The Synthesis and Purification of 4-[^{14}C]- and 7α[^{3}H]-androsta-4,16-dien-3-one

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SUMMARY

The synthesis of radioactive androsta-4,16-dien-3-one from both 4-[^{14}C]- and 7α-[^{3}H]-testosterone has been achieved using modifications of a method due to Henbest. Contrarily to this earlier report, yields of purified androstadienone were approximately 6 %. Other compounds, which may be ring D isomers, have been separated by column chromatography on silicic acid impregnated with silver nitrate. Labelled 17-epitestosterone acetate is also formed in this reaction, and may be readily isolated.

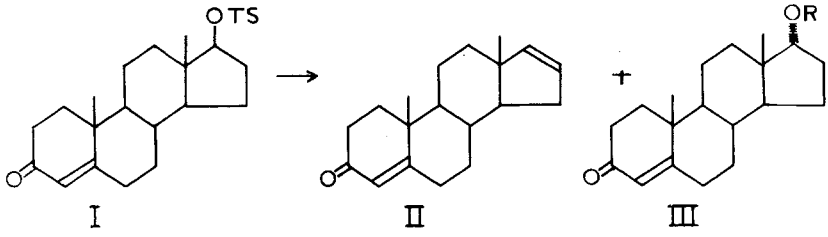
Androstadienone (II) (androsta-4,16-dien-3-one) is now known to be biosynthesised in boar testis from pregnenolone [1], and is formed *in vitro* from androsta-5, 16-dien-3β-ol in the presence of NAD^+ [2]. It is also formed from testosterone in very small yields in human liver and rat testis preparations [3,4]. More recently the compound has been isolated from human male peripheral blood plasma [5].

Labelled androstadienone was required in order to study its metabolism in more detail and for use as a tracer in extractions of this and other Δ^{16}-steroids from plasma.

The practical value of a synthesis of any compound labelled to high activity is enhanced if the chemical transformations involved be few and of a simple nature, since of necessity the manipulation of very small quantities of highly radioactive material is called for. With these limitations in view, a careful study of the available synthetic routes [6-15,18] to Δ^{16}-androstene derivatives revealed only two [15,18] seemingly practical approaches to the preparation of androsta-4,16-dien-3-one (II) from labelled precursors.

Since labelled testosterone was readily available the methods of Miescher [6] and of Prelog [7,8] were not considered, because they required 17-epitestosterone. The procedures according to Sondheimer [9], Barton [10], Caglioti [11-13] and Shapiro [14] involved three- or four-step syntheses and were rejected as being impractical on a micro scale.

We investigated a reported [15] dehydration of testosterone to give androstadienone using the N-bromoacetamide-pyridine - sulphur dioxide reagent [16a], but in our hands this procedure did not yield androstadienone.



I II III

a.R = *t*-Bu
b.R = $COCH_3$

This failure led us to adapt a method described by Brown [17] for the preparation in high yield of 3-methylcyclopentene from *trans*-2-methylcyclopentyl tosylate. Though this type of tosylate is stereochemically analogous to 17α-testosterone tosylate, other reports [16b] describing eliminations in the A and C rings encouraged us to attempt the reaction by heating 17β-testosterone tosylate (I) with potassium *t*-butoxide in *t*-butanol. The results were discouraging; the only product observed was chromatographically distinct from androstadienone and may be testosterone-17α-*t*-butyl ether (III*a*).

Finally we turned our attention to a preparation [18] of androstadienone from the high-temperature acetolysis of 17β-testosterone tosylate (I) using tetra-*n*-butyl ammonium acetate [19,20] in N-methylpyrrolidone. We could not reproduce the yield quoted [18] but under very carefully controlled conditions a 38 % yield of crystalline product was obtained. However, although this material was homogeneous by thin layer chromatography it slowly underwent partial decomposition when stored at room temperature (approximately seven days). Three more recrystallisations were necessary with only minor change in melting point, to obtain crystals which did not subsequently so decompose. The overall yield was thus reduced to around 6 %. We have shown that the initially obtained 'pure' sample of androstadienone was heavily contaminated with material which we tentatively regard as olefinic ring-D rearrangement products.

