THE METABOLISM OF TRITIATED TESTOSTERONE BY A WETHER

By W. B. Osborne,* P. K. Wong,† and J. L. Garnett‡

[*Manuscript received August* 19, 1965]

Summary

An experiment to study the metabolism of exogenous testosterone and the formation of tissue residues in a wether is described. Testosterone labelled with tritium by a catalytic process was injected intramuscularly. Rapid metabolism resulted in the excretion of 74·5% of the injected radioactivity in 4 days and complete elimination (101·4%) in 31 days, of which the urine accounted for 59·6%, faeces 41·0%, and expired air 0·8%.

No evidence was found for the formation of tissue residues of testosterone or its radioactive metabolites. The principal urinary metabolite identified was androsterone which accounted for 30% of the radioactivity excreted in the urine in the 5 days following injection. Butanol extracts of the urine over the same period of time accounted for 55% of the excreted radioactivity.

I. Introduction

The administration of hormones to animals that may be destined for human consumption is a practice that has come under close scrutiny by health authorities in many countries. However, whilst oestrogenic substances have been thoroughly investigated there are few reports in the literature of studies on steroid residues in tissues of animals treated with androgens. This situation is largely due to the fact that androgens have, until recently, found little use in procedures to improve animal production. However, as a consequence of the use of testosterone propionate for the treatment and control of ovine posthitis in wethers (Watson and Murnane 1958; Southcott 1962) there is a need for an assessment of the extent to which wethers are capable of metabolizing exogenous testosterone. In particular, there is a need to determine the extent of tissue storage and the rate of excretion of exogenous testosterone and its metabolites.

Whilst chemical assay procedures for testosterone in blood and urine based on isotope dilution methods with [4-^{14}C]testosterone have high sensitivity (Lindner 1961; Dulmanis *et al.* 1964) they are not only laborious to perform but yield no information about the metabolites of testosterone. Less specific assay procedures for 17-ketosteroids (Miller and Turner 1961) suffer the disadvantage that metabolites such as androsterone§ are derived from precursors other than testosterone (Vande Wiele

* School of Wool Technology, University of New South Wales, Kensington, N.S.W.

† Department of Physical Chemistry, University of New South Wales; present address: Department of Chemistry, University of Wisconsin, Madison, Wisconsin, U.S.A.

‡ Department of Physical Chemistry, University of New South Wales, Kensington, N.S.W.

§ The following trivial names are used in this paper:
testosterone: androst-4-en-17β-ol-3-one; androstenedione: androst-4-ene-3,17-dione; androsterone: 5α-androstan-3α-ol-17-one; aetiocholanolone: 5β-androstan-3α-ol-17-one.

et al. 1962, 1963). Radiochemical techniques employing isotopically labelled testosterone combine simplicity with high sensitivity, detection of tissue residues is greatly simplified, and the presence of the label makes it possible to distinguish metabolites from those of endogenous origin (Gallagher *et al.* 1951). The lack of specificity in the technique facilitates the calculation of a "hormone balance" between administered, stored, and excreted radioactivity. A fundamental assumption is that the label does not become detached from the testosterone during metabolism and, further, that the label does not cause any distortion of the molecule likely to affect its metabolism. Labelling of the testosterone molecule may be effected with either ^{14}C or tritium. Tritium labelling has the advantage that it involves a simple, one-step procedure utilizing preformed testosterone whereas the former requires the synthesis of the testosterone molecule. The choice of the tritium labelling procedure used with steroids is critical for with certain methods degradation may readily occur (J. O'Keefe and J. L. Garnett, unpublished data), resulting in the contamination of the steroid with traces of compounds of similar biochemical reactivity but having an exceptionally high specific activity (Wilzbach 1961).

This study describes the fate of a dose of tritiated testosterone administered to a wether.

II. Materials and Methods

(*a*) *Experimental Design*

A mature Merino wether of approximately 55 kg live weight was placed in a metabolism cage. During the preliminary stage the wether was fed *ad libitum* on a pelleted ration of lucerne and wheatmeal (60 : 40 w/w). To reduce the volume of faeces to manageable proportions feeding was reduced to maintenance levels during the experimental period.

