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EVALUATION OF THE SPECIFIC ACTIVITY OF BIOCHEMICALLY-PREPARED CARRIER-CRYSTALLIZED ANDROGEN RADIOMETABOLITES BY A NEW PROCEDURE*

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SUMMARY

Cytoplasmic fractions of livers of male and female rats have been used as enzyme source for the preparation of labelled 5α- and 5β-androstane and 5α-estrane derivatives. Incubation conditions are given for the preparation of labelled 5α-dihydrotestosterone, 5α-dihydro-19-nortestosterone, androsterone, *iso*androsterone,‡ 5α-androstane-3, 17-dione, 5α-androstane-3α, 17β-diol, 5α-androstane-3β, 17β-diol and 5β-androstane-3α, 17β-diol. Radiohomogeneity of the purified products has been established by crystallization with carrier to constant S.A. A new procedure which avoids direct weighing is described for evaluating the S.A. of the radiometabolites. For each crystallization step, two factors, determined by measuring the radioactivity and g.l.c. response to identical portions of crystals and mother liquors, permit the computation of the S.A. of both fractions based on the known S.A. of the preceding solution. The versatility, accuracy and limitations of the methods are discussed.

INTRODUCTION

INVESTIGATIONS OF the mechanism of action of the circulating androgens in target tissue have demonstrated extensive hormone metabolism to a multiplicity of intracellular C_{19}-steroids[1–7]. The need has therefore arisen for the availability of these metabolites as radioactive substrates or reference compounds of the same S.A. as their commercially available precursors. Hepatic microsomes of adult female rats contain substantial levels of 4-ene-3-oxosteroid-5α-reductase[8, 9] and 3α-hydroxysteroid oxido-reductase[10] activities. In contrast, hepatic microsomes of adult male rats have a much lower 5α-reductase content[9] but a high level of 3β-hydroxysteroid oxido-reductase[10]. The hepatic cytosol fraction of male rats differs from that of female rats in having considerable 4-ene-3-oxosteroid 5β-reductase activity[11]. We utilized these findings to establish

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†Part of the work reported here was carried out while on leave from Faculté de Médecine de Brest, and is contained in a thesis presented to the University of Paris in partial fulfillment of the requirements for the degree of Doctorat ès Sciences. Reprint requests should be directed to R. F. Morfin.

‡The following trivial names are used in this paper: 19-nortestosterone, 17β-hydroxy-4-estren-3-one; 5α-dihydro-19-nortestosterone, 17β-hydroxy-5α-estran-3-one; 5α-dihydrotes-tosterone, 17β-hydroxy-5α-androstan-3-one; *iso*androsterone, 3β-hydroxy-5α-androstan-17-one.

incubation conditions for the reproducible preparation in high yield of 5α- and 5β-reduced derivatives of isotopically-labelled testosterone, 19-nortestosterone and androstenedione. The radiosteroids were extracted from the digests and resolved by t.l.c. The purity of individual products was determined by crystallization to constant S.A. Criteria for establishing radiohomogeneity, and thereby definitive identification, by crystallization to constant S.A. have been extensively discussed by Axelrod *et al.*[12]. We now present a modification of the evaluation procedure in which determination of the S.A. of crystals and mother liquor is based on their radioactivities, on the ratio of the weights as measured by g.l.c. and on the S.A. of the fraction that was crystallized.

MATERIALS AND METHODS

Isotopically labelled and carrier steroids. [4-^{14}C]-Testosterone (40-50 mCi/mmol), [1,2-^{3}H]-testosterone 45 Ci/mmol) and [4-^{14}C] androstenedione (40–50 mCi/mmol) were purchased from New England Nuclear Corporation; [4-^{14}C] 19-nortestosterone (50·2 mCi/mmol) was obtained from Nuclear Chicago Corporation. [7β-^{2}H]-testosterone was donated by Dr. James C. Orr. The isotopically labelled steroids were purified by t.l.c. before use.

Carrier steroids: androsterone, *iso*androsterone, 5α-androstane-3,17-dione, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17β-diol and 5β-androstane-3α, 17β-diol were commercial samples. 5α-dihydro-19-nortestosterone was synthesized according to Bowers *et al.*[13] and crystallized once before use, m.p. 129–130°C. Purity of the carrier steroids was checked by t.l.c. and g.l.c. All solvents were distilled before use and checked by g.l.c.

