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Studies on the Metabolism of Testosterone *Trans*-4-*n*-butylcyclohexanoic Acid in the Cynomolgus Monkey, *Macaca fascicularis*

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Metabolism of intravenously administered testosterone *trans*-4-*n*-butylcyclohexanoate (T bucyclate), a potent, long-acting androgen, was studied in cynomolgus monkeys (*Macaca fascicularis*). About 5% of the radioactivity of a dose of doubly labeled ester (^{14}C, ^{3}H) was excreted via the gastrointestinal tract. Most of the administered radioactivity was excreted in the urine within 120 h. No intact T bucyclate was recovered from either compartment. Tritium attributed to bucyclic acid and its metabolites was excreted rapidly (peak excretion was at 6 h after injection), while ^{14}C excretion, attributed to testosterone and its metabolites, extended over 4 days. Testosterone metabolites were excreted predominantly as sulfate esters. Analysis of urinary products derived from the bucyclic acid moiety of T bucyclate showed no products susceptible to glucuronidase treatment, and showed a mixture of unidentified solvolyzable and unconjugated products. No unmetabolized *trans*-4-*n*-butylcyclohexanoic acid was detected in urine or feces. It is concluded that metabolism of testosterone bucyclate is initiated *in vivo* in cynomolgus monkeys by hydrolysis of ester to testosterone and bucyclic acid. The bucyclate side chain is rapidly cleared, and the testosterone is retained in the circulation.

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INTRODUCTION

Among the androgenic variants that have been investigated as potential long acting therapeutic replacements for testosterone in men have been the testosterone esters. Though some have proved to be clinically useful, those currently available have not succeeded in overcoming the critical problem of providing a reservoir of steroid capable of maintaining physiological levels of testosterone over extended periods of time. It has been found recently that the ester testosterone-17β *trans*-4-*n*-cyclohexyl carboxylic acid (T bucyclate, testosterone buciclate, CDB-1781, 20Aet-1) displays pharmacokinetic behavior that achieves that goal, permitting the maintenance of circulating testosterone for 16 weeks or longer in men and experimental primates [1–3]. Consistent with these observations, we find that the *in vitro* hydrolysis of T bucyclate to testosterone and bucyclic acid (*trans*-4-*n*-cyclohexanoic acid) by cynomolgus monkey liver is slow, and that the metabolism of the bucyclic acid hydrolysis product is slow as well, generating a small number of metabolites [4]. In this paper, we report the *in vivo* metabolism of T bucyclate in cynomolgus macacques.

EXPERIMENTAL

Unlabeled testosterone, androstenedione, androstanediol, and estradiol were bought from Steraloids (Wilton, NH). 5α-Dihydrotestosterone-17β-*trans*-4-*n*-butylcyclohexanoate was provided by Dr P. N. Rao (San Antonio, TX); T bucyclate and bucyclic acid were provided by Dr Jerry Reel (Rockville, MD). Sep-pak cartridges were purchased from Waters Chromatography Division, Millipore Corp. (Milford, MA). Polyethyleneiminesilane cartridges (PEI) were purchased from J. T. Baker Inc. (Phillipsburg, NJ). Solvents were all HPLC grade (J. T. Baker). Solvolysis was performed using solvolysis reagent (ethyl acetate–methanol 8:2, v/v, containing 1 drop of H_2SO_4 per 25 ml) with hydrolysis being performed at 56°C for 2 h

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or 37°C overnight. Testosterone and *trans*-4-*n*-cyclohexanoate were found to be stable to this procedure.

Normal phase TLC was performed using plastic-backed Polygram Sil G (0.25 mm) with indicator UV_{254} (Polygram Sil G/UV_{254}, Brinkmann Instruments Co., Westbury, NY), in ethyl acetate–chloroform–acetic acid, 49.5:49.5:1 (by vol) unless otherwise stated. Spray reagents used to identify both steroid and side chain moieties included acid charring (5% H_2SO_4 in methanol) and vanillin reagent [1.5 g vanillin in absolute alcohol (99 ml) containing H_2SO_4 (1 ml)]. Plates were sprayed and heated at 300°C.

