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Is Testosterone Glucuronoside Uniquely Derived from Plasma Testosterone? *

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The isotope dilution method has been useful in measuring either secretion rates or production rates of several important steroid hormones. This technique consists of measuring the dilution of a tracer amount of hormone into the plasma pool from the cumulative specific activity of a unique urinary metabolite of that pool. If the plasma pool is derived entirely from glandular secretion of the hormone, then a secretion rate is measured. If, however, both glandular secretion and peripheral conversion of one or more precursors contribute to this pool, then a production rate is measured. In either case the validity of the method depends strictly upon the presence of a urinary metabolite uniquely derived from the pool in question. Since it has generally been assumed that a steroid hormone conjugate would be such a unique metabolite, secretion rates of aldosterone and, in women, estradiol have been measured in this way.

In discussing an isotope dilution method (1) for measuring the testosterone production rate (TPR), we considered the possibility that testosterone glucuronoside (TG) may not be a unique metabolite of the plasma testosterone (T) pool. This situation could arise as follows: It has been shown that T can be synthesized peripherally from both androstenedione (Δ) and dehydroepiandrosterone (D) (2–5). Presumably this transformation occurs in the liver, and indeed this has been demonstrated by perfusion studies with dog liver (6). When T is synthesized either in the liver or at any other peripheral site, if conjugation occurs before entry of this T into plasma some TG will be excreted that is not derived from plasma T. This concept is diagrammed in Figure 1. The portion within the dotted line represents that hypothetical moiety of T derived from Δ, which is conjugated immediately. We have designated this route the Δ → TG pathway.

The following studies were undertaken to test for the existence of this pathway and evaluate its quantitative significance. Androstenedione-H^3 and T-C^{14} were infused, and the H^3/C^{14} ratios of free and conjugated T were compared. If all the T derived from Δ re-entered the plasma as free T, then the H^3/C^{14} ratio of free and conjugated T should become equal as metabolism proceeds towards completion. Conversely, if all the T synthesized from Δ underwent immediate conjugation, plasma free T would contain no tritium. Our results indicate that the Δ → TG pathway exists, that it may contribute substantially to urinary TG, and therefore that urinary TG is not a unique metabolite of the plasma T pool. Thus the TPR must overestimate production of plasma T.

Methods

The methods of isolating urinary T and measurement of the TPR have been described (1, 7). Testosterone-4-C^{14} [1] was chromatographed on paper in the Bush type system, ligroin : methanol : water (10 : 7 : 3), followed by thin-layer chromatography in the system, benzene : ethyl acetate (3 : 2). Androstenedione-1,2-H^3 [1] was purified by thin-layer chromatography in benezene : ethyl acetate (3 : 2) before use.

Free T was isolated from plasma as follows: Plasma was separated from red cells within an hour of completion of the experiment, and 200 μg of T was added to each sample. After the addition of 1 ml of 1 N NaOH per 40 ml plasma, the plasma was extracted twice with 3 vol ether : chloroform (3 : 1). After washing with $\frac{1}{2}$ and $\frac{1}{10}$ vol of H_2O, the ether-chloroform was dried over Na_2SO_4 and evaporated. The free T was then isolated by thin-layer and paper chromatography as described for urinary T.

To isolate TG the spent plasma and combined aqueous washes were mixed with 5 vol of absolute ethanol. After 16 hours at 23° C, the white flocculent precipitate

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[1] New England Nuclear Corp., Boston, Mass.