To carry out this preparation on labelled starting material on a micro scale, in the absence of air and under anhydrous conditions, a standard vacuum

manifold system was employed. In general, the reaction mixtures yielded pale yellow solutions quite different in appearance from those of larger experiments which were invariably dark brown. On occasion, the micro preparations were themselves unusually dark in colour, and this had a profound effect on the ease of purification of androstadienone. In addition, preliminary experiments using low activity 7α-[^{3}H]-testosterone tosylate revealed that, under these rather severe reaction conditions, approximately 15 % of the tritium was lost, probably by exchange with the solvent.

Benzene extracts from non-radioactive preparations were subjected to preparative thin layer chromatography, and a band corresponding to authentic androstadienone could easily be separated from the accompanying 17-epitestosterone acetate (III*b*). However, careful analysis by gas-liquid chromatography demonstrated the presence of at least four compounds, including androstadienone. Subsequent investigation showed these to be separable on a column of silicic acid impregnated with silver nitrate, pure androstadienone (II) being obtained finally in approximately 6 % yield.

Materials and Methods.

Materials. — Benzene, light petroleum (b.p. 80°-100°) and other solvent used in chromatography were purified as described earlier [21]. Pyridine, N-methylpyrrolidone and *t*-butanol were all redistilled and stored over 4A molecular sieve (BDH Chemicals Ltd., Poole, Dorset, England—1/16″ pellets) which had been activated at 320° for 3hrs. 7α-[^{3}H]-testosterone (specific activity 172.8 mC/mM) was synthesised [22] at the Imperial Cancer Research Fund; 4-[^{14}C]-testosterone (specific activity 58.2 mC/mM) was from The Radiochemical Centre, Amersham, Bucks., England. Toluene *p*-sulphonyl chloride was purified before use by the method of Pelletier [23]; tetra-*n*-butyl ammonium acetate was prepared by the method of Luder [19].

Methods. — Thin layer chromatography (TLC) was performed with plates spread with a layer (0.5 mm thick) of Kieselgel G or with Kieselgel H impregnated with $AgNO_3$ [24]. Column chromatography was performed (*a*) on silicic acid impregnated with $AgNO_3$; columns were prepared as previously described [2] using benzene—ethyl acetate (1 : 2, v/v) as eluting solvent or (*b*) on alumina as described before [21] with benzene—light petroleum (b.p. 80°-100°) (1 : 1, v/v) as eluting solvent.

Radioactivity in column fractions was measured with a Beckman Automatic Scintillation spectrometer. Counting efficiencies for ^{14}C and ^{3}H were 83 % and 43 % respectively. Weight of carrier androstadienone was determined by gas liquid chromatography (GLC) using glass columns (length 1.5 m, i.d. 3 mm) packed with silanised Gas Chrom Q (100-120 mesh) which was coated with cyclohexane dimethanol succinate (0.6 %) plus methyl polysiloxane gum (JXR) (0.75 %). Column temperature was 196° and carrier gas flow

rate was 50 ml/min. Cholestane was used as internal standard. Under these conditions the retention time of androstadienone was approximately 8.0 min.

TLC plates were automatically scanned for activity using a Radiochromatogram Scanner system (Panax Equipment Ltd., Redhill, Surrey, England). Efficiencies were approximately 10 % and 1 % for ^{14}C and ^{3}H respectively. Non-radioactive androstadienone was detected on TLC plates with UV light, wavelength 254 nm.

EXPERIMENTAL.

Testosterone-17β-tosylate (I).

Large scale. — Preparations (1-5 g) were carried out using the method of von Schleyer [16c].

Micro scale. — 7α-[^{3}H]-testosterone (3.0 mg, 1.80 mC) and toluene *p*-sulphonyl chloride (9.5 mg) were quickly weighed into the same 5 ml conical flask and treated with dry pyridine (0.25 ml). After storage at room temperature (21°), in the dark, for two days, the reaction mixture was diluted with water (1.0 ml) and extracted with chloroform (4 × 1 ml). The chloroform extracts were washed with ice-cold 2N sulphuric acid (4 × 1.5 ml), saturated sodium bicarbonate solution (2 × 2 ml) and water (2 × 2 ml) respectively, before drying over sodium sulphate. This solution was then transferred to the reaction vessel (as described below) and evaporated in a stream of nitrogen. Final drying was accomplished by storage over P_2O_5.