Scintillation counting was performed using a Tricarb 460-C liquid scintillation system (Packard, Meriden, CT) in 20 ml vials (VWR, Media, PA) using Readysafe scintillation fluid (Beckman, Somerset, NJ). For dual label analysis, composite ^3H and ^{14}C spectra were decomposed by computer generated calculation, with channel A set at 0–12 keV and channel B set at 19–156 keV.

Administration of test compounds

Two separate studies, designated DVS-55 and DVS-60, were performed using the same four intact adult male *Macaca fascicularis* monkeys for each study. Each monkey was housed individually in a metabolism cage. Urine collected for 24 h prior to the start of each experiment was used as a reference point to ascertain when post-treatment radioactivity returned to 4 times background. For each study, a total of eight vials were prepared for i.v. injection:

(a) Four vials containing 0.7 ml of T bucyclate (0.5 mg/ml) in 65.4% aqueous ethanol and approx. 5 μCi ^3H-labeled T bucyclate (testosterone 17-β-*trans*-4-*n*-butyl-2,3-[^3H]cyclohexanyl carboxylate, 18.71 μCi/mmol) [5].

(b) Four vials containing 0.7 ml of T bucyclate (0.5 mg/ml) in 65.4% aqueous ethanol and approx. 3 μCi ^{14}C-labeled T bucyclate ([^{14}C]testosterone 17β-*trans*-4-*n*-butyl-cyclohexanoate, 50 mCi/mmol) [5].

Collection of excreta

Injection of radioactive material and collection of urine and feces were performed at the California Regional Primate Research Center, University of California (Davis, CA). Our objective was to obtain consecutive 24 h urine and fecal samples, terminating collection when the urine radioactivity was $<4 \times$ background. Four monkeys were dosed between 9 and 9.10 a.m. of the initial day of the study. Two i.v. doses of a total of 5 mg/ml of T bucyclate were administered, with the vehicle being 65.4% ethanol in sterile water. Five μCi of the tritium labeled copound were injected first, followed by 3 μCi of the ^{14}C-labeled compound. Urines and feces were collected for 6 (DVS-55) or 3 days (DVS-60), frozen, and sent to the Population Council for further analysis. A detailed description of urine volumes and fecal weights accompanied the samples. Treatment of urine was designed to separate neutral and acidic radioactive metabolites, and to hydrolyze conjugates (glucuronides, sulfates). The procedure is outlined in the accompanying flowsheet (Fig. 1). Preparations were cochromatographed with authentic compounds as standards. To quantitate radioactive spots, corresponding positions were cut out and ^3H and ^{14}C were measured by scintillometry.

Analysis of fecal samples

A 1 g aliquot of fecal matter from each monkey was taken for analysis. It was necessary to solubilize the dry material in order to extract the radiolabel present. No external or internal standard was available to determine extraction efficiency, and an extraction protocol had to be developed.

Total solubilization. NCS solubilizer (Amersham Corp, Arlington Heights, IL), a mixture of quaternary

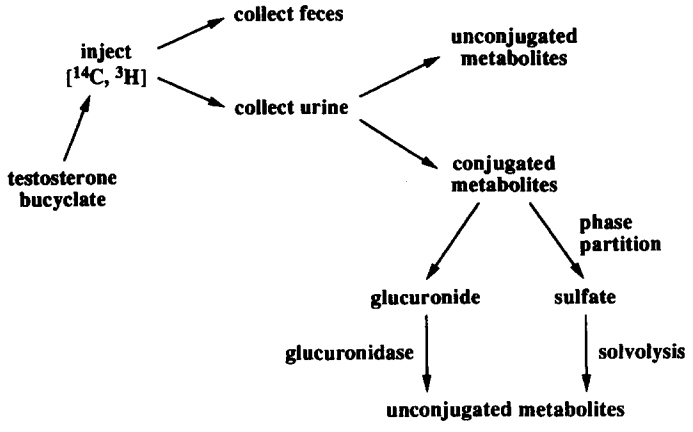

Fig. 1. Flow chart illustrating fractionation procedure for separating radioactive components in cynomolgus excreta as detailed in the text.

