series of determinations.

17-Ketosteroid determinations. The 17-ketosteroid content of incubation extracts was determined by the Callow, Callow & Emmens (1938) modification of the Zimmermann (1936) reaction. A 1% (w/v) solution of purified *m*-dinitrobenzene in absolute ethanol was used. Optical densities at 440, 500, 520, 540 and 600 $\text{m}\mu$. were measured (Unicam SP. 600 spectrophotometer) against a reagent blank. The 17-ketosteroid content of a standard solution was measured with each series of unknowns. The optical density of the reagent blank at 470 $\text{m}\mu$. is a measure of the concentration of interfering impurities in the reagents.

Separation of C_{19} -steroids by adsorption chromatography on alumina. The Dingemans, Huis in't Veld & De Laat (1946) separation of urinary 17-ketosteroids suggested itself as a means of estimating the polarity range of the transformation products produced by incubating testosterone with liver tissue. The micro procedure described by Edwards, Kellie & Wade (1953) was found to be suitable for this purpose. With benzene, 0.1–0.3% ethanol in benzene, and

Fig. 1. Resolution of a mixture of androst-4-ene-3:17-dione (0.4 μmole) (peak a) and testosterone (0.4 μmole) (peak b). $F = 2 \log (I_0/I)_{240 \text{ m}\mu} - [\log (I_0/I)_{230 \text{ m}\mu} + \log (I_0/I)_{250 \text{ m}\mu}]$ measured in 95% ethanol (5 ml.). Column 10 $\times$ 1.0 cm. diam. Hold-up, 5 ml. Standard solutions of the two steroids gave $F = 0.672$ and 0.632 respectively.

0.5–1.0% ethanol in benzene as eluents, known urinary C_{19} -ketosteroids fell into three groups. Benzene eluted the diketones 5 α - and 5 β -androstane-3:17-diones and androst-4-ene-3:17-dione; androsterone, testosterone and 3 α -hydroxy-5 β -androstane-17-one were in the second group; the third included alcoholic 11-oxygenated 17-ketosteroids. A micro procedure for the determination of 17-ketosteroids (Edwards *et al.* 1953) and infrared spectroscopy were employed to analyse the chromatogram fractions.

Separation of C_{19} -ketosteroids by partition chromatography. Hyflo-Supercel columns (10 $\times$ 1.0 cm. diam.) were packed in the manner described by Butt, Morris, Morris & Williams (1951) and Taylor (1954). The system ethylene glycol (stationary phase; 0.5 ml./g. supporting phase) and cyclohexane (mobile phase) was effective in separating androst-4-ene-3:17-dione from testosterone (Fig. 1). The flow rate was adjusted to 5 ml./hr. by means of a head of mobile phase; fractions (1 ml.) were collected with a siphon-operated fraction cutter. The mixture (0.4 μmole of each

component) dissolved in equilibrated mobile phase (0.5 ml.) was added and the tube which had contained the extract and the walls of the column rinsed with another 3×0.25 ml. The solvent was removed in a stream of N_2 and the steroid content of the fractions determined by the methods described. The proportions recovered from the column (single determination) were: androst-4-ene-3:17-dione, 95%; testosterone 91%.

Separation of C_{19} -ketosteroids by paper chromatography. The paper-chromatography system of Savard (1953) was adopted with minor modifications. Light petroleum (b.p. $100-120^\circ$) was the moving phase, and methanol-propylene glycol (1:1, v/v) the stationary phase. Whatman no. 1 filter paper was used without preliminary solvent extraction. A mixture of androst-4-ene-3:17-dione (7.5–15 μ g.) and testosterone (2.5–5 μ g.) could be separated and identified after running the moving phase for 5 hr.

Incubation of testosterone with rat-liver tissue

Incubation conditions. Male white rats on the stock diet (no. 86, North Eastern Agricultural Cooperative Society, Aberdeen), average weight 170 g. and about 6 weeks old, were killed by decapitation followed by exsanguination. The livers were removed as rapidly as possible and covered with isotonic ice-cold saline till minced. For comparative purposes the preparation of the liver mince and the incubation procedure were those described by Samuels *et al.* (1947). The medium consisted of a solution (25 ml.) of DPN (6 μ moles) and 0.04M nicotinamide in 0.04M phosphate buffer (KH_2PO_4 – Na_2HPO_4 ; pH 7.8). Samples (1 ml.) of an ethanolic solution of testosterone (1 μ mole/ml.) were pipetted into 150 ml. conical stoppered flasks. The solvent was removed in a stream of air. Flasks containing no testosterone (blanks) were also included in the series. The residues were then dissolved in ethanol (0.2 ml.) and freshly prepared mince (500 $\pm$ 5 mg.) rinsed into the incubation flask with the medium. The flasks were then filled with O_2 , attached to a rotating drum in a water tank kept at $37.5 \pm 0.1^\circ$ and incubated for 1 hr. The flasks were removed, octan-2-ol (0.2 ml.) was added to each and the contents were boiled for 5 min. to stop enzyme action; the flasks were then allowed to cool to room temperature. Controls were treated similarly, immediately after addition of the mince and before incubation, to obtain recovery data. Ether (20 ml.) was then added, the stoppers were rinsed with 95% ethanol (2 ml.) and the flasks stored in the refrigerator overnight.