Tritiated testosterone (10 mg, 36·7 μc) was weighed into a glass viule and sealed with a rubber plunger. The viule was then fitted into a syringe and peanut oil (2 ml) was drawn up. Solution of the testosterone was effected by warming. This solution was injected intramuscularly into the wether and the syringe rinsed with peanut oil ($2 \times 0 \cdot 5$ ml): the rinsings were also injected into the wether. The wether was slaughtered (on the 38th day) when the level of radioactivity in the excreta did not differ significantly from background for three successive samplings.

Counts obtained on the urine and faeces excreted prior to treatment ranged from 3 to 8% above that obtained on the scintillation liquid alone. The upper value has been taken to set the limit of significance of the radioactivity excreted in the terminal stages of this study. The average counting rate of the scintillators used was (1) dioxan 32 counts/min, (2) toluene gel 61 counts/min, both having a standard error of $\pm 1\%$.

(*b*) *Preparation of Tritiated Testosterone*

Two labelling methods were examined: (1) gas irradiation (Wilzbach 1961) and (2) exchange on a pre-reduced catalyst. In the former method testosterone (0·5 g) was exposed, under vacuum, to tritium gas (1 curie, ex-Amersham, U.K.A.E.A.) for 1 week. The testosterone used was supplied by Roussel–UCLAF (France) and melted

at 152·5–153·5°C. After labelling, the tritium was pumped off and the tritiated testosterone dissolved in ethanol and the resulting solution passed into a column (10 by 1 in.) of alumina (B.D.H.). The testosterone was eluted with acetone, the solution evaporated to dryness, and the solid consecutively recrystallized from methanol–water until the specific activity was constant ($\pm 0 \cdot 5\%$) in two successive recrystallizations. Precautions were taken to minimize losses by steam volatilization. The chemical purity of the labelled compound was confirmed by infrared and nuclear magnetic resonance techniques (m.p. 152·5–153·5°C). In the second method, tritium oxide (1 ml, 1 c/g specific activity, ex-Amersham, U.K.A.E.A.) was added to a mixture of testosterone (1 g) and pre-reduced platinum oxide (0·1 g) (Calf and Garnett 1964) in a glass tube. The tube was evacuated and out-gassed by several freeze–thaw cycles and finally sealed at a pressure of about 0·01 mmHg. The reaction vessel was equilibrated with shaking for 2 days at a temperature sufficiently high (approximately 135°C) for the material to be liquid during exchange. The testosterone was recovered and purified in the same manner as outlined above for the Wilzbach irradiation. The physical properties of the labelled material were again the same as those of the original compound.

Testosterone labelled by both methods was used and consistent results were obtained in separate metabolism trials. The results reported here were obtained with testosterone labelled by the latter (catalytic) method.

(*c*) *Collection of Samples*

Total 24-hr collections of urine and faeces were made on days 1–8 and 48-hr collections from day 9 onwards. The urine was collected over 6N HCl and separately from the faeces.

A face mask was fitted to the wether for 100 min each afternoon and the respired air drawn under light vacuum through a series of three jacketed coil condensers through which was circulating brine at 2°C. The condensate was assumed to be 0·07 of the total daily loss of water by the lungs. No correction was applied for atmospheric moisture: calculations based on the wether's respiration rate and the wet and dry bulb air temperatures on two separate occasions suggested that the atmospheric contribution to the condensate was of the order of 0·25 ml.

During post-mortem examination of the carcass, samples of the liver, kidney, kidney fat, prostate and adrenal glands, and muscle from the injection site were taken.

(*d*) *Measurement of Radioactivity*

All samples were counted in a Packard model 314 EX Tri-Carb liquid scintillation spectrometer set for balance point operation for the particular scintillator system used. Each sample was counted for sufficient time to accumulate at least 10,000 counts. A correction for quenching was obtained by recounting each sample after the addition of standard tritiated water ($2 \cdot 28 \times 10^6$ disintegrations/min) in such an amount as to yield at least 10 times the original counting rate.