ammonium bases in toluene, was used to solubilize the fecal samples. However, the brown suspensions obtained after 1 h at 50°C were not miscible with scintillation fluid (Beckman "Ready Safe" scintillation cocktail for aqueous samples). The mixtures were bleached with a variety of agents including glacial acetic acid, benzoyl peroxide, and sodium hypochlorite. It was not possible to count smaller aliquots of the fecal extracts because small aliquots contained too few counts.

Solid–liquid extraction. To extract maximum radioactivity and minimum color, fecal samples were first wetted with water (0.5 ml per g solids) or retained as received, and extracted with 5 ml of methanol or ethyl acetate (1 h with gentle agitation). The suspensions were centrifuged (2000 *g*, 10 min) and the organic phase was aspirated. Methanol extracts were easier to bleach with 5% aqueous sodium hypochlorite than ethyl acetate extracts, and methanol was retained as the solvent of choice.

Wetting the feces prior to extraction increased overall recovery, but while levels of ^{14}C (indicating steroid) remained low and constant, successive extractions with fresh aliquots of methanol indicated poor overall extraction of the ^{3}H present. Effective recovery (greater than 85–94% in first aspirate, $n = 6$) was achieved by altering the pH of the wetted feces to around 10 (5 M NaOH). Acidifying the slurry (pH 2, 6 M HCl) did not have an expected similar effect. These procedures generated strong chemiluminescence. It was necessary to keep the samples in the dark in the scintillation counter for 48 h in order to suppress spurious scintillation events.

Analytical procedure for fecal samples. The protocol adopted for the analysis of fecal samples from experiments DVS-55 and DVS-60 was as follows:

One gram of frozen fecal matter (duplicates) was weighed into 15 ml polypropylene tubes. A volume of 0.6 ml of 5 M NaOH was added to fully wet the material; the suspension was vortexed and 5 ml methanol were added. The tubes were gently agitated on a rocking platform for 1 h, centrifuged at 2000 *g* for 10 min, and the methanol was aspirated into 20 ml disposable scintillation vials. The eluates were decolorised using sodium hypochlorite (5% v/v) and scintillant cocktail (15 ml Readysafe, Beckman) was added. Samples were counted 24 h after extraction, then stored in the dark at room temperature for 48 h and recounted to establish if chemiluminescence had receded. Background was established using alkalinized water/methanol mixed together with extracts from the individual monkey fecal samples obtained 24 h prior to injection.

Analysis of urine samples

Straight counts. Five ml of urine were found to be quite effectively counted without additional treatment in 15 ml of scintillant cocktail. This expedient method of establishing radioactive content was adopted together with a solid phase extraction technique (Waters, Sep-pak) as comparison. Decolorization using sodium hypochlorite was only used with deeply colored samples in straight count work.

Solid phase extraction. It has been established that the lipophilic properties of the C18 resin are effective in quantitatively removing steroids from aqueous solution. Their usefulness in isolating the ^{3}H corresponding to the 4-*n*-butyl cyclohexanoic acid moiety remained to be established. When aliquots of urine were directly applied to an activated Sep-pak cartridge (washed sequentially with methanol and water, 5 ml each, cartridge never allowed to dry out completely between applications), between 59 and 78% ($n = 4$) of the ^{3}H was recovered in the aqueous eluate or discard fraction. By successively lowering the pH of the urine to pH 4.5 (ion suppression), losses of ^{3}H appeared to be reduced, although a significant level (10–40%) remained unretained.

Greater than 90% of the ^{14}C moeity was accounted for in the methanol used to strip the cartridge of hydrophilic species. The resultant fraction did not require decolorization prior to counting. Hexane was used as an intermediary step to dry the cartridge prior to the addition of methanol, and for both ^{3}H and ^{14}C, only between 1–5% of total activity was seen in this phase.