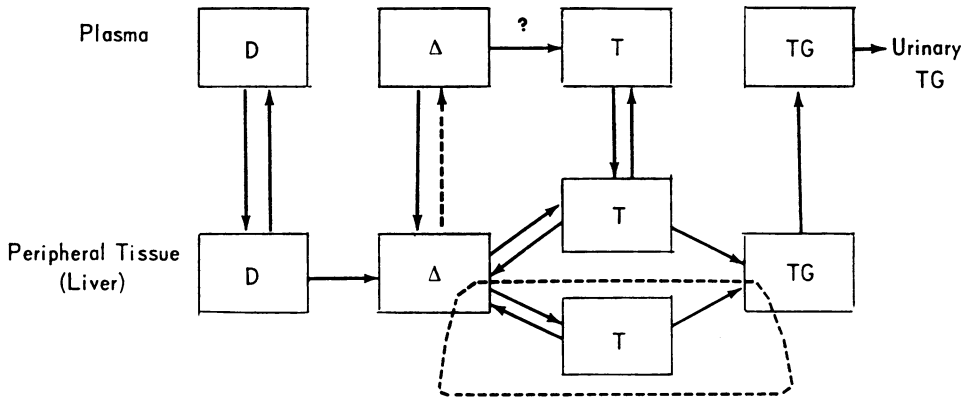

FIG. 1. MODEL COMPARTMENTAL DISTRIBUTION OF TESTOSTERONE (T), ANDROSTENEDIONE (Δ), DEHYDROEPIANDROSTERONE (D), AND TESTOSTERONE GLUCURONOSIDE (TG) IN PLASMA AND PERIPHERAL TISSUES. The broken arrow represents a pathway that has not been demonstrated. The area enclosed by the dotted line represents the formation of T and conjugation before entry into plasma (Δ → TG pathway).

was removed by vacuum filtration and the supernatant fluid taken as nearly as possible to dryness by vacuum distillation at 50° C. The samples were diluted with water to the initial volume of plasma, acidified to pH 5.0, buffered with $\frac{1}{10}$ vol 1 M acetate buffer, pH 5.0, and incubated with β-glucuronidase, 600 U per ml, at 37° C for 72 hours. After adding 200 μg of T, the steroids were extracted twice with 3 vol of ether: chloroform (3:1). The organic phase was washed twice with water, dried with Na_2SO_4, and evaporated. The dry lipid extract was dissolved in 200 ml of 70% methanol, placed at −14° C for 16 hours, and centrifuged at 10,000 *g* at −14° C for 1 hour. The T was isolated from the supernatant liquid as above.

Testosterone was oxidized to Δ with 0.2% chromic acid: acetic acid (wt/vol) at 23° C for 100 minutes. Androstenedione was reduced to 3β,17β-dihydroxyandrost-4-ene with 200 mg potassium borohydride in 2 ml methanol at 23° C for 15 minutes. After decomposing the borohydride with 2 drops of glacial acetic acid, the samples were dried, taken up in chloroform, and washed three times with H_2O to remove salts. The 3β,17β-diol is formed in over 90% yield. Androstenediol was converted to the diacetate by overnight incubation with acetic anhydride in pyridine. Androstenediol and Δ were isolated by thin-layer chromatography in the system, benzene: ethyl acetate (3:2), and androstenediol diacetate similarly in benzene: ethyl acetate (4:1).

Simultaneous counting of H^3 and C^{14} was carried out in the Packard Tri-Carb liquid scintillation spectrometer, model 314 EX, in toluene, 2,5-diphenyloxazole, and 1-4-bis-2-(5-phenyloxazolyl) benzene fluor with discriminator and gain settings such as to allow less than 0.3% of the H^3 counts to enter the C^{14} channel. Unless otherwise indicated, the errors of the H^3/C^{14} ratios were less than 5% at the 95% confidence limits. On occasion, because of low counting rates, the total sample was used and the labeled steroid recovered from the counting vials by thin-layer chromatography and used for further analyses.

The reverse isotope dilution experiments were performed by adding 200 μg of T to 10% aliquots of the urine samples, isolating the urinary T in the usual fashion, and assaying for radioactivity and recovery. Since TG was not available, we assumed 100% hydrolysis of urinary TG in our calculations.

After the simultaneous intravenous administration of Δ-H^3 and of T-C^{14}, blood was removed by plasmapheresis into heparinized bags, centrifuged, and the plasma extracted on completion of the experiment. Urine was collected for the subsequent 48 hours and stored at −14° C until processed. Details of the time of plasmapheresis and the amounts of plasma obtained are given in Tables I, II, and IV.

Subject H.F. was a 36-year-old man with generalized psoriasis who was otherwise in good health. M.L., a 23-year-old woman, was in good health 4 months after chemotherapy for persistent hydatidiform mole. C.T. was a 36-year-old man with a 1-year diagnosis of mycosis fungoides. There was no evidence of systemic disease or hepatic damage. J.P., a 54-year-old man with panhypopituitarism, was receiving replacement doses of cortisone, desiccated thyroid, and fluoxymesterone.