A thixotropic gel scintillator (Harlan 1961; Handler 1963) was used for the urine samples to avoid the progressive decline in counting rate observed when low-activity urine was counted over long periods. This decline was considered to be

associated with the slow formation of a precipitate due to the action of dioxan on the urine. This effect has been ascribed to adsorption of tritium on to the walls of the counting vial. This scintillator was prepared by loosely filling the counting vial with a finely divided silicon dioxide powder* then adding 15 ml of a solution made up as follows: toluene 300 ml; PPO (2,5-diphenyloxazole) 2 g; POPOP [1,4-bis-2(5-phenyl-oxazolyl)-benzene] 0·1 g; naphthalene 35 g; methanol 190 ml. Analytical grade reagents were used as delivered. Commercial grade naphthalene was recrystallized thrice from benzene before use.

The oxidized faecal and tissue samples and respiratory water samples were counted in a dioxan scintillator (Bray 1960). The fat samples were counted in the toluene scintillator.

(*e*) *Preparation of Samples for Counting*

(i) *Urine.*—The radioactivity in duplicate 50-ml samples of the first 5 days collection of urine was fractionated by exhaustive extraction with three volumes of methylene dichloride for 24 hr in a continuous, all-glass extractor. The extracted urine was acidified to pH 1 (pH-meter), saturated with ammonium sulphate, and extracted with an equal volume of n-butanol by shaking for 10 min. Aliquots of the methylene dichloride extract ("free steroid fraction"), n-butanol extract (conjugated fraction), and urine residue were counted in the gel scintillator. A second set of duplicate 50 ml samples of this urine were subjected to β-glucuronidase hydrolysis and extraction using the methods of Bush (1961).

Aliquots (0·5 ml) were taken in duplicate and transferred to a standard 4-dram, low-potassium glass counting vial containing 15 ml of gel scintillator. The pipette was rinsed with the scintillator to ensure complete transfer and the vial shaken vigorously.

(ii) *Faeces.*—Water, equal to 2·5 times the total weight of faeces collected, was added. The mass was placed in a 5-gallon jar then tumbled end-over-end for 12 hr. Duplicate 5-g samples were taken before sedimentation could occur: distilled water (0·25 ml) was added to one and standard tritiated water (0·25 ml, $2{\cdot}28\times10^6$ disintegrations/min) was added to the other. Both samples were subjected to the wet oxidation procedure of Belcher (1960) except that the initial heating with nitric acid was done under reflux and, after oxidation with perchloric acid, exactly 5 ml of distillate was collected. Aliquots (0·5 ml) of the neutralized, diluted distillate were counted. The average recovery of added tritium following wet oxidation of the faecal samples was 93·8%.

(iii) *Respired Water.*—Aliquots (0·5 ml) of the daily sample were counted in the dioxan scintillator without further treatment.

(iv) *Tissue.*—Duplicate 2-g samples of each tissue except fat were prepared by the technique of Belcher (1960) and counted in the dioxan scintillator. Duplicate 0·25-g samples of fat were dissolved in the toluene scintillator and counted without the addition of Aerosil.

* Aerosil 2491: Degussa, Frankfurt, Germany.

(*f*) *Identification of Steroids*

The methylene dichloride extracts of urine were washed with 1N NaOH (3×5 ml), saturated NaCl (2×1 ml), and then evaporated to dryness under vacuum. The residues were taken up in methanol (1 ml) and applied to 40 by 5 cm strips of Whatman No. 4 paper. After equilibration overnight these were run in the Bush A

TABLE 1

EXCRETION OF RADIOACTIVITY BY A WETHER FOLLOWING THE INTRAMUSCULAR INJECTION OF TRITIATED TESTOSTERONE

10 mg (36·7 μc) of tritiated testosterone injected

Days after Injection	Percentage of Administered Radioactivity		
	Urine	Faeces	Total
1	28·4	4·9	33·3
2	12·6	10·1	22·7
3	3·9	8·9	12·8
4	2·9	2·8	5·7
5	2·3	2·1	4·4
6	1·4	2·1	3·5
7	1·0	0·9	1·9
8, 9	2·5	2·0	4·5
10, 11	0·9	1·2	2·1
12, 13	0·8	1·2	2·0
14, 15	1·4	0·7	2·1
16, 17	0·3	1·7	2·0
18, 19	0·0	1·0	1·0
20, 21	0·2	0·3	0·5
22, 23	0·2	0·1	0·3
24, 25	0·0	0·0	0·0
26, 27	0·0	0·4	0·4
28, 29	0·4	0·3	0·7
30, 31	0·4	0·3	0·7
32, 33	0·0	0·0	0·0
34, 35	0·0	0·0	0·0
36, 37	0·0	0·0	0·0
Totals	59·6	41·0	101·4*