Fractionation of conjugates

Urines (5 ml, duplicates) adjusted to pH 6.8 were incubated with β-glucuronidase for 2 h at 56°C, cooled, and applied to activated Sep-pak columns. The unconjugated and glucuronide fractions were eluted using diethyl ether (5 ml) while the sulfoconjugates were isolated using methanol (5 ml). Comparative studies where samples were not subjected to glucuronidase treatment prior to Sep-pak extraction were performed. Using this methodology an estimate of conjugation of the ^{3}H and ^{14}C metabolites were made.

RESULTS

Fecal extraction

^{3}H metabolites. Fecal excretion of tritiated material derived from T bucyclate is summarized in Fig. 2. The data indicates that the amount of ^{3}H excreted into the gastrointestinal tract of cynomolgus monkeys is not quantitatively significant. In Table 1 is estimated the percent of tritium found in the fecal matter of the monkeys to whom were administered 5 μCi of tritiated steroid ester. The technical difficulties inherent in measuring ^{3}H in the solid excreta have been discussed in the Experimental section. The values given are therefore approximations. Nevertheless, the values in the two sets of experiments indicate that no more than 5% of the radioactive steroid ester or its components were excreted via the gastrointestinal tract.

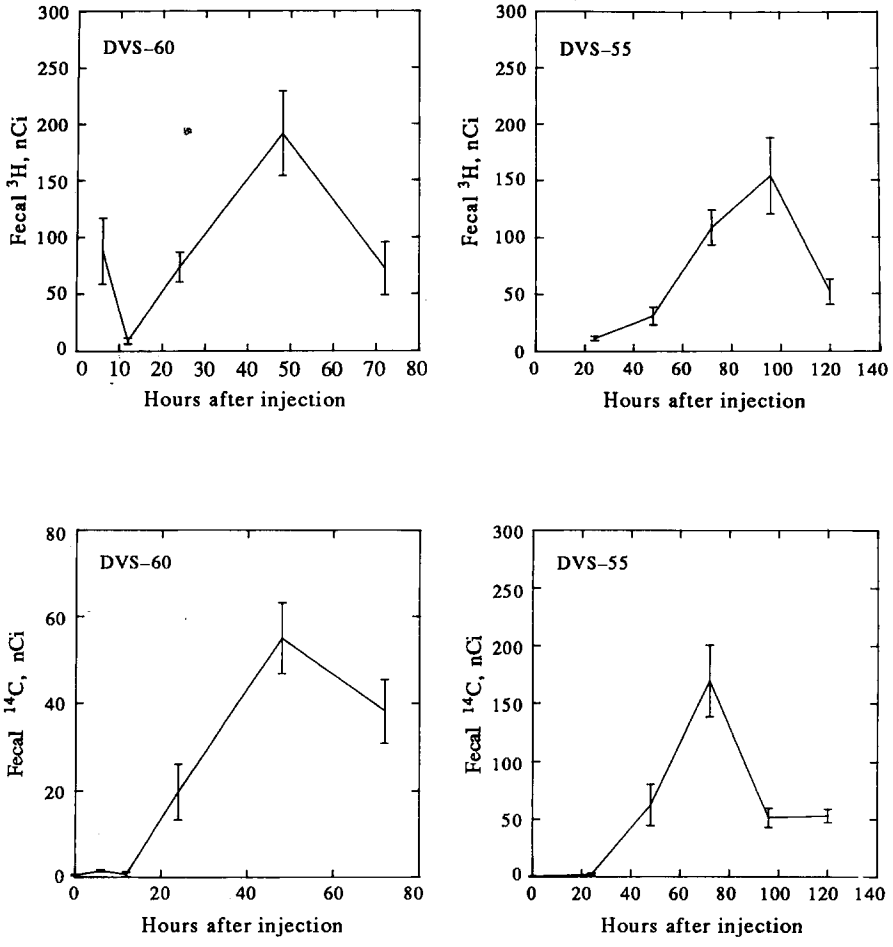

Fig. 2. Fecal excretion of ^{3}H and ^{14}C metabolites of [^{14}C]T [^{3}H]bucyclate. Each point represents means ± SEM for 6 monkeys.