Results

Experiment 1: H.F. This study was designed to compare the H^3/C^{14} ratio of urinary TG with the maximal plasma T ratio. Accordingly, after the administration of 6×10^7 dpm of Δ-H^3 and 2×10^6 dpm of T-C^{14}, plasma was collected at intervals between 10 and 58 minutes (Table I), and urine was collected for 48 hours. A constant

TABLE I

Successive H^3/C^{14} ratios in plasma and urinary testosterone after administration of androstenedione-1,2-H^3 and testosterone-4-C^{14} in subject H.F.

	Interval after injection	Plasma volume	Androstenedione: Total plasma H^3/C^{14}	Androstenedione: Ratio H^3/C^{14}	Androstenediol: Total plasma H^3/C^{14}	Androstenediol: Ratio H^3/C^{14}	Androstenediol diacetate: Total plasma H^3/C^{14}	Androstenediol diacetate: Ratio H^3/C^{14}
	minutes	*ml*	*dpm*		*dpm*		*dpm*	
Plasma	10–15	109	1,850/840	2.2	556/266	2.1	167/73	2.3
	21–27	110	2,054/254	8.1	790/92	8.6	224/31	7.3
	30–40	84	1,636/184	8.9	576/73	7.9	210/27*	7.8
	41–58	154	1,935/276	7.0	1,450/254	5.7	127/18†	7.1
Urine	0–48 hours			11.5‡				

* Error at the 95% confidence limits = ± 6.2%.
† Error at the 95% confidence limits = ± 8.4%.
‡ H^3/C^{14} of the urinary testosterone was 12.1.

and maximal H^3/C^{14} ratio in plasma T should be attained when Δ metabolism is largely complete and both isotopes are undergoing the same exponential decline. In Table I, it is apparent that by 21 minutes the final ratio had been reached. In general, the H^3/C^{14} ratios of successive derivatives agreed closely. The H^3/C^{14} ratio of urinary TG determined from the 48-hour collection was 11.5. This is significantly higher than the average final plasma ratio of 7.4, indicating that some H^3 had entered urinary TG without passing through the plasma T pool. From the excess of H^3 in the urinary TG it was calculated that about one-third of the urinary TG derived from Δ was formed via the Δ → TG pathway.

Experiment 2: M.L. and C.T. The contribution of the Δ → TG pathway to the TPR was studied by measuring the H^3/C^{14} ratios in plasma T, plasma TG, and urinary TG, as well as determining the TPR and the excretion of urinary TG. After the administration of 6×10^7 dpm of Δ-H^3 and 2×10^6 dpm of T-C^{14}, plasma was obtained as indicated in Table II, and urine was collected for the first 8 and subsequent 40 hours. Samples from each urinary collection were used to measure the total T excretion by reverse isotope dilution.

In Table II, the H^3/C^{14} ratios in plasma T and TG are presented for both C.T. and M.L. at two time intervals. In each TG fraction the H^3/C^{14} was four to seven times that of the corresponding T fraction, indicating that 60 to 87% of that TG derived from Δ must have been formed via the Δ → TG pathway. It is of little importance that

TABLE II

H^3/C^{14} ratios of plasma testosterone (T) and testosterone glucuronoside (TG) after administration of androstenedione-1,2-H^3 and testosterone-4-C^{14}

			Testosterone*: Androstenedione		Testosterone*: Androstenediol		Testosterone glucuronoside*: Androstenedione		Testosterone glucuronoside*: Androstenediol		
Subject, age, sex	Interval after injection	Plasma volume	Total plasma H^3/C^{14}	Ratio H^3/C^{14}	Total plasma H^3/C^{14}	Ratio H^3/C^{14}	Total plasma H^3/C^{14}	Ratio H^3/C^{14}	Total plasma H^3/C^{14}	Ratio H^3/C^{14}	TG not derived from T
	minutes	*ml*	*dpm*		*dpm*		*dpm*		*dpm*		%
C.T., 36, M	20–30	170	2,090 / 1,650	1.3	940 / 788	1.2	224 / 33.8	6.6†	146 / 20.9	7.0†	83
	30–40	186	1,350 / 968	1.4	483 / 340	1.4	224 / 43.7	5.6†	164 / 27.6	6.0†	60
M.L., 23, F	20–30	186	3,420 / 3,050	1.1	1,670 / 1,670	1.0	953 / 122.5	7.8	730 / 94.5	7.8	87
	30–40	176	1,530 / 1,020	1.5	705 / 440	1.6	176 / 19.4	9.1‡	126 / 13.0	9.7†	84