4-[^{14}C]-Testosterone-17β-tosylate was prepared similarly from 4-[^{14}C]-testosterone (2.47 mg; 0.5 mC).

Interaction of Testosterone-17β-tosylate (I) and potassium t-*butoxide* (cf. ref. 17).

(*a*) *In* t-*butanol.* — The tosylate (I) (114 mg) was suspended in *t*-butanol (5 ml) and stirred, with the temperature at 50°. A 1.0N solution of potassium *t*-butoxide in *t*-butanol (1.0 ml) was added dropwise, producing a yellow solution. Stirring was continued for 2 hr before diluting the reaction mixture with water (5 ml). After acidifying with conc. hydrochloric acid the mixture was extracted with ether (4 × 10 ml). The extracts were washed to neutrality with satd. sodium bicarbonate solution (2 × 5 ml) and water (2 × 5 ml) before drying over sodium sulphate. TLC (benzene-ethyl acetate 3 : 1, v/v) showed the solution to contain two components in approximately equal amounts, one of which was starting material (R_f, 0.45). The other component (R_f, 0.70) was not androstadienone (II) (R_f, 0.53) and it seems likely that this is testosterone-17α-*t*-butyl ether (III*a*).

Repetition of this experiment at the b.p. (83°) for 5 hr gave the same result.

(*b*) *In dimethyl sulphoxide at room temperature.* — The experiment in part (*a*), above, was repeated using dimethyl sulphoxide (2 ml) as solvent. The reaction was set aside at room temperature for 20 hr. Following work-up, TLC showed an almost quantitative recovery of starting material.

Unlabelled androsta-4,16-dien-3-one (II), based on the method of Henbest and Jackson [18]. — Tetra-*n*-butyl ammonium acetate was recrystallised from benzene before use and stored over P_2O_5, under vacuum, overnight. N-methylpyrrolidone (30 ml) was refluxed in an atmosphere of dry nitrogen to remove any dissolved air. After cooling under nitrogen, tetra-*n*-butyl ammonium acetate (6.3 g) and testosterone-17β-tosylate (I, 1.5 g) were added rapidly and the reaction mixture was heated to 160° ± 2° under nitrogen in a pre-heated metal bath. Heating was continued for 4 hr in which time the colour of the solution became dark brown. After diluting with water (50 ml), the mixture was extracted with ether (4 × 10 ml). The extracts were washed with water and dried over sodium sulphate. TLC showed this solution to contain an approximately equal mixture of androstadienone (II) and 17-epitestosterone acetate (III*b*). Evaporation to dryness yielded a pale yellow mobile oil. A solution in benzene was chromatographed on alumina (Martindale Samoore, Grade 2; column 70 × 2.5 cm). Elution with benzene—light petroleum (40°-60°) (4 : 1, v/v) (1,000 ml) yielded the androstadienone (350 mg, 38 %) as colourless needles, m.p. 130-4° (Lit. 131°-3°) [18]. On storage over P_2O_5 these needles became slightly sticky and pale yellow in colour. Recrystallisation (× 3) from a mixture of light petroleum (60°-80°)—isopropyl ether (1 : 1, v/v) gave colourless flat prisms, m.p. 135-6° (53 mg; 6 %), unaffected by prolonged storage.

7α-[^3H]- and 4-[^{14}C]-androsta-4,16-dien-3-one. — The reaction vessels were thick-walled glass tubes (i.d. 1.5 cm, length 16 cm) fitted with a B14 cone, and having a constriction (0.5 cm i.d.) below the cone to facilitate sealing. Standard high vacuum procedures were employed.

The reaction vessels (A), containing the tosylates (see above), were charged with tetra-*n*-butyl ammonium acetate (20 mg) and then pumped down on the vacuum line to 5 × 10^{-5} torr. The evacuated system was isolated from the vacuum pump and N-methylpyrrolidone, stored over 4A molecular sieve and previously de-gassed in the usual way [22], was distilled (1.0 ml) by allowing it to condense into A, previously cooled in liquid nitrogen. Before allowing A to reach room temperature, the constriction was sealed. The reaction vessels were removed from the line, immersed in an oil bath, and heated at 160° for 4 hr.