^{14}C metabolites. Fecal excretion of ^{14}C was greater than that of tritium. Our data (Fig. 2) indicate that excretion peaked at between 48 and 72 h. Table 1 summarizes the total ^{14}C excreted in each study based on the data in Fig. 2. The lower excretion for DVS-60 may indicate incomplete collection of radioactivity, since the time interval of collection (72 h) was less than that for DVS-55.

Urinary excretion

^{3}H metabolites. Urinary excretion of ^{3}H was determined at 24 h intervals in series DVS-55. Maximum accumulation in the urine occurred at 24 h, followed by rapid decay, so that little additional radioactivity was found at later intervals. To determine the rapidity of excretion more closely, measurement of urinary ^{3}H was made at shorter intervals in series DVS-60. The peak of excretion occurred at 6 h. Figure 3 shows the mean ^{3}H excretion data for the monkeys in the two series of experiments.

Table 1. Fecal excretion of T bucyclate metabolites

	DVS-55	DVS-60
Collection interval	120 h	72 h
Fecal ^{3}H (nCi)	265	108
Proportion of ^{3}H (%)	5.3	2.2
Fecal ^{14}C (nCi)	315	115
Proportion of ^{14}C (%)	10.5	3.8

^{14}C metabolites. In series DVS-55, maximum urinary ^{14}C excretion occurred within the first 24 h after injection of steroid ester. Based on the data obtained in the subsequent DVS-60 series (Fig. 3), this represented the cumulative effect of a slowly decreasing excretion of ^{14}C from a maximum value at 6 h. In an independent study performed with the same animals, it was found that ^{14}C (attributed to testosterone and its metabolites) was excreted into the urine more slowly than the ^{3}H representing the cyclohexanoic acid moiety (Fig. 4). It was concluded that the low rate of excretion of testosterone metabolites in these two studies was caused by retention of steroid by the animals.

The evidence shows that T bucyclate and its metabolites, including those derived both from the steroid and the side chain, are excreted overwhelmingly through the kidney in the cynomolgus monkey. Tritiated metabolites are excreted early and rapidly, while ^{14}C metabolites are excreted more slowly over an