* Counted as the derivatives, androstenedione and androstenediol.
† Error of the 95% confidence limits between 5 and 10%.
‡ Error at the 95% confidence limits = 12.6%.

TABLE III

Analysis of radioactivity and testosterone recovered in urine after administration of androstenedione-1,2-H^3 and testosterone-4-C^{14}

Subject	Interval	Testosterone glucuronoside excretion*	Excretion in testosterone*		H^3/C^{14}	TPR†	Conversion of androstenedione to TG	Excretion of T as TG
			H^3	C^{14}				
	hours	*μg/2 days*	*dpm*	*dpm*		*mg/day*	%	%
C.T.	0–8	13.4	206,000	22,000	9.4			
	8–48	59.3	13,900	1,130	12.8‡			
Totals		72.7	219,900	23,130	9.5	3.2	.37	1.16
M.L.	0–8	4.3	126,000	13,200	9.5			
	8–48	13.0	18,300	410	39			
Totals		17.3	144,300	13,610	10.5	1.3	.24	.68

* As determined by reverse isotope dilution.
† TPR = testosterone production rate.
‡ Error of 6% at the 95% confidence limits.

the final H^3/C^{14} ratio may not have been attained, since in every interval the excess of H^3 in plasma TG was apparent. From the reverse isotope dilution studies we obtained the total excretion of TG and of H^3 and C^{14} as TG (Table III). The TPR and the percentage conversion of Δ and T to TG were then determined. In the first 8 hours, 94 and 87% of the radioactivity in T were excreted. The urinary H^3/C^{14} ratios at this time were 9.4 and 9.5. In M.L., this agreed with the final plasma ratio of 9.4, whereas in C.T. the plasma TG ratio was only 7.8. The TPR's of 3.2 and 1.3 mg per day were within the expected ranges. The total conversion of Δ to TG was 0.37% and 0.24% in C.T. and M.L., respectively, with corresponding T to TG conversions of 1.16% and 0.68%.

The elevated H^3/C^{14} ratios in the urinary TG excreted between 8 and 48 hours after isotope administration indicate that some of the Δ metabolized to T was slowly released from some intracellular pool. This component constituted only a small fraction of the total radioactivity excreted.

Experiment 3: J.P. In this study after the administration of 4×10^7 dpm of Δ-H^3 and 10^6 dpm of T-C^{14}, successive plasma H^3/C^{14} ratios were compared with the ratio in urine after prior extraction of urinary free T with ether. The maximal plasma T H^3/C^{14} ratio was reached more slowly in this subject (Table IV). Additional derivatives of plasma T could not be made because of low counting rates. Nevertheless the ratios as T and Δ agreed well. The ratio in urinary TG was the same as that of the last plasma sample, indicating that the Δ → TG pathway did not contribute significantly to urinary TG. The TPR of 0.68 mg per day was compatible with the diagnosis of hypopituitarism.

TABLE IV

H^3/C^{14} ratios in plasma testosterone and urinary testosterone glucuronoside after the administration of androstenedione-1-2-H^3 (Δ) and testosterone-4-C^{14} (T)

Material	Interval after injection	Derivative	
		T H^3/C^{14}	Δ H^3/C^{14}
Plasma	10–18.5 min	0.90	0.83
	20–29 min	2.2*	1.6
	33–41 min	7.8	6.3†
Urine	0–48 hrs	6.7	6.9

* Error at the 95% confidence limits ± 7.3%.
† Error at the 95% confidence limits ± 5.8%.