Purification. — In pilot experiments using unlabelled testosterone as starting material, reaction mixtures were diluted with water (5 vols.) and then extracted with benzene (3 × 0.5 ml), the extracts dried over Na_2SO_4 and then evaporated

to dryness under reduced pressure (water pump vacuum) at a temperature not exceeding 45°. Earlier experiments with Δ^{16}-steroids have shown that considerable losses were incurred if the temperature was allowed to rise higher than this [5]. A portion of this extract was subjected to TLC on Kieselgel G using benzene - ether (9 : 1, v/v) [25] as solvent and double running. GLC of the zone running with the mobility of authentic androstadienone (11.5 mm from the origin), revealed that at least three compounds were present together with androstadienone. In this system 17-epitestosterone acetate, another product of the reaction [18] ran 7.5 mm from the origin and could be detected in UV light (254 nm). TLC of portions of the reaction mixture on silicic acid impregnated with $AgNO_3$ and with benzene-ethyl acetate (2 : 1, v/v) as solvent, achieved a separation of androstadienone (R_f, 0.27) from three impurities which ran much faster (R_f, 0.71). In this system 17-epitestosterone acetate had R_f, 0.13. Although this method achieved an excellent separation of androstadienone from other products of the reaction, recoveries of only 30 % from the plates were obtained by elutions with solvents such as chloroform or methylene dichloride (3 × 2 ml). If the appropriate zone was sucked off the plate and treated with distilled water (1.5 ml) and 2N sodium hydroxide (0.5 ml), extraction of the mixture with ether (3 × 2 ml) gave recoveries of 85-90 % [2]. Treatment of androstadienone with sodium hydroxide solution, however, seemed to cause extensive decomposition, as shown later when the eluted

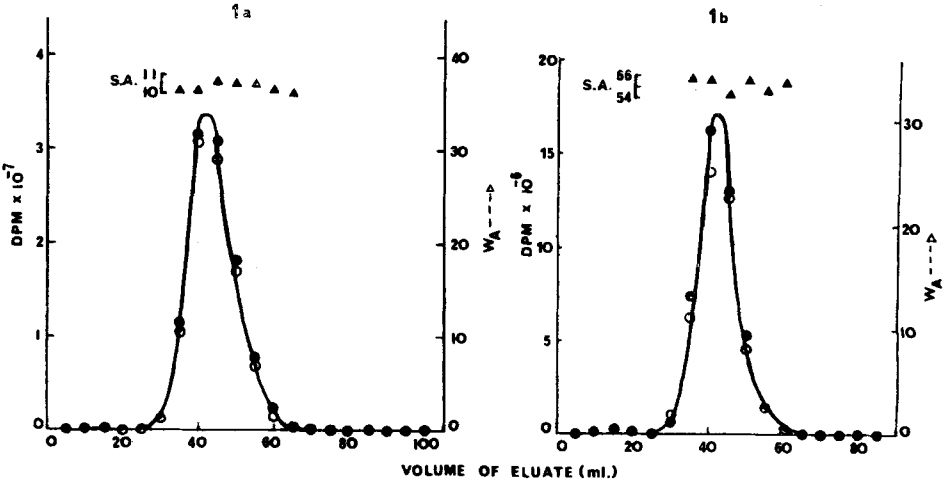


FIG. 1. Alumina column chromatography with light petroleum-benzene (1 : 1, v/v) as eluant of (*a*) 7α-[^{3}H] androstadienone and (*b*) 4-[^{14}C] androstadienone synthesised from radioactive testosterone. Initial purification was achieved by chromatography on columns of silicic acid impregnated with $AgNO_3$ (see text). The weight of androstadienone, W_A (μg) (O) was determined by gas-liquid chromatography and radioactivity (●) by liquid scintillation counting. (▲), specific activity for (*a*) dpm × 10^{-5} μg and for (*b*) dpm × 10^{-4}/μg.

material was re-run on Kieselgel G plates. This method was therefore abandoned as a means of purification.