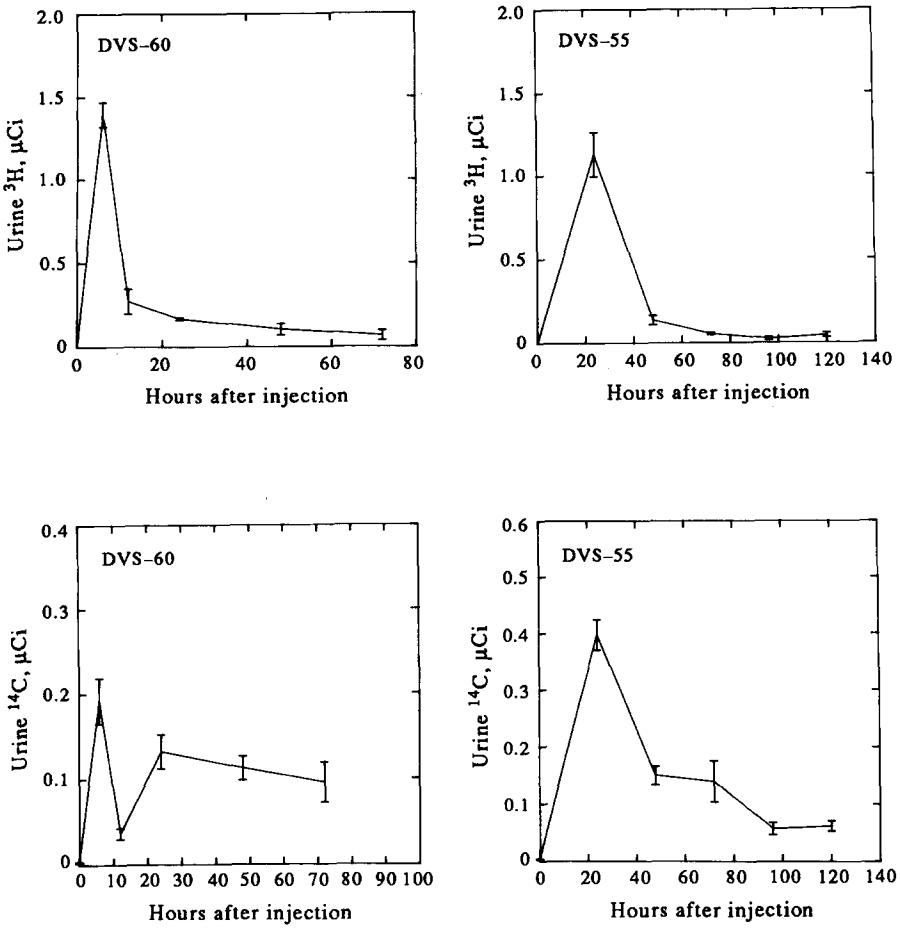

Fig. 3. Urinary excretion of ^{3}H and ^{14}C metabolites of [^{14}C]T [^{3}H]bucyclate. Each point represents means ± SEM for 6 monkeys.

extended interval of time. About 90% of the administered radio tracers were recovered in the urine. The tritium excreted in 24 h in DVS-55 approximates to the sum of the means of the 0–6, 6–12 and 12–24 h values obtained for DVS-60.

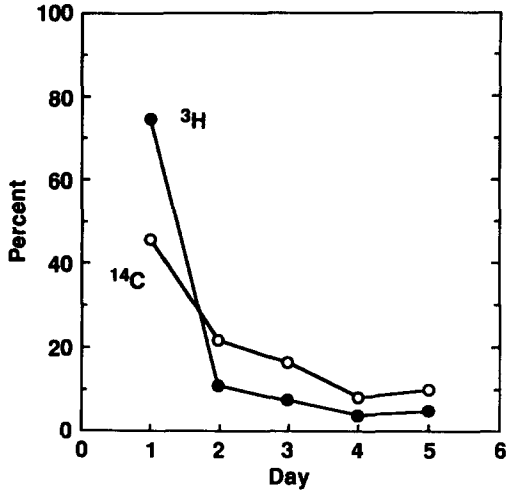

Fig. 4. Excretion of ^{3}H and ^{14}C into urine after bolus i.v. injection of [^{14}C]T [^{3}H]bucyclate into cynomolgus monkeys. Each point represents the daily percent of total radioactivity excreted.

Distribution of free and conjugated metabolites in urine. **^{14}C metabolites**: Figure 5 summarizes the results of studies on the distribution of conjugated and unconjugated urinary metabolites of T bucyclate corresponding to [^{14}C]testosterone. Excreted ^{14}C, representing testosterone metabolites, was found overwhelmingly in the conjugated fraction. This indicates extensive A ring reduction, as was found in the *in vitro* studies [4]. Total ^{14}C absorbed to Sep-pac was 98% of what was introduced. Of this, 13.1% was eluted in the unconjugated fraction, and probably represents testosterone and/or androstenedione.