* Including expired air (0·8%).

system (Bush 1952) at 25°C alongside testosterone, androstenedione, and androsterone: development was stopped when the solvent front ran off the sheet. After drying the chromatogram was dipped through freshly prepared 2N NaOH then oven-dried on a sheet of glass (Bush 1961). The chromatogram was read under ultraviolet light.

The washed butanol extract of the enzyme-hydrolysed urine was evaporated to dryness under vacuum and the residue taken up in ethanol (1 ml). The entire extract was applied to a 5 by 40 cm strip of Whatman 3 MM paper and run in a system

containing equal volumes of 85% acetic acid and a 1 : 1 mixture of light petroleum and toluene (Bush 1961). The preliminary chromatogram so obtained was cut into three segments corresponding to the fractions likely to contain androsterone, testosterone, and the corticosteroids. The fractions were determined by running standards concurrently on separate strips. The fractions were eluted and the eluates taken up in ethanol (1 ml). Half of each was applied to 40 by 2·5 cm strips of Whatman No. 2 and run as described by Bush (1961) whilst the other half was added to 15 ml of the dioxan scintillator and counted. Identification of the steroids was by means of Zimmerman's reagent and the ultraviolet fluorescence reaction. The spots were concentrated by "running up" (Bush 1961), cut out of the strip, and then placed in the dioxan scintillator for counting.

III. Results

Essentially complete recovery of the administered radioactivity was achieved over a period of 31 days (Osborne, Wong, and Garnett 1966). The faeces yielded 41%, urine 59·6%, and respired air 0·8%. A third of the total activity was excreted in the first 24 hr and most of this was in the urine (28·4%). Thereafter similar amounts were excreted in urine and faeces. After 4 days 74·5% had been recovered. These data are set out in Table 1.

None of the tissues examined had counting rates significantly greater than background.

Methylene dichloride extraction yielded an average of 40·9% of the total activity contained in the first 5 days urine. Butanol extraction yielded some 55·6% of the activity whilst the urine residue retained 3·5%. The only recognizable steroid obtained by chromatography of the methylene dichloride extract was androsterone. Androsterone was also the principal steroid identified from the hydrolysed urine and accounted for some 30% of the total activity of the urine. Testosterone and androstenedione each accounted for 1% of the total activity. The remainder of the active material was not identified.

IV. Discussion

The validity of conclusions reached as a result of studies with labelled steroids depends on the veracity of the assumptions that (1) during the radioactive labelling process traces of degradation products of high specific activity are not formed, and (2) that the label remains attached to the steroid nucleus during metabolism. With respect to the first assumption, Wilzbach, in a review (Wilzbach 1961), has shown that the tritium gas irradiation technique can induce saturation of double bonds. In the case of testosterone saturation of the A ring could lead to the formation of impurities having specific activities up to 100 times that of the parent compound. For this reason an alternative technique using a transition metal catalysed exchange with tritium oxide (Garnett 1962) was also used.

However, the gas irradiation procedure combined with extensive chemical purification could still be used to produce a tritium-labelled testosterone but of lower activity (J. O'Keefe and P. K. Wong, unpublished data). With respect to the second

assumption the appearance of traces of radioactivity in the expired air is regarded as evidence that at least some of the label is involved in metabolic changes: e.g. the formation of some TOH would seem likely following the catabolism of testosterone to androstenedione for instance. Such a mechanism could explain the appearance of radioactivity in the expired air. The yield of TOH is unlikely to account for more than a small fraction of the total activity as much of the label would remain attached to the steroid nucleus except under conditions that would lead to the destruction of the nucleus itself.