Cytoplasmic fractions. Hepatic microsomes from adult male and female Sprague–Dawley rats were prepared as described previously[14], Biuret assay on the washed microsomal pellet derived from livers of female rats weighing 200–250 g indicated 20–30 mg protein per g of hepatic tissue. Male rats of the same weight yielded 30–35 mg microsomal protein and 70–76 mg cytosol protein per gram of hepatic tissue.

Preparative incubations [4-^{14}C]-5α-*dihydrotestosterone and* [4-^{14}C, 7β-^{2}H]-5α-*dihydrotestosterone.* The solution of purified [4-^{14}C] testosterone substrate (14 μg) with or without 7β-^{2}H carrier was evaporated to dryness under nitrogen in the bottom of the incubation tube. Phosphate buffer solution (5 ml, 0·067 M, pH 7·4) containing NADPH (1·5 mg) was added, followed by the suspension of freshly prepared hepatic microsomes of female rat (2·5 mg protein). After incubation for 7·5 min at 37°C, the enzymatic reaction was stopped by vigorous shaking with ethyl acetate (10 ml) and the tube stored at − 17°C.

[1,2-^{3}H]-5α-*dihydrotestosterone.* Tritiated product was prepared in the same manner by incubating 3·2 μg substrate in 1·5 ml with 0·57 mg microsomal protein and 1·0 mg NADPH for 7 min.

[4-^{14}C]-5α-*androstane*-3α,17β-*diol.* This radiosteroid was prepared from [4-^{14}C] testosterone (14 μg in 5 ml) in the same manner except that the amount of microsomal protein was increased to 5 mg and the incubation time extended to 20 min.

[1,2-^{3}H]-5α-*androstane*-3α,17β-*diol.* Changes in incubation conditions were as follows: [1,2-^{3}H] testosterone (3·2 μg in 1·5 ml), NADPH (1·0 mg), microsomal protein (0·57 mg), incubation time (18 min).

[4-^{14}C]-5α-*androstane*-3,17-*dione and* [4-^{14}C]-*androsterone.* [4-^{14}C]-

Androstenedione (14 μg in 5 ml) was incubated for 10 min in the presence of NADPH with 1·5 mg of hepatic microsomal protein from female rats. Operations were performed as described above.

[4-^{14}C]-iso*androsterone*. Biosynthesized [4-^{14}C]-5α-androstane-3,17-dione was used as substrate after careful purification and proof of radiochemical purity by crystallization to constant S.A. The substrate (14 μg) was incubated in phosphate buffer (5 ml) at pH 7·4 for 3 min with 12·5 mg hepatic microsomal protein from male rats in the presence of NADPH (1·5 mg).

[4-^{14}C]-5α-*androstane*-3β,17β-*diol*. This steroid was prepared by incubating biosynthesized, carefully purified [4-^{14}C]-5α-dihydrotestosterone (14 μg in 5 ml) for 3 min at pH 7·4 with NADPH and 12·5 mg hepatic microsomal protein of male rats.

[4-^{14}C]-5β-*androstane*-3β,17β-*diol*. [4-^{14}C]-Testosterone (14 μg in 5 ml) was incubated for 10 min with NADPH and high-speed-supernatant fraction (25 mg protein) derived from the liver of male rats. Cytosol containing 0·25 M sucrose was diluted to 0·08 M in the incubation mixture.

[4-^{14}C]-5α-*dihydro*-19-*nor testosterone*. [4-^{14}C]-19-Nortestosterone (14 μg in 5 ml) served as substrate in a 5 min incubation with NADPH (1·5 mg) and liver microsomes (2·5 mg protein) of female rats.

Extraction of radiometabolites. After removing the supernatant ethyl acetate extract, the frozen digest was thawed and extracted twice with the same solvent (10 ml). The extracts were pooled, solvent dried under nitrogen and the mixture of radiometabolites resolved by t.l.c.