Extraction and examination of incubation products

Procedure 1. The inactivated tissue suspensions were transferred to heavy-walled centrifuge tubes (100 ml.). Extraction was effected by vigorous agitation with ether (5×20 ml.) followed by centrifuging at low speed and separation of the ether layers. These were combined and the solvent was evaporated. The crude extracts were purified by chromatography on alumina of the *n*-hexane solutions, in which nicotinamide remained undissolved, followed by partition between *n*-hexane and 70% ethanol (Samuels *et al.* 1947). The purity of the final extracts was adequate for measuring the Δ^4 -3-ketosteroid and the 17-ketosteroid content against an incubation blank. Testosterone was the standard for the Δ^4 -3-ketone, androst-4-ene-3:17-dione for the 17-ketosteroid determinations. Values are expressed as

$10^4 \times \mu$ mole steroid/ μ mole testosterone incubated/0.5 g. tissue (wet wt.)/hr.; range in brackets (3 experiments): controls, 91 ± 0.9 (90–93) (Δ^4 -3-ketone); incubation, 44 ± 2.9 (39–49) (Δ^4 -3-ketone), 54 ± 2.6 (51–58) (17-ketone). Levedahl & Samuels (1950) find 0.50–0.76 μ mole 17-ketosteroid (as androsterone)/2 μ moles testosterone incubated/g. of tissue/hr.

The infrared spectrum of the purified incubation extract had three peaks at 1703, 1740 and at 1676 cm^{-1} , in order of decreasing intensity. The liver blank gave no violet colour with alkaline *m*-dinitrobenzene and showed marked, weak and no absorption at 1703, 1740 and at 1676 cm^{-1} respectively. A portion of the incubation extract in benzene was chromatographed on alumina (300 mg.) prepared in benzene. The eluting solvents were benzene (28 ml.), 0.1% ethanol in benzene (34 ml.), 0.5% ethanol in benzene (28 ml.) and ethanol (10 ml.) (cf. p. 289). Of the fractions collected (2 ml.) alternate ones were tested for the presence of 17-ketosteroids (Fig. 2). Those of the remaining fractions

Fig. 2. Qualitative elution pattern of 17-ketosteroid metabolites. E_g values are 'absorption coefficients' measured with a Spekker photoelectric absorptiometer and an Ilford Spectrum Green filter (maximum transmission at 520 $m\mu$). Cells of 0.5 cm. thickness and an androst-4-ene-3:17-dione standard were employed (17-ketosteroid microprocedure) (Edwards *et al.* 1953).

eluted with the same solvent were combined and the infrared spectra of the CS_2 -soluble residues taken. The benzene fractions contained material absorbing strongly at 1676 and 1742 and very weakly at 1709 cm^{-1} ; fractions eluted with 0.1% ethanol in benzene absorbed weakly at 1743 cm^{-1} , with 0.5% ethanol in benzene strongly at 1742 cm^{-1} . Portions of the control extracts were converted into the 2:4-dinitrophenylhydrazones which were eluted from alumina with benzene– $CHCl_3$ (3:1) ($\lambda_{max}^{CHCl_3}$ 390 $m\mu$). Testosterone recoveries (Table 2) were in good agreement with the values obtained on the original extract by u.v. absorption measurements. Two small, pale bands were eluted from alumina with benzene, when the 2:4-dinitrophenylhydrazone of the blank extract was similarly chromatographed; the eluate with benzene– $CHCl_3$ (3:1)

remained colourless. Chromatography of the 2:4-dinitrophenylhydrazones of the incubation extract gave, under identical conditions, a benzene fraction ($\lambda_{\text{max}}^{\text{CHCl}_3}$ 380 m μ .) and another eluted with benzene- CHCl_3 (3:1) ($\lambda_{\text{max}}^{\text{CHCl}_3}$ 375 m μ .), of approximately equal colour intensities.