^{3}H metabolites: Multiple metabolites containing ^{3}H were present in urine. A small proportion (11.2%) of the injected cyclohexanoate moiety was excreted as unconjugated tritium-containing metabolites (Fig. 6). Of the proportion of the total retained on the column, most was eluted in the conjugated fraction (37% of total added ^{3}H; 85.5% of bound ^{3}H). Of the urine passed through Sep-pac at pH 7, 52% of the ^{3}H did not bind (effluent). When the unbound fraction was adjusted to pH 2 and subjected to solvolysis, 74% of this

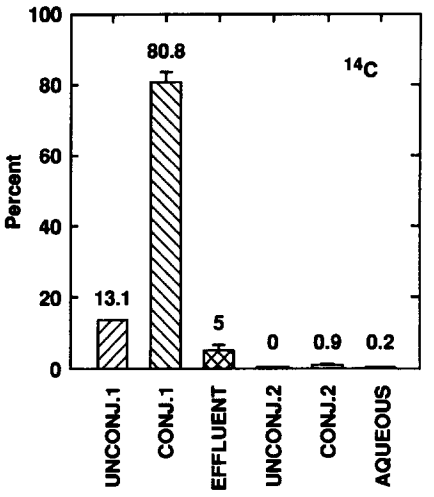

Fig. 5. Distribution of conjugated and unconjugated species of ^{14}C metabolites after fractionation of urine. Bar marked *effluent* indicates ^{14}C that was not bound to the Sep-pac absorption column. This was extracted with ethyl acetate and the organic phase was refractionated (unconj. 2, conj. 2).

fraction (42% of total radioactivity) was extracted into ethyl acetate. The remaining aqueous fraction was partitioned on Sep-pac. Little unconjugated metabolite was found (Unconj. 2). Most of the remainder was eluted in the conjugate fraction. A small residue of water-soluble radioactivity remained.

Thus, the partition data indicated that the testosterone and bucylic acid metabolites originating from T bucyclate migrated in distinct compartments, consistent with their independent metabolism. The partition of ^{3}H indicated the formation of metabolites extending over a range of polarities.

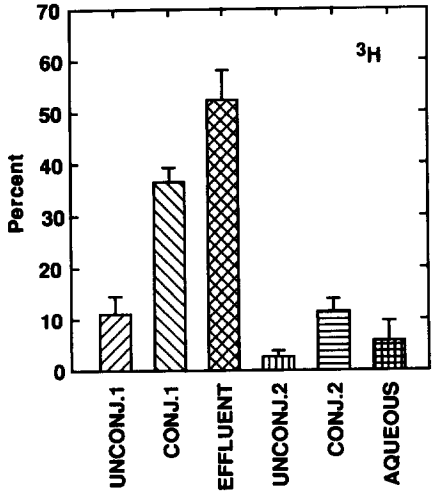

Fig. 6. Distribution of conjugated and unconjugated species of ^{3}H metabolites after fractionation of urine. Bar marked effluent indicates ^{3}H that was not bound to the Sep-pac absorption column. This was extracted with ethyl acetate and the organic phase was refractionated (unconj. 2, conj. 2).

Analysis of conjugated metabolites

The strategy used to analyze the conjugated metabolites of testosterone is based on the assumption that the ligand is either glucuronic or sulfuric acid. Treatment of the conjugate with glucuronidase and rechromatography on Sep-pac permits the determination of the proportion of metabolite conjugated with glucuronic acid. Subsequent solvolysis permits the determination of sulfate conjugates.

Based on the changes in chromatographic mobility, solvolysis of ^{14}C-labeled conjugates was $91.5 \pm 3.0\%$ (mean $\pm$ SEM for 4 monkeys) complete. The amount of ^{14}C remaining at the origin after chromatography was $8.5 \pm 3.0\%$ of the total radioactivity. Solvolysis released $23.3 \pm 8.2\%$ (mean $\pm$ SEM for 4 monkeys) of tritiated urinary metabolites under the same conditions, leaving $76.7 \pm 8.2\%$ at the origin. Further identification of the ^{14}C- and ^{3}H-labeled metabolites was not made. None of the tritiated metabolites released after solvolysis corresponded with *trans*-4-*n*-butylcyclohexanoic acid.

DISCUSSION

In this study, we have shown that T bucyclate introduced into the bloodstream of the cynomolgus monkey is rapidly hydrolyzed. The bucyclic acid side chain is readily metabolized and excreted. The testosterone thus released is retained in the circulation. We find no evidence of metabolic products that contained both ^{3}H and ^{14}C. Unlike some other species (e.g. raabbit, guinea, pig, rat), where cleavage of T bucyclate by blood may play an important role in releasing biologically active androgen, this process occurs in the liver of the primate, rather than the blood [4]. In these studies we did not attempt to identify the ^{14}C metabolites in urine.