Chromatography. A first fractionation was done on silica gel G using a single development with chloroform–ether (7:3, v/v) (system 1). Radiosteroids were located by autoradiography with Kodak BB-54 X-ray film over a period of 48 h. Appropriate zones were eluted. When necessary, the eluate and residues were subjected to a second t.l.c. on Alumina G. Double development in dichloro methane-ether (9:1, v/v) (system 2) separated androsterone from a mixture of 5α-dihydrotestosterone and *iso*androsterone. Recently, we achieved complete separation of the 3 constituents either in dichloroethane–chloroform–ether (7:7:6, v/v) on 40 cm plates covered with thin layers of silica gel G or in benzene–ether (6:4, v/v) on 40 cm aluminum foil pre-coated with silicia gel (Merck). Benzene–ethanol–water (97:2·85:0·15, v/v) was effective in separating 5α-androstane-3α,17β-diol from its 3β-epimer after a single development on freshly activated alumina G (system 3).

Addition of carrier. Sufficient carrier was added to provide a S.A. of at least 500 d.p.m./μmol. The steroid was weighed in a glass vial on a Mettler microbalance and dissolved in the solvent used for the first crystallization. The solution was then quantitatively added to the dried residue of the eluate. A portion of the mixture was counted and S.A. expressed in d.p.m./μmol. The volume of the remaining solution was reduced under nitrogen at 40–50°C before the first crystallization.

Crystallization. The solution was concentrated at 30–40°C to a small volume. The second component of the solvent pair was then added with constant agitation, a few drops at a time, to the point of turbidity. One drop of the first solvent was added to redissolve. The tube was then stoppered and refrigerated. When an adequate amount of crystals had formed, the mother liquor was aspirated through a thin capillary tube and the crystals washed once with the second ice-cold solvent.

Pooled washings and the mother liquor were made up to volume in a volumetric flask. Solution of the crystals in the first solvent was transferred to an identical flask.

Determination of steroid-weight ratio by g.l.c. Samples were injected with a 0·01 ml Hamilton syringe with or without Chaney adaptor. Syringes were repeatedly rinsed with solvent after each use. Determination of ratios of steroid weights was carried out using (i) a Jarrell–Ash chromatograph, model 29–700 and a coiled glass column 6 ft long, with either 3% QF-1 or 1% OV-1 on 50–60 mesh Anachrom ABS as stationary phase (ii) a Pye-104 chromatograph fitted with a 5-ft-long glass column containing Corning glass-beads coated with 0·2% OV-1; both chromatographs were equipped with a flame ionization detector and nitrogen was used as carrier gas. An Infotronis Integrator CRS-104 attachment was used for computing peak areas.

At least 3 equal portions (2–5 μl) of crystal and mother liquor solutions were injected into the gas chromatograph. To increase accuracy it was desirable to obtain peak heights of at least 80 mm and reproducible retention times. Data were averaged and peak heights/μl were then calculated. Injection of known quantities of steroids showed that measurement of peak area ($\pm$ 1·5%) was somewhat less variable than measurement of peak height ($\pm$ 2·0%).

Liquid-scintillation spectrometry. Radioactivity determinations were carried out in 20 ml scintillation vials. Equal portions of solutions of crystals and mother liquor were evaporated to dryness at 40–50°C under a stream of nitrogen and scintillation fluid was added. All count rates exceeded 5 times the background. The counting error was not allowed to exceed 0·5%. Radioactivity in d.p.m. of crystals and mother liquor was determined from these data and external standard ratios. An error of $\pm$ 0·8% was calculated for these determinations.

Determination of S.A. In general:

$$\frac{s}{S} = \frac{c+m}{C+M}.$$

Where s = radioactivity in the original solution, S = weight of carrier in the original solution, c = radioactivity in the crystals, C = weight of carrier in the crystals, m = radioactivity in the mother liquor, M = weight of carrier in the mother liquor.

From this equation we can derive the S.A. of crystals and mother liquor as:

$$\frac{c}{C} = \frac{s}{S} \times \frac{c}{c+m} \times \frac{C+M}{C}$$

$$\frac{m}{M} = \frac{s}{S} \times \frac{m}{c+m} \times \frac{C+M}{M}.$$

The last term of these equations is conveniently determined by g.l.c. using the flame-ionization-detection response to identical portions of both fractions. Peak heights or peak areas are then sufficient to establish the desired ratio.