The absence of significant amounts of radioactivity in the tissues examined and particularly in those having an affinity for androgens, such as the prostate and kidney, suggests that exogenous androgens are not stored in the body. This view is supported by the complete recovery of the administered radioactivity in the excreta.

High recoveries of radioactivity from rats treated with physiological doses of [4-^{14}C]testosterone in ethanol were reported by Ashmore *et al.* (1953). It was found that from 86 to 104% of the ^{14}C injected was accounted for in the excreta after 4 days: 44–72% appeared in the faeces and 23–44% in the urine. Similar results with mice were reported by Gallagher *et al.* (1951): from 86 to 105% of administered radioactivity was recovered from the excreta in the first 24 hr following treatment with 113 μg testosterone in aqueous propylene glycol. Studies on steers (Gassner *et al.* 1960) yielded high recoveries (98·4%) although the rate of excretion was very much slower than noted above: only 8·4% being recovered from the urine and 90·0% from the faeces after 120 days. In this experiment the single dose of 120 mg of testosterone in polyglycollic paste was injected subcutaneously whereas the others used intraperitoneal (Gallagher *et al.*), intragastric, or intramuscular injection (Ashmore *et al.*). These authors commented that absorption from the intramuscular site was much slower than from the intestinal tract. Absorption from a subcutaneous site would likely be even slower whereas absorption from an intraperitoneal route would be very rapid (Deanesly and Parkes 1937). Hence the differences in the excretion rates between the work reported here and the several studies referred to above may be explained by the different routes employed in the administration of the testosterone: these differences would have been enhanced by the carriers chosen in each case (Deanesly and Parkes 1937).

The principal steroid excretory route in the sheep is biliary and enterohepatic circulation operates to maintain high urinary levels (H. Lindner, personal communication). Although this mechanism could explain the high urinary levels reported here, it seems probable that other factors may influence the mode of excretion. Restrictions on food intake, by slowing the rate of passage of ingesta, could lead to greater absorption from the intestine (Blaxter, Wainman, and Wilson 1961). Dodds *et al.* (1958) observed that the excretion of radioactivity from microgram doses of hexoestrol by the rabbit was in greater concentration (up to 4 times) in the faeces than in the urine although in some experiments more was excreted in the urine than faeces. Ogilvie *et al.* (1962) reported higher urinary excretion of radioactivity from rabbits treated with progesterone whilst 17α-acetoxy-6α-methylprogesterone reversed the pattern. Evidently more is involved in determining the excretory pattern than species differences.

The finding that labelled androsterone constituted 75% of the "free steroid" fraction and that 55·6% of the urinary radioactivity was in the "conjugated fraction" leads to the deduction that wethers are able to metabolize exogenous testosterone. Evidently this metabolism is quite rapid as 76% of the administered radioactivity was recovered within 4 days. The principal known urinary steroid metabolites of testosterone in man are aetiocholanolone and androsterone: these two metabolites excreted in the first 24 hr together accounted for 40% of the radioactivity administered as [4-^{14}C]testosterone (Gallagher *et al.* 1951). Evidently aetiocholanolone is not found in sheep's urine (H. Lindner, personal communication): the results reported here are in general agreement with this.

V. Acknowledgments

Grateful acknowledgment is made of the assistance rendered by Mr. D. Paix and the Department of Haemotology, Prince Henry Hospital, for the use of the liquid scintillation spectrometer.

VI. References

Ashmore, J., Elliott, N. M., Doisy, E. A. Jr., and Doisy, E. A. Sr. (1953).—Excretion of the metabolites of testosterone-4-C^{14} in the rat. *J. Biol. Chem.* **200**: 661–8.

Bagget, B., Ergel, L. L., Savard, K., and Dorfman, R. I. (1956).—The conversion of testosterone-3-C^{14} to C^{14}-estradiol-17β by human ovarian tissues. *J. Biol. Chem.* **221**: 931–6.

Belcher, E. H. (1960).—The assay of tritium in biological material by wet oxidation with perchloric acid followed by liquid scintillation counting. *Physics Med. Biol.* **5**: 49–56.

Blaxter, K. L., Wainman, F. W., and Wilson, R. S. (1961).—The regulation of food intake by sheep. *Anim. Prod.* **3**: 51–61.