Procedure 2. The incubated contents of 20 flasks (5.77 mg. testosterone) were boiled and separately extracted with ether as described above. The residues were triturated with *n*-hexane (20 ml.), the nicotinamide suspensions pooled and centrifuged at low speed. The sediment was resuspended in *n*-hexane (300 ml.), triturated vigorously and centrifuged. The clear *n*-hexane solutions were combined and shaken twice with 70% ethanol (500 ml.), the ethanolic solution was back-extracted with *n*-hexane (2 $\times$ 100 ml.) and water added to reduce the ethanol content to 30%. Extraction by CHCl_3 (3 $\times$ 200 ml.) of the milky-white emulsion, followed by evaporation under reduced pressure below 50°, yielded a yellow-brown, semi-solid oil; weight, after drying over CaCl_2 *in vacuo* overnight, 128.5 mg. 17-Ketosteroid determination indicated a 37.5% conversion by comparison with an androst-4-ene-3:17-dione standard. The extract dissolved in methanol (5 ml.)-acetic acid (0.38 ml.) containing Girard's reagent T (300 mg.) was left to stand overnight at room temperature and was then separated into ketonic and non-ketonic fractions in the usual way. The ketonic fraction, a pale-yellow, partly crystalline solid, weighed 16.7 mg. The coloured solution with *m*-dinitrobenzene and alkali now gave a symmetrical absorption curve between 440 and 600 m μ . with the peak at 520 m μ . [35% conversion (7.05 μ moles) based on the androst-4-ene-3:17-dione standard]. The purified extract gave rise to three peaks in the ketone region of the infrared spectrum, those at 1743 and 1708 cm^{-1} of equal intensity, the third at 1674 cm^{-1} half their value. Optical density measurements at 240 m μ . indicated the presence of 38.3% Δ^4 -3-ketosteroid (7.65 μ moles).

Orange-red 2:4-dinitrophenylhydrazones were precipitated from a portion (3.6%) of the purified extract in 95% ethanol. Adsorption chromatography using two different elution systems was employed in attempts to separate the crude 2:4-dinitrophenylhydrazone mixtures into their constituents (Fig. 3). In system 1 the residue dissolved in (1:1) *n*-hexane-benzene (5 ml.) was chromatographed under 90 mm. pressure Hg on a column of alumina (1.5 g.; 8.5 $\times$ 0.4 cm. diam.) prepared in that solvent mixture. Separation was effected by elution with benzene and benzene-ethanol mixtures. In system 2 the residue likewise dissolved in (1:1) *n*-hexane-benzene (5 ml.) was transferred to a column of alumina (3 g.; 5 $\times$ 1 cm. diam.) and eluted under gravity with benzene, benzene- CHCl_3 , and CHCl_3 -methanol mixtures. The yields of material behaving as androst-4-ene-3:17-dione isolated as the bis-2:4-dinitrophenylhydrazone were 20 and 16% respectively. A number of additional bands with more polar elution characteristics and absorption maxima at lower wavelengths were obtained; testosterone 2:4-dinitrophenylhydrazone was not found.

A portion (10.8%) of the ketonic extract dissolved in benzene containing 0.2% ethanol (1 ml.) was chromatographed under 90 mm. pressure Hg on alumina (1 g.; 0.4 cm. diam.) prepared in this solvent mixture. The extract was eluted from the column with 0.2% ethanol in benzene (64 ml.), 0.75% ethanol in benzene (36 ml.) and 20% ethanol in benzene (10 ml.). The first seven fractions were

combined, the solvent removed in a stream of N_2 , the residue was dissolved in (1:1) *n*-hexane-benzene (1 ml.) and rechromatographed on alumina (300 mg.) prepared in *n*-hexane-benzene (1:1). The 17-ketosteroid content of the fractions (2 ml.) was determined by the micro procedure of Edwards *et al.* (1953); the benzene eluates were determined against an androst-4-ene-3:17-dione standard, and

Fig. 3. Adsorption chromatography of the ketosteroid 2:4-dinitrophenylhydrazones of a portion (3.6%) of the purified incubation extract. (a) System 1: (1:1) *n*-hexane-benzene (20 ml.); benzene (20 ml.); 0.2% ethanol in benzene (60 ml.); 0.75% ethanol in benzene (30 ml.); 5% ethanol in benzene (10 ml.). (b) System 2: (1:1) *n*-hexane-benzene (20 ml.); benzene (14 ml.); (9:1) benzene- CHCl_3 (12 ml.); (3:1) benzene- CHCl_3 (8 ml.); (1:1) benzene- CHCl_3 (8 ml.); (3:1) CHCl_3 -methanol (10 ml.). Values in m μ . are λ_{max} . A, peaks ascribed to androst-4-ene-3:17-dione.

all other fractions against a dehydroepiandrosterone standard (Fig. 4). The presence of saturated diones in benzene fractions 4-7 and of androst-4-ene-3:17-dione (main benzene peak) was indicated. On this basis a 20% conversion into the unsaturated dione was calculated.