Standardization of results. We propose to redistribute the S.A. of the mother liquor fractions based on the weight of the crystals. This device of equalizing weights will allow a direct comparison of S.A. of crystals and mother liquors. The

redistribution of S.A. of the mother liquor (m^1/M^1) may be calculated as follows:

$$\frac{m^1}{M^1} = \frac{s}{S} + \left[\left(\frac{m}{M} - \frac{s}{S}\right) \times \frac{M}{C}\right].$$

In a well conducted crystallization without extraneous contamination, the S.A. of the crystals and the redistributed S.A. of the mother liquor must differ by an equal amount from s/S.

RESULTS

Incubation of testosterone with hepatic microsomes of the female rat

5α-dihydrotestosterone was isolated in 80% yield. Other radiosteroids present in the crude extract in significant quantities had chromatographic mobilities identical to those of authentic androstenedione, 5α-androstane-3,17-dione, androsterone, 5α-androstane-3α,17β-diol and $C_{19}O_3$-steroids. There was no marked difference in yield, whether fresh microsomes or once frozen-and-thawed preparations (stored frozen for 5 days) were used. In prolonged incubations, 5α-androstane-3α,17β-diol was formed in increased yield and could be readily separated from the 3β-epimer by chromatography in the t.l.c. system 3. Under the specified conditions the two epimers were present in the ratio of 3α/3β = 19·4.

Incubation of 19-nortestosterone with hepatic microsomes of the female rat

After development of the solvent extract in t.l.c. system 1, 5α-dihydro-19-nortestosterone was isolated in 74% yield. The autoradiogram showed the presence of unmetabolized 19-nortestosterone but no trace of dioxo or dihydroxy-derivatives. Chromatography of the eluted 5α-dihydro-19-nortestosterone on alumina G in t.l.c. system 2 did not provide evidence for 17-oxo contaminants.

Incubation of androstenedione with hepatic microsomes of female rat

Chromatography of the extract in t.l.c. system 1 yielded significant quantities of the radiosteroids in the region of 5α-androstane-3,17-dione and androsterone which accounted for 60 and 15%, respectively, of the incubated radioactivity. The remaining 25% were polar metabolites. Androsterone was separated from 5α-dihydrotestosterone and *iso*androsterone by chromatography of alumina G in t.l.c. system 2.

Incubation of testosterone with 105,000 g supernatant fraction derived from the liver of male rats

Resolution of metabolites on silica G in the t.l.c. system 1 demonstrated the presence of many radioactive zones. After elution a second chromatography in the t.l.c. system 2 resolved radiometabolites isopolar with 5α-dihydrotestosterone into androsterone and *iso*androsterone with almost no trace of the 17β-hydroxy-steroid. Chromatography in the t.l.c. system 3 resolved steroids originally isopolar with testosterone and the 5α-androstanediols into the well separated metabolite pairs testosterone/etiocholanolone and 5α-androstane-3α, 17β-diol/5α-androstane-3β, 17β-diol, respectively; radiosteroid isopolar with 5β-androstane-3α,17β-diol remained as a single component in this system and was generally obtained in 8–10% yield.

Incubation of 5α-dihydrotestosterone with hepatic microsomes of male rat

The crude extract was chromatographed on silica G in t.l.c. system 1. Three main radioactive components corresponded to 5α-dihydrotestosterone, the 5α-androstanediols and $C_{19}O_3$-metabolites. 5α-androstane-3α, 17β-diol and 5α-androstane-3β, 17β-diol (27% yield) were separated as described in the ratio $3\alpha/3\beta = 0{\cdot}44$.

Incubation of 5α-androstane-3, 17-dione with hepatic microsomes of male rat

Resolution of the mixture of extracted metabolites on silica G in the t.l.c. system 1 gave two main radioactive components, one corresponding to 5α-androstane-3, 17-dione, the other to *iso*androsterone. The latter (52% yield) was resolved on alumina G with t.l.c. system 2 into androsterone and *iso*androsterone. The epimer ratio ($3\alpha/3\beta$) was 0·31.

Determination of S.A.