The separation of 7α-[^{3}H]-androstadienone was achieved instead by applying dry benzene extracts (0.5, 0.25, 0.25, and 0.25 ml) to a column of silicic acid impregnated with silver nitrate. Such a column has recently been found effective in separating androsta-5, 16-dien-3β-ol from 5α-androst-16-en-3β-ol [2,26]. The analogous purification of the 4-[^{14}C] labelled preparation required two such columns, since the product was contaminated with far more of the fast-running impurities than was the tritiated preparation. This difficulty was no doubt encountered as a result of the unusually darkened colour of this particular reaction mixture. The coincidence of radioactivity and mass peaks in each case suggested that radioactive androstadienone had been separated, although the high specific activities in the first two fractions of each peak relative to those in later fractions implied contamination from earlier-running impurities. The first two fractions were therefore excluded and only fractions with specific activities of the mean $\pm$ 1 S.D. were pooled and subjected to further chromatography on columns of alumina [21]. Figures 1*a* and 1*b* show that the radioactive androstadienone samples so obtained were now pure, with coincidence of radioactivity and mass peaks and constant specific activities over those peaks. Yields of purified tritiated and [^{14}C]-androstadienone were 6.3 % and 5.0 % respectively.

The purity of both labelled androstadienone samples (stored in anhydrous benzene at 6° for 6 weeks) was further checked by TLC and by GLC with gas fraction collection (GFC).

(*a*) By TLC : Carrier androstadienone (40 μg) was added to a portion of each labelled preparation and the mixtures applied quantitatively to a TLC plate ("F 254" pre-coated, Merck-Darmstadt). Pure unlabelled androstadienone was applied to a third lane. After running once in the system benzene-ether (9 : 1, v/v), the plate was scanned for radioactivity. This revealed that the purity of the tritiated preparation was 97.3 and that of the ^{14}C-labelled preparation was 99.2 %. Both peaks of radioactivity coincided exactly with the position of carrier androstadienone (detected in UV light, wavelength 254 nm). Furthermore, autoradiography revealed a single spot of radioactivity in the ^{14}C-preparation which again coincided exactly with the position of the carrier. After elution of the radioactive steroids from the relevant zones, the specific activities were determined and both agreed with the calculated values (Table 1).

(*b*) By GFC : Mixtures of carrier androstadienone with a portion of each of the labelled preparations were prepared to give products with the same calculated specific radioactivities as in (*a*). These were subjected to GFC on a column which was connected to a Splitter (Pye-Unicam, Cambridge, England) so that most of the column effluent could be collected while approximately 10 % passed into the flame ionization detector [27,28]. Collections were made, when the androstadienone was being eluted from the column

TABLE I. Determination of the purity of 4-[^{14}C]- and 7α-[^{3}H]-androsta-4,16-dien-3-one.

	Specific radioactivity (μC/μg)			
	Initial ± S.D.	After addition of carrier (40 μg) (calculated)	After TLC (observed)	After GFC (observed)
4-[^{14}C]-androstadienone	0.274 ± 0.02	1.05×10^{-3}	1.03×10^{-3}	1.04×10^{-3}
7α-[^{3}H]-androstadienone	0.460 ± 0.01	2.74×10^{-3}	2.98×10^{-3}	2.32×10^{-3}

in glass U-tubes, packed with silanized glass beads [27,28]. The steroid was eluted with acetone (4 × 1 ml), the acetone evaporated to small bulk and the weight and radioactivity of suitable portions measured. The specific radioactivities so determined agreed with the calculated values.

DISCUSSION.

We have failed to reproduce the yields of androstadienone, quoted by Henbest and Jackson [18], even under carefully controlled conditions. The impurities successfully separated from apparently pure androstadienone have not been identified, but we think that these may be ring D olefinic rearrangement products. In general, steroidal toluene *p*-sulphonates may readily be converted into olefins by elimination reactions, though these are always accompanied by S_{N2} nucleophilic substitution of the toluene *p*-sulphonate group [29]. Further, rearrangements under these conditions are known to take place involving the 18-methyl group and the 16-17 double bond [6,29*b*-32].

Despite the low yields obtained, the method used is readily adaptable for the synthesis of both androstadienone and 17-epitestosterone acetate (III*b*) labelled to any required level of activity.

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