The fate of the released testosterone presumably proceeds along a conventional course, since it is not likely that it could be metabolically distinguished from endogenous circulating steroid. The use of the i.v. route of administration to the monkeys was decided by the observation that a depot of T bucyclate is transferred to the circulation over many weeks [1–3, 6–8]. In this sense, our choice of administering the ester intravenously, though artifactual, was dictated by practical considerations. The results of these experiments show that, even under these extreme conditions, little of either steroid or side chain is excreted through the gastrointestinal tract. Processing by the liver (i.e. hydrolysis, metabolism and conjugation) is more thorough than would have been predicted by our *in vitro* studies. The side chain, bucyclate, is metabolized, conjugated, and excreted into the urine significantly more rapidly than is testosterone. Thus, the rapid clearance of the side chain result in the selective retention of the steroid. We conclude that the fate of

the ester is partitioned: active testosterone persists in the circulation and the side chain is excreted into the urine. The fecal data, though involving much lower levels of tracer, leads to essentially the same conclusion. Bucyclic acid itself is absent from the urine, suggesting that *in vivo*, in contrast with *in vitro*, the substrate is extensively metabolized before excretion. Identification of these urinary metabolites remains a significant challenge.

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REFERENCES

1. Behre H. M. and Nieschlag E.: Testosterone buciclate (20Aet-1) in hypogonadal men: Pharmacokinetics and pharmacodynamics of the new long-acting androgen ester. *J. Clin. Endocr. Metab.* 75 (1992) 1204–1210.
2. Weinbauer G. F., Marshall G. R. and Nieschlag E.: New injectable testosterone ester maintains serum testosterone of castrated monkeys in the normal range for four months. *Acta Endocr. (Copenh)* **113** (1986) 128–132.
3. Rajalakshmi M. and Ramakrishnan P.: Pharmacokinetics and pharmacodynamics of a new long-acting androgen ester: maintenance of physiological androgen levels for 4 months after a single injection. *Contraception* **40** (1989) 399–412.
4. Monder C., Vincze I., Blye R. and Iohan F.: Metabolism of testosterone *trans*-4-*n*-butylcyclohexanoate (T bucyclate), a high potency androgen, in rodents and primates: *in vitro* studies. *J. Endocr.* **140** (1994) 465–473.
5. Acosta C. K., Cessac J. W., Rao P. N. and Kim, H. K.: Tritium labeled *trans*-4-*n*-butyl-2,3-^{3}H-cyclohexane carboxylic acid. Preparation of testosterone 17β-*trans*-4-*n*-butyl-2,3-^{3}H-cyclohexane carboxylate and [4-^{14}C]-testosterone 17β-*trans*-4-*n*-butylcyclohexane carboxylate. *J. Labeled Comp. Radiopharm.* **28** (1990) 1201–121.
6. Weinbauer G. F., Göckeler E. and Nieschlag E.: Testosterone prevents complete suppression of spermatogenesis in the gonadotropin-releasing hormone antagonist-treated nonhuman primate (*Macaca fasicularis*). *J. Clin. Endocr. Metab.* **67** (1988) 284–290.
7. Weinbauer G. F., Khurshid S., Fingscheidt U. and Nieschlag E.: Sustained inhibition of sperm production and inhibin secretion induced by a gonadotropin-releasing hormone antagonist and delayed testosterone substitution in non-human primates (*Macaca fascicularis*). *J. Endocr.* **123** (1989) 303–310.
8. Rajalakshmi M., Ramakrishnan P. R., Kaur J., Sharma D. N. and Pruthi J. S.: Evaluation of the ability of a new long-acting androgen ester to maintain accessory gland function in castrated rhesus monkey. *Contraception* **43** (1991) 83–90.