Analysis of error. The crystallization steps accumulated errors since the determination was based on the S.A. of the preceding fraction. The overall error of the specific activity determinations were calculated and expressed according to Axelrod *et al.*[12]. The error of ±0·8% for the starting solution increased to ±2·5%, ±3·5%, ±4·2%, ±4·8% and ±5·3% for the first, second, third, fourth and fifth crystallizations, respectively. The contributions of different factors to these errors are presented in Table 1. Due to the observed increase in the overall error, it was not practical to proceed beyond the fifth crystallization. If more crystallizations are needed to attain constant S.A., it is advisable to use substantial amounts of carrier, the weight of which can be determined with accuracy. One should continue with the present procedure until the error introduced by consecutive weighings exceeds 5%.

Accuracy and sensitivity. Table 1 demonstrates that the accuracy of the method is limited by the accumulation of errors in S.A. determinations. Since flame-ionization-detection gas chromatography permits accurate measurement of quantities of carrier as small as 500 ng with an error of 1·5%, the present method improves the overall sensitivity of S.A. determination.

Radiohomogeneity of biosynthesized steroids. Data on crystallizations of carbon 14-labelled androsterone and *iso*androsterone to constant S.A. are given

Table 1. Per cent relative contribution of the individual measurements to the overall error on S.A. determination in five crystallizations

	O.S. ±0·8%	C_1 ±2·5%	C_2 ±3·5%	C_3 ±4·2%	C_4 ±4·8%	C_5 ±5·4%
g.l.c. measurement		69·7	36·7	25·0	18·9	15·2
dpm measurement	38·0	7·8	4·1	2·8	2·1	1·7
preceding S.A.		10·1	52·6	67·8	75·7	80·4
volume measurement	62·0	12·4	6·6	4·4	3·3	2·7

S.A. were determined for crystals and mother liquors of each of five crystallizations. The following abbreviations are used: O.S. = original solution, C_1 to C_5 = first crystallization with calculated overall error.

in Table 2. Crystallizations were performed after addition of 86·2 and 69 μmoles of the respective authentic carriers.

Table 3 presents data on crystallization of ^{14}C-labelled 5α-dihydrotestosterone, 5α-androstane-3, 17-dione and 19-nor-5α-dihydrotestosterone, carried out after addition of 54·1, 69·5 and 69·5 μmoles of the respective authentic carriers. Data concerning crystallization of carbon 14-labelled 5α-androstane-3β, 17β-diol (152 μmoles), 5α-androstane-3α, 17β-diol (113·4 μmoles) and 5β-androstane-3α, 17β-diol (102·7 μmoles) are shown in Table 4.

Crystallization of tritium-labelled 5α-dihydrotestosterone and 5α-androstane-3α, 17β-diol were performed after addition of 35·1 and 36·4 μmoles of the respective authentic carriers (Table 5). Figure 1 illustrates a compact form of documenting the attainment of radiohomogeneity for all biosynthesized radiosteroids described in this paper. By adopting a logarithmic scale of presentation, wide disparities and changes in crystal and redistributed mother liquor S.A. can be readily accommodated.

DISCUSSION

Use of subcellular fractions of rat ventral prostate and human hypertrophied prostate for the preparation of labelled 5α-dihydrotestosterone and 5α-androstane-3α, 17β-diol in amounts suitable for metabolic investigations have been reported [14–17]. Thus the former steroid was obtained by incubating labelled testosterone

Table 2. Crystallization of carbon 14-labelled androsterone and *iso*androsterone to constant S.A.

Carrier steroid	$\frac{s}{S}$	Solvent mixture	d.p.m.	Peak height (mm)	S.A.	Redistributed S.A. of MLQ
*Iso*androsterone						
1st CR			24280	100·5	1290	
1st MLQ	1330	EA/H	32180	127·0	1355	1362
2nd CR			18740	116·3	1278	
2nd MLQ	1290	A/H	1600	8·6*	1095	1273
3rd CR			7870	184·0*	1102	
3rd MLQ	1278	A/Wa	6250	102·0	1585	1448
4th CR			2710	78·0	1068	
4th MLQ	1102	M/Wa	3605	98·6	1122	1127
Androsterone						
1st CR			11135	238·0*	498	
1st MLQ	586	EA/H	8122	113·4	762	656
2nd CR			5530	289·1*	475	
2nd MLQ	498	A/H	2508	114·1	522	507
3rd CR			2002	196·2*	488	
3rd MLQ	475	E/H	1257	132·0	454	461

*G.l.c. peak height based on matching aliquot.