The remainder of the extract was chromatographed on alumina (3 g.) in the same manner and an identical elution pattern established by determining the 17-ketosteroid content of every third fraction. Where a peak was obtained, a number of fractions were combined and the infrared spectra of their residues taken. The second benzene fraction, while negative in the alkaline *m*-dinitrobenzene reaction, gave rise to a strong peak at 1739 cm^{-1} , with lower subsidiaries at 1675 and 1775 cm^{-1} , but no corresponding ester absorption between 1220 and 1250 cm^{-1} . As the

main peak of the benzene eluate was approached, the band at 1675 increased in intensity compared with that at 1739 cm^{-1} , until, at the centre, the two were of almost equal height. Sharp, high-intensity bands at 1739 cm^{-1} were characteristic of peak material eluted with 0.75 and 20% ethanol in benzene.

Fig. 4. Differential analysis of 17-ketosteroid metabolites, portion (10.8%) of incubation extract. (a) 0.2% ethanol in benzene (64 ml.); 0.75% ethanol in benzene (36 ml.); 20% ethanol in benzene (10 ml.). (b) The first 7 fractions of (a) re-chromatographed: (1:1) *n*-hexane-benzene (8 ml.); benzene (28 ml.); 0.2% ethanol in benzene (4 ml.).

The residues of the Zimmermann-positive fractions were subjected to paper chromatography. The Bush (1951) system A was used. Two chromatograms were run with the same fractions. I am indebted to Mr E. R. Smith for these paper-chromatography results. After the paper (Whatman no. 2) had been dried, substances were located by dipping the first paper in 2,4-dinitrophenylhydrazine reagent (Savard, 1953) and spraying the second with methanolic sodium hydroxide (Bush, 1951). The presence of androst-4-ene-3:17-dione in the fractions of the main benzene peak was indicated on comparison with an authentic specimen used as a marker. Identical R_F values, orange-yellow 2,4-dinitrophenylhydrazone spots and dull, yellow-brown areas of sodium hydroxide-fluorescence (viewed under u.v. light) were obtained. A number of other substances, with lower R_F values, were located in these and later fractions, reflecting the complexity of the ketonic fraction of the extract. Yellow 2,4-dinitrophenylhydrazone spots close to the solvent front pointed to the presence of saturated diones in the earliest Zimmermann-positive fractions of the benzene eluate. Later chromatogram fractions were resolved more satisfactorily with the *B* (1) system Bush (1951), but identification and proof of derivation from substrate was not possible with the quantities available. The non-ketonic fraction of the incubation extract was not investigated.

Incubation of [4- ^{14}C]testosterone with rat-liver mince

[4- ^{14}C]Testosterone. Labelled hormone with an activity of 42 $\mu\text{C}/\text{m-mole}$ was synthesized by the method of Fujimoto (1951). The crude product of the Grignard reaction of methyl iodide (2.6 mg.; 0.1 mc), diluted to 2 m-moles, and the enol lactone of 17 β -acetoxy-5-oxo-4-nor-3:5-*seco*andro-

stan-3-oic acid was hydrolysed and ring closure effected simultaneously by treatment with ethanolic NaOH. After adsorption of the crude product (210 mg.; 36% yield) on neutralized alumina (4 g.) and elution with benzene the yield of [4- ^{14}C]testosterone (160 mg.; m.p. 152.5–155°) was 28% of the theoretical. A mixture of purified testosterone (95 mg.; m.p. 156.5–157°) and [4- ^{14}C]testosterone (15.6 mg.) in benzene (5 ml.) was chromatographed on alumina (2 g.) prepared in benzene and eluted with the same solvent. Of the fractions (10 ml.) collected the residues with the highest melting points were dissolved in ether and combined, and the solvent was removed cautiously. The white, crystalline material (64 mg.; m.p. 155–156°) thus obtained had an activity of $20.4 \times 10^{-3} \mu\text{C}/\text{mg}$. In 95% ethanol solution it showed the characteristic u.v. absorption maximum at 240 $\text{m}\mu$. and its thiosemicarbazone showed the expected peak at 300 $\text{m}\mu$. The corresponding values of ϵ , for the thiosemicarbazone the ratio ($E_{300 \text{ m}\mu}/E_{265 \text{ m}\mu}$), and the infrared spectrum (in a KBr window) were in good agreement with those of the testosterone standard. Paper chromatograms (cf. p. 290), in contact with the moving phase for 5, 10, and 29 hr., demonstrated the coincidence of the radioactive zone with the blue alkaline-*m*-dinitrobenzene zone and agreement of the R_F value with that of a testosterone marker. In contrast with a recent report by Savard, Wotiz, Marcus & Lemon (1953), who used a tracer of higher specific activity, no residual radioactivity could be detected at or near the starting line. The radioactivity along the paper strip was measured by dragging it at a steady known speed between oppositely placed thin end-window Geiger counters.