Bray, G. A. (1960).—A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Analyt. Biochem.* **1**: 279–85.

Bush, I. E. (1952).—Methods of paper chromatography of steroids applicable to the study of steroids in mammalian blood and tissues. *Biochem. J.* **50**: 370–8.

Bush, I. E. (1961).—"The Chromatography of Steroids." (Pergamon Press: Oxford.)

Calf, C. E., and Garnett, J. L. (1964).—The use of sodium borohydride in catalytic deuterium exchange reactions. *J. Phys. Chem.* **68**: 3887–90.

Deanesly, R., and Parkes, A. S. (1937).—Factors influencing the effectiveness of administered hormones. *Proc. R. Soc.* B **124**: 279–98.

Dodds, E. C., Folley, S. J., Glascock, R. F., and Lawson, W. (1958).—The excretion of microgram doses of hexoestrol by rabbits and rats. *Biochem. J.* **68**: 161–7.

Dulmanis, A., Coghlan, J. P., Wintour, M., and Hudson, B. (1964).—The estimation of testosterone in biological fluids. II. Testosterone in urine. *Aust. J. Exp. Biol. Med. Sci.* **42**(3): 385–400.

Gallagher, T. F., Fukushima, D. K., Barry, M. C., and Dobriner, K. (1951).—Studies with isotopic steroid hormones. *Rec. Prog. Horm. Res.* **6**: 131–53.

Garnett, J. L. (1962).—Catalytic tritium labelling attractive for organics. *Necleonics* **20**(12): 86–91.

Gassner, F. X., Martin, R. P., Shimoda, W., and Algeo, J. W. (1960).—Metabolism of radioactive steroid esters in the bovine male and female. *Fert. Steril.* **11**: 49–73.

Handler, J. A. (1963).—A liquid-scintillation method for determining tritium in the tissues of animals dosed with tritium-labelled vitamin A. *Analyst* **88**: 47–55.

Harlan, J. W. (1961).—Liquid scintillation counting of $C^{14}O_2$ in aqueous carbonate solutions. *Atomlight* **19**: 8–11.

Lindner, H. R. (1961).—Androgens and related compounds in the spermatic vein blood of domestic animals. I. Neutral steroids secreted by the bull testis. *J. Endocr.* **23**: 139–59.

MILLER, W. R., and TURNER, C. W. (1961).—Hydrolysis, extraction, and fractionation of the biliary androgens in the female bovine. *J. Dairy Sci.* **44**: 1356–63.

OGILVIE, M. L., CASSIDA, L. E., FIRST, N. L., and HOEKSTRA, W. G. (1962).—Excretion and tissue distribution of tritium labelled progestions in rabbits and sheep. *J. Anim. Sci.* **21**: 262 (abstr.).

OSBORNE, W. B., WONG, P. K., and GARNETT, J. L. (1966).—The use of testosterone labelled with tritium to determine steroid residues in animals. *Aust. J. Sci.* **28**(12): 463–5.

SOUTHCOTT, W. H. (1962).—The prevention and treatment of ovine posthitis with testosterone propionate. *Aust. Vet. J.* **38**(2): 33.

VANDE WIELE, R. L., MACDONALD, P. C., BOLTE, E., and LIEBERMAN, S. (1962).—Precursors of the urinary 11-desoxy-17-ketosteroids: estimation of the secretory rate of dehydro-iso-androsterone. *J. Clin. Endocr.* **22**: 1207–13.

VANDE WIELE, R. L., MACDONALD, P. C., GURPIDE, E., and LIEBERMAN, S. (1963).—Studies on the secretion and interconversion of the androgens. *Rec. Prog. Horm. Res.* **19**: 275–310.

WATSON, R. H., and MURNANE, D. (1958).—Non-contagious ovine posthitis ("sheath rot"): some aspects of its course and etiology. *Aust. Vet. J.* **34**(5): 125–36.

WILZBACH, K. E. (1961).—Gas exposure method for tritium labelling. Proc. Int. Atomic Energy Agency Conf., Vienna. Vol. 2 p. 3–10.