The following abbreviations are used: CR = crystals, MLQ = mother liquor, EA = ethyl acetate, H = n-hexane, A = acetone, M = methanol, Wa = water, E = ether, s/S = S.A. of the preceding fraction that was crystallized expressed as the ratio of the d.p.m. of its solution (s) to its weight in μmoles (S).

Table 3. Crystallization of carbon 14-labelled 5α-dihydrotestosterone, 5α-androstane-3, 17-dione and 5α-dihydro-19-nortestosterone to constant S.A.

Carrier steroid	$\frac{s}{S}$	Solvent mixture	d.p.m.	Peak height (mm)	S.A.	Redistributed S.A. of MLQ
5α-dihydrostestosterone						
1st CR	1837	EA/H	76432	915·1*	1789	
1st MLQ			12938	134·2	2057	1869
2nd CR	1789	A/H	39444	312·0*	1815	
2nd MLQ			17118	137·0	1769	1785
5α-Androstane-3, 17-dione						
1st CR	10180	EA/H	180040	78·5	10680	
1st MLQ			302840	143·0*	9870	9616
2nd CR	10680	A/H	77000	91·2	10430	
2nd MLQ			84500	96·0	10900	10910
5α-dihydro-19-nortestosterone						
1st CR	13750	EA/H	513000	70·5	14150	
1st MLQ			638000	92·5	13450	13356
2nd CR	14150	A/H	263500	84·0	14050	
2nd MLQ			225500	71·0	14200	14209

*G.l.c. peak height based on matching aliquot.
For abbreviations, cf. Table 2

with microsomes of human prostate[14] and nuclei of the rat tissue[15]; the diol was prepared by incubating [1,2-^{3}H] 5α-dihydrotestosterone with the 105,000 *g* supernatant fraction of rat ventral prostate[18, 19]. Proof of identity was not presented.

Table 4. Crystallization of carbon 14-labelled 5α-androstane-3β, 17β-diol, 5α-androstane-3α, 17β-diol and 5β-androstane-3α, 17β-diol to constant S.A.

Carrier steroid	$\frac{s}{S}$	Solvent mixture	d.p.m.	Peak height (mm)	S.A.	Redistributed S.A. of MLQ
5α-androstane-3β, 17β-diol						
1st CR	970	A/H	38160	69·0	910	
1st MLQ			45400	83·6	1038	1052
2nd CR	910	E/H	22960	160·0*	907	
2nd MLQ			9960	69·0	915	912
5α-androstane-3α, 17β-diol						
1st CR	5430	A/H	188670	88·0	5320	
1st MLQ			180530	81·3	5670	5652
2nd CR	5320	EA/H	53900	108·5	5325	
2nd MLQ			112700	228·0*	5340	5362
5β-androstane-3α, 17β-diol						
1st CR	1248	A/Wa	16700	33·8	1097	
1st MLQ			70460	121·6	1365	1668
2nd CR	1097	M/Wa	7910	67·0	1090	
2nd MLQ			7150	66·0	1105	1105

*G.l.c. peak height based on matching aliquot.
For abbreviations, cf. Table 2.

Table 5. Crystallization of tritium-labelled 5α-dihydrotestosterone and 5α-androstane-3α, 17β-diol to constant S.A.

Carrier steroid	$\frac{s}{\bar{S}}$	Solvent mixture	d.p.m.	Peak height (mm)	S.A.	Redistributed S.A. of MLQ
5α-dihydrotestosterone						
1st CR	2398	EA/H	66390	692·5*	2309	
1st MLQ			8670	68·3	3069	2465
2nd CR	2309	A/H	45410	785·0*	2165	
2nd MLQ			7326	78·0	3509	2427
3rd CR	2165	E/H	29736	450·2*	2089	
3rd MLQ			14504	196·5	2329	2236
5α-androstane-3α, 17β-diol						
1st CR	2520	EA/H	42120	264·1*	2383	
1st MLQ			24990	135·0	2746	2633
2nd CR	2383	A/H	34470	348·9*	2280	
2nd MLQ			7260	59·0	2859	2449
3rd CR	2280	E/H	12116	376·0*	2209	
3rd MLQ			7745	223·0	2371	2333

*G.l.c. peak height based on matching aliquot.
For abbreviations, cf. Table 2.