Radioactivity measurements. When sufficient material was available, radioactivity measurements were made by plating the sample at infinite thickness on polythene disks (1 cm^2 area) and recording the activity with an end-window Geiger counter. Radioactivity measured at infinite thickness was related to counts at infinite thinness as follows: a methanolic standard solution (10 ml.) of the diluted [4- ^{14}C]testosterone (3.31 mg.; $20.4 \times 10^{-3} \mu\text{C}/\text{mg}$.) was prepared, portions were pipetted on to thin lens paper covering the circular plating area (2 cm^2) of polythene disks and the solvent was cautiously evaporated under an infrared lamp. The radioactivity (counts/min. at infinite thinness) was simply proportional to the volume of standard solution taken and the graph relating them was practically a straight line between 0.05 and 0.5 ml.; the activities of the small portions were too low, possibly owing to pipetting errors: 0.05 ml. (61), 0.10 ml. (125), 0.20 ml. (282), 0.50 ml. (748) (no. of counts/min. at infinite thinness in parentheses). The data represent the mean of three results (range $\pm 2\%$). Extracts and residues to be counted *in toto* were dissolved in CHCl_3 or methanol (0.2 ml.), the solutions and several rinses were transferred to the disk, 0.2 ml. at a time, and the solvent was evaporated.

Partition chromatography of [4- ^{14}C]testosterone. The labelled hormone (1.45 mg.; $20.4 \times 10^{-3} \mu\text{C}/\text{mg}$. (6180 counts/min.)) was dissolved in equilibrated cyclohexane (0.5 ml.) and transferred with several rinses ($4 \times 0.25 \text{ ml.}$) to a partition chromatography column (12 $\times$ 1 cm. diam.) (p. 289). The fractions were combined as follows (counts/min. in parentheses): nos. 1–6 (0), 7–26 (200), 27–49 (45), 50–79 (5290), 80–110 (270), total eluate (5805). A paper chromatogram of the remainder (one-half) of fractions 7–26 containing radioactive contaminant was left in contact with the moving phase for 5 hr., air-dried and treated with

alkaline-DNB reagent; a brown spot, with a slightly lower R_F than the value for androst-4-ene-3:17-dione, developed and faded rapidly.

Preparation of [$4\text{-}^{14}\text{C}$]testosterone solution. [$4\text{-}^{14}\text{C}$]Testosterone (10 mg.) was added to 0.04M phosphate buffer ($\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$, pH 7.4), the suspension triturated vigorously at 0° in a motor-driven glass homogenizer of the Potter-Elvehjem type and centrifuged, until an optically clear supernatant solution resulted. The concentration of the solution (assuming $\epsilon_{248\text{m}\mu}^{\text{H}_2\text{O}}$ 15 500) was 86 $\mu\text{g.}/10\text{ ml.}$, equivalent to 365 counts/min. Extraction of portions (10 ml.) with ether ($3 \times 10\text{ ml.}$) and CHCl_3 ($2 \times 10\text{ ml.}$) and plating of the concentrated extracts yielded 341 counts/min. (three determinations). Since aqueous solutions of testosterone kept at 0° appeared to encourage the growth of fungus after quite short periods of storage (48 hr.), the substrate solution was used immediately after preparation.

Incubation conditions. The livers of four male hooded rats (160 g. average weight), maintained on the Institute

diet (no. 41), were minced as described by Samuels *et al.* (1947). Fresh liver mince ($500 \pm 5\text{ mg.}$; 152 mg. dry weight) was rinsed into each of ten 150 ml. incubation flasks with a 0.04M phosphate buffer solution (20 ml.; $\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$, pH 7.4) containing DPN (6 μmoles), nicotinamide (0.08M) and penicillin G (10 000 i.u.). [$4\text{-}^{14}\text{C}$]Testosterone solution (20 ml.) was then added and a stream of oxygen passed over the surface of the incubation medium for 1 min. The flasks were incubated for 1 hr. at $37.5 \pm 0.1^\circ$ as described (cf. p. 290) and enzyme action stopped by rapid heating to the boiling point. The contents of 10 control flasks containing buffer, DPN, nicotinamide and penicillin G, but no substrate solution, were boiled immediately after addition of the liver mince; [$4\text{-}^{14}\text{C}$]testosterone solution (20 ml.) was added and the suspensions of inactivated tissue were oxygenated and boiled for another 5 min. Fresh penicillin G (10 000 i.u.) was added, at room temperature, to each of the incubation and control flasks. They were then stored at 0° overnight and the extraction was carried out on the following morning.

Fig. 5. Method of extraction and preliminary separation of products from boiled digests. I and C denote incubation and control respectively; no. of counts/min. at infinite thinness in parentheses. Total extracted: incubation (5380), control (5275).

Extraction and examination of the incubation products

Extraction and preliminary separation. The contents of the incubation and control flasks were bulked separately, the flasks rinsed with water and the two boiled digests extracted in identical fashion. The inactivated minces were dispersed in the medium in a Townson and Mercer homogenizer run at high speed. Details of the extraction and preliminary separation of the products are given in Fig. 5. The CHCl_3 , acetone and fat extracts (C, A and F) of the incubation digest were dissolved in CHCl_3 and combined; the solution was, however, lost during manipulation.

Partition chromatography of extracts. (i) *Purified ether extract, incubation.* The extract, dissolved in equilibrated cyclohexane (1 ml.), was transferred to a Hyflo-Supercel column (12 × 1 cm. diam.). The residues of every third fraction were counted *in toto*. Sharp peaks were obtained at fractions 15 (fractions 9–12 form a shoulder) and 120. Elution with methanol stripped the stationary phase from the column; as the CHCl_3 solutions of these fractions were washed with water to remove ethylene glycol, some highly polar material might have been lost in the process. Peak fractions were combined, the radioactivity of portions was measured (Table 3), residues were examined by paper

Table 3. Partition chromatography of purified ether extracts, $[4\text{-}^{14}\text{C}]$ testosterone, incubation and control experiments

Moving phase: fractions 1–110, cyclohexane; fraction 111–128, methylene chloride; fractions 129–141, methanol.

Fraction numbers	Incubation (PE_I) (counts/min. at infinite thinness)	Control (PE_C) (counts/min. at infinite thinness)
Extract	3600	3060
1–6	0	0
7–26	985	165
27–49	Negligible*	64
50–79	Negligible*	2260
80–110	Negligible*	75
111–128	1210	200
129–141	170	48
Total	2365	2812

* Individual fractions in these groups did not exceed 8 counts/min. The fractions were not pooled and counted *in toto*, as they were required for paper chromatography.

chromatography and the thiosemicarbazones prepared (Fig. 6). A paper chromatogram of fraction 7, portions of combined fractions 11, 13, 14, 16, 17, 19, fractions 24–100 and authentic androst-4-ene-3:17-dione (25 $\mu\text{g.}$) was scanned for radioactivity and then developed with alkaline *m*-dinitrobenzene. The marker and the samples produced violet spots of comparable colour stabilities at the same distance from the starting line and the radioactivities were concentrated in the coloured zones. Fraction 7 gave one weak violet spot (androstenedione) superimposed on a rapidly fading brown one; no Zimmermann-positive ketosteroids could be detected on the chromatogram of fractions 24–100. Portions of the pooled methylene chloride fractions containing peak radioactivity were run with two systems; the positions of the alkaline-*m*-dinitrobenzene

stains on the chromatograms are shown in Figs. 7 and 8. $2\alpha:17\beta$ -Dihydroxyandrost-4-en-3-one did not show up with alkaline DNB, but gave rise to yellow soda-fluorescence. Radioactivity was associated with the area of the strip occupied by the three spots (Fig. 7), but resolution into sharp peaks could not be obtained. The Bush chromatogram (Fig. 8) was scanned unsuccessfully after dipping in alkaline *m*-dinitrobenzene, owing to spread of the radioactivity over the entire paper strip.

(ii) *Ether extract, control.* The extract was subjected to partition chromatography, fractions (1 ml.) were combined in groups and their radioactivities determined (Table 3). The paper chromatogram of fractions 7–26 (one-half) (thiosemicarbazone spectrum, Fig. 6) gave rise to a rapidly fading brown spot with an R_F value slightly lower than that of androst-4-ene-3:17-dione (cf. p. 293). Portions of fractions 50–79 and the methylene chloride fractions 111–128 (*in toto*) showed the characteristic blue reaction of testosterone at the same positions on the chromatogram as an authentic specimen. Fractions 27–49 and 80–110 gave blank chromatograms after exposure to the alkaline-*m*-dinitrobenzene reagent.

Fig. 6. Ultraviolet absorption of the thiosemicarbazones of $[4\text{-}^{14}\text{C}]$ testosterone metabolites in methanol measured against reagent blanks. (a) ●, Standard androst-4-ene-3:17-dione (0.2 μmole ; 15 ml.); ×, fractions 111–128 (6.6%; 5 ml.) PE_I ; ○, fractions 8 and 10 (*in toto*; 15 ml.) PE_I ; (b) ×, fractions 7–26 (8.4%; 5 ml.) PE_I ; ●, fractions 7–26 (12.5%; 5 ml.) PE_C ; ○, reagent blank (15 ml.) read against methanol.

(iii) *Combined F-, C- and A-extracts, control.* A preliminary purification of the dark-brown oily residue was effected by reversed-phase partition chromatography. Hyflo-Supercel, treated with dimethyldichlorosilane (kindly given by Dr A. T. James), was impregnated with 0.5 ml. equilibrated stationary phase (CCl_4)/g. supporting phase; the moving phase was equilibrated with 80% methanol. The extract in methanol (0.5 ml.; 1640 counts/min.) was stirred into a paste with the Hyflo-Supercel (2 g.) and

transferred to the top of the column (12 × 1 cm. diam.) with 80 % methanol (3 × 1 ml.). A total of 1480 counts/min. were recovered from the column in six consecutive fractions (1 ml.). The first and last three fractions were separately partitioned between *n*-hexane and 70 % ethanol as described and the CHCl_3 extracts of the ethanolic (30 %) solutions subjected to paper chromatography. The characteristic, rapidly fading blue stains were found at the same distance from the starting line as the spot produced by the testosterone marker.