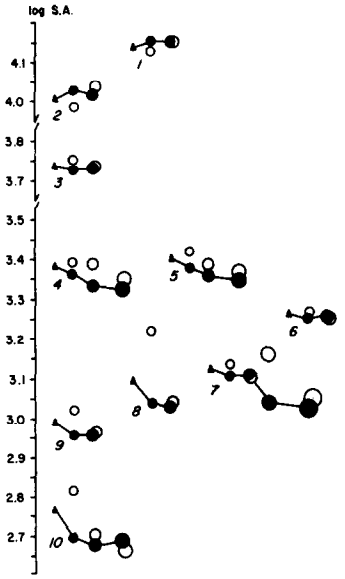

Fig. 1. Crystallization to constant S.A. The figure presents data on the following: 1) [4-^{14}C] 5α-dihydro-19-nortestosterone; 2) [4-^{14}C]-5α-androstane-3,17-dione; 3) [4-^{14}C]-5α-androstane-3α,17β-diol; 4) [1,2-^{3}H]-5α-dihydrotestosterone; 5) [1,2-^{3}H]-5α-androstane-3α,17β-diol; 6) [4-^{14}C]-5α-dihydrotestosterone; 7) [4-^{14}C]-*iso*androsterone; 8) [4-^{14}C]-5β-androstane-3α,17β-diol; 9) [4-^{14}C]-5α-androstane-3β,17β-diol; 10) [4-^{14}C]-androsterone. The symbols denote: ▲, free steroid with carrier before crystallization; ●, crystals; ○, mother liquor residue. The size of the symbols is directly related to the overall error on S.A. determination.

Our study of the metabolism of testosterone and 5α-dihydrostestosterone in the canine[18] and human[19] prostate required appreciable quantities of labelled C_{19}-steroids. Our previous experience[14] and the cited literature pointed to rat liver as a convenient source of the required enzymes. After the present work had been completed, Arimasa and Kochakian[20] published an extensive study of the subcellular localization of hepatic 4-ene-3-oxo-C_{19}-steroid 5α-reductase of female rats. Applying the available information, we have used cytoplasmic fractions of livers of male and female rats under optimal conditions as enzyme source for efficient preparation of labelled 5α- and 5β-androstane and 5α-estrane derivatives. 5α-reduction of the 4-ene-3-oxo-steroid substrates was effected by NADPH-supplemented hepatic microsomes of female rats; if incubation was prolonged 3α-hydroxysteroids of the 5α-androstane series accumulated. NADPH-mediated reduction of 5α-dihydrotestosterone by hepatic microsomes of male rats yielded a mixture of epimeric diols, with 5α-androstane-3β, 17β-diol predominating. The relative abundance of the two diols can be explained by the work of Gustafsson *et al.*[21] who found no further hydroxylation of 5α-androstane-3β, 17β-diol in contrast to 2β-hydroxylation for the 3α-epimer. Incubation of testosterone with hepatic NADPH-fortified cytosol of male rats yielded 5β-androstane-3α, 17β-diol but no 17β-hydroxy-5α-androstan-3-one. Chromium-trioxide oxidation of 5β-reduced radiometabolites furnishes 5β-androstane-3, 17-dione in high yield.

The new method of evaluating S.A. in tests of radiosteroid homogeneity by carrier crystallization complements the methodology of Axelrod *et al.*[12] in the following respects:

Direct weighing of crystals and mother liquor is eliminated by the use of g.l.c. which removes impurities that might accumulate during successive crystallizations. Measurement of peak-height ratios avoids use of standard curves as described by Chamberlain *et al.*[22].

Efficiency in removal of weightless radioactive contaminants of closely related structure from the carrier was established by cross-crystallization experiments in which we confirmed that a given radiosteroid did indeed separate from a foreign carrier.

In practice our method cannot accommodate more than five crystallizations since the standard error for S.A. determinations increases with the number of crystallizations. The method is preferred when small quantities of carriers are to be crystallized three or four times. One is then only limited by crystallization of the carrier and the response of the gas-chromatograph.

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