Fig. 7. Polar transformation products of $[4\text{-}^{14}\text{C}]$ testosterone (PE_1 , fractions 111–128; cf. Table 3) and reference compounds run with the modified Savard system. 6T, 6 β :17 β -dihydroxyandrost-4-en-3-one; T, testosterone; M, metabolites, Ad, adrenosterone; 2T, 2 α :17 β -dihydroxyandrost-4-en-3-one; St, starting line; B, blue, V, violet alkaline DNB colours; S, soda-fluorescence.

DISCUSSION

This paper reports a preliminary investigation of the products of the *in vitro* metabolism of testosterone by rat-liver mince under conditions favouring 17-ketosteroid formation. This choice provides a suitable starting-point for pathway studies, since evidence has been presented that 17-ketones are intermediates in the *in vitro* transformation of the hormone to non-ketonic substances (Samuels & West, 1952); a number of experimental methods are now available for the identification and determination of microgram quantities of ketosteroids in tissue extracts, whereas the analysis of alcoholic, non-ketonic steroids is much more difficult. Testosterone has been incubated for 1 hr. (cf. Samuels, Sweat, Levedahl, Pottner & Helmsreich, 1950) in a simple phosphate buffer ($\text{KH}_2\text{PO}_4\text{--Na}_2\text{HPO}_4$) supplemented with

DPN and nicotinamide (cf. Mann & Quastel, 1941), in the absence of heavy metals (to minimize attack of the substrate due to processes of oxidative phosphorylation, Friedkin & Lehninger, 1949), and at a pH (7.8) close to the optimum established with a purified, cell-free preparation of the DPN-linked liver dehydrogenase (Sweat, Samuels & Lumry, 1950). By employing concentrations of

Fig. 8. Polar transformation products of $[4\text{-}^{14}\text{C}]$ testosterone (PE_1 , fractions 111–128; cf. Table 3) and reference compounds run on Whatman no. 2 paper with the system toluene-ligroin-methanol-water 10:10:9:1 for 2.5 hr. after equilibration for 3 hr. at 31°. 6T, T, M, Ad, 2T, St, B, V, as in Fig. 7. 11A, 11 β -hydroxyandrost-4-ene-3:17-dione; 11Te, 3 α :11 β -dihydroxy-5 β -androst-17-one; 11T, 11 α :17 β -dihydroxyandrost-4-en-3-one; 17T, 17 α -hydroxyandrost-4-en-3-one *cis*-testosterone; Te, 3 α -hydroxy-5 β -androst-17-one; An, androst-17-one; D, dehydroepiandrosterone; BV, blue-violet alkaline DNB colour. I am indebted to Dr I. E. Bush for these paper-chromatography results.

testosterone which are in solution in the aqueous medium, substrate:tissue ratios which approach physiological conditions have been possible. In the first isolation experiment the hormone was dissolved in a small quantity of ethanol (1 μ mole testosterone/0.2 ml. ethanol/25 ml. medium). $[4\text{-}^{14}\text{C}]$ Testosterone has been incubated in an entirely aqueous medium at pH 7.4.

Substantial loss of $\alpha\beta$ -unsaturation and formation of material reacting as 17-ketosteroids with alkaline *m*-dinitrobenzene are confirmed (Samuels *et al.* 1947), but these structural changes cannot be explained by those authors' assumptions (Samuels & West, 1952). Only trace amounts of 3-hydroxy-17-ketones (or of other alcoholic ketosteroids of similar polarity) have been found in the incubation extracts and no unchanged testosterone has been identified. The recovery of micromolar quantities of testosterone from boiled controls has been satisfactory (91%). Enzymic transformation into androst-4-ene-3:17-dione accounts for at least 20% of the added hormone, and this metabolite is largely responsible for the remaining ultraviolet absorption peak at 240 $m\mu$. The evidence that androst-4-ene-3:17-dione is a metabolic product is as follows: the elution characteristics of the free metabolite in adsorption chromatography and column and paper partition chromatography systems and of the bis-2:4-dinitrophenylhydrazones in adsorption chromatography agree with those of an authentic specimen. With alkaline *m*-dinitrobenzene the metabolite reacts as a typical 17-ketone both in solution and on paper. The thiosemicarbazone and the 2:4-dinitrophenylhydrazone derivatives have the expected maxima in the ultraviolet and visible regions of the spectrum respectively, and the free metabolite shows the required peaks in the ketone region of the infrared spectrum. Less polar 17-ketosteroids, possibly saturated 3:17-diones, appear to be formed in trace amounts. Incubation of [4- 14 C]testosterone has confirmed these findings.

Evidence is now presented that compounds more polar than testosterone are major metabolites. Incubation of the 14 C-labelled compound has demonstrated that these substances are in fact derived from the substrate. On adsorption and partition chromatography they show elution characteristics similar to 11-oxygenated 3-hydroxy-17-ketosteroids and in solution give violet colours with *m*-dinitrobenzene and ethanolic alkali. Paper chromatography of the [4- 14 C]testosterone incubation extract points to the presence of two polar 17-ketosteroids and a Δ^4 -3-ketosteroid or saturated 3-ketosteroid without carbonyl at C-17; 3 α :11 β -dihydroxy-5 β -androstan-17-one has a slightly higher R_f value than that of the less polar of the two 17-ketones. After considerable manipulative and one accidental loss of material, radioactivity measurements accounted for 30% of the added [4- 14 C]testosterone as androst-4-ene-3:17-dione (11%) and unidentified, polar ketosteroids (19%). These results show that it is not permissible to measure the 17-ketosteroid content of the incubation extract against a single standard; since the colorimetric equivalents (at 520 $m\mu$)/mole for

androst-4-ene-3:17-dione, androsterone and 3 α :11 β -dihydroxy-5 β -androstan-17-one are 127, 100 and 65 respectively (personal communication from Dr A. E. Kellie; cf. Wilson, 1954), a differential analysis of the extract is required.

Samuels *et al.* (1947) have observed that the addition of citrate enhances the destruction of the $\alpha\beta$ -unsaturation of testosterone by liver tissue. It is not clear whether this action is specific. The mechanism of action of citrate may be explained by the coupling of the aconitase reaction and the TPN-specific oxidation of isocitric acid with Δ^4 -3-ketone reduction. A stereospecific addition of hydrogen (cf. Loewus, Westheimer & Vennesland, 1953) catalysed by two enzymes would account for the isolation of labelled androsterone and 3 α -hydroxy-5 β -androstan-17-one (aetiocholanolone) in varying ratios from urine extracts after administration of labelled testosterone (Fukushima, Bradlow, Dobriner & Gallagher, 1954). As members of the citric acid cycle have been excluded from the experiments now reported, mediation of hydrogen transfer to the Δ^4 -3-ketone group by reduced triphosphopyridine nucleotide is limited by the concentration of endogenous substrates. As expected, enzymic oxidation of the 17 β -hydroxyl group occurs readily under the conditions of the experiments now reported. Whether the reduced DPN formed by dehydrogenase action at C-17 is required and available as a cofactor for the reduction of the $\alpha\beta$ -unsaturation remains to be tested by experiment.

Hydroxylation reactions are postulated to account for the formation of the polar 17-ketosteroids. C_{19} -Steroids with hydroxyl groups at C-11 or C-16 have been isolated from human urine (Samuels & West, 1952; Hirschmann, 1943; Lieberman, Praetz, Humphries & Dobriner, 1953); a steroid oxygenated at C-7 has also been found but may be an artifact (Fukushima, Kemp, Schneider, Stokem & Gallagher, 1954). It has generally been assumed that 11-oxygenated steroids have an adrenal origin (Dobriner & Lieberman, 1950). The mechanism of this hydroxylation reaction has been widely studied (Sweet, 1951; Hayano, Dorfman & Yamada, 1951; Kahnt & Wettstein, 1951; Brownie & Grant, 1954); the adrenal enzyme appears to be concentrated in the mitochondria and can be primed by any citric acid cycle intermediate, preferably fumarate or α -oxoglutarate. Little information is available as to the site of 16-hydroxylation (cf. Schneider & Mason, 1948). The possibility that 16-hydroxy-17-ketosteroids may have been formed under the conditions described above is of particular interest, as further metabolism of these compounds may result in the rupture of the steroid D-ring. It is suggested that a search among the steroids of the more highly oxygenated urinary fractions for excretory end-

products of testosterone metabolism of this type may prove fruitful.

Further investigation is required to identify these polar compounds and to account for the testosterone added in terms of its major metabolites.

SUMMARY

1. The metabolism of testosterone by rat-liver mince *in vitro* has been studied at concentrations ensuring aqueous solution of the hormone and under conditions favouring 17-ketosteroid formation.

2. Testosterone has been incubated in phosphate buffer (pH 7.8) supplemented with diphosphopyridine nucleotide and nicotinamide, but in the absence of heavy metals. At least 20 % of the substrate is converted into androst-4-ene-3:17-dione. Enzymic transformation into unidentified polar ketosteroids accounts for a large fraction of the incubated hormone. Hydroxylation reactions are postulated to explain their formation. Androst-4-ene-3:17-dione, 3 α -hydroxy-5 β -androst-17-one, and residual testosterone have not been detected in the extracts.

3. Incubation of [4-¹⁴C]testosterone at pH 7.4 has confirmed these findings.

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