Discussion

The production of T from Δ in peripheral tissues has been inferred by Vande Wiele, MacDonald, Gurpide, and Lieberman (8) in their studies based on the specific activities of urinary steroid metabolites and has been directly demonstrated by Mahesh, Greenblatt, Aydar, and Roy (2) and by Baulieu and Robel (3). In all four of our studies, H^3 was found in plasma T, confirming the peripheral production of a hormone from an inactive precursor.

The excess of H^3 in the plasma TG in both

subjects indicated that most of the TG synthesized from Δ had been derived via the Δ → TG pathway. This demonstrates that some T formed peripherally is conjugated immediately and thus never enters the plasma as T. Therefore, urinary TG is not a unique metabolite of plasma T.

Lindner (9) has found that T may be synthesized from Δ by incubation of Δ with human whole blood. If this occurred to any significant extent in our studies, then the effect would be to increase the H^3/C^{14} ratio of plasma T and therefore decrease the relative proportion of the Δ → TG pathway. Since plasma TG had a much higher H^3/C^{14} ratio than plasma T, it is unlikely that much Δ-H^3 was directly converted to T in the plasma during the course of the study.

To illustrate the disproportionate influence of the Δ → TG pathway on the estimate of T production by the urinary isotope dilution method, we have assumed Δ production rates of 3,000 μg per day for M.L. and 5,000 μg per day for C.T. and calculated T secretion and production rates (Figures 2 and 3). In the case of M.L., since 0.24% of administered Δ was excreted as TG, 7.2 μg per day of her urinary TG would have been derived from Δ and 1.45 derived from T secretion. From the plasma T H^3/C^{14} of 1.6 and urinary ratio of 10.5, we calculated that the Δ → TG pathway was responsible for 85% of the Δ excreted as TG or 6.13 μg. The remaining 1.07 μg, derived from Δ, passed through the plasma T pool. Since 0.68% of T-C^{14} was excreted as urinary TG (Table III), the T secretion would be 214 μg (1.45/0.0068) and the T production from Δ 158 μg (1.07/0.0068). The estimated total production of T of 214 + 158 = 372 μg per day was much lower than the estimate of 1.3 mg per day obtained by the dilution of T-C^{14} in urinary TG.

The data in Figure 3 were derived similarly except that an Δ production rate of 5 mg per day was assumed. With these data, the production of plasma T was 1.77 mg per day, whereas the TPR measured by urinary dilution was 3.2 mg per day.

In both cases, the H^3/C^{14} ratio of plasma TG was approaching that of urinary TG. Therefore the free plasma T ratio is not far from its maximal value. Since the percentage of TG not derived from T was calculated by the formula,

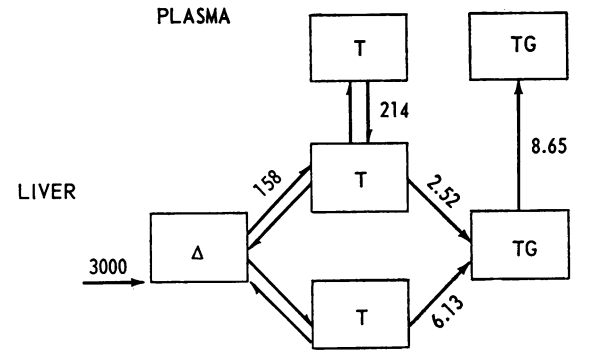

FIG. 2. M.L. TESTOSTERONE SECRETION AND PRODUCTION RATES ASSUMING A PRODUCTION OF ANDROSTENEDIONE OF 3,000 μG PER DAY. All rates are in micrograms per day.

$(R_{TG} - R_T \times 100)/R_{TG}$, where $R = H^3/C^{14}$, it is clear that this value is rather insensitive to small changes in R_T when R_{TG} is much higher than R_T.

Several assumptions have been made in the construction and interpretation of the models in Figures 2 and 3. These assumptions are: *1*) The Δ production rate values of 3 and 5 mg per day are reasonable. Vande Wiele and his associates (8) estimated Δ production rates in several subjects and arrived at higher values than those we used. Any increase in the estimated Δ production rate would tend to increase the contribution of the Δ → TG pathway to urinary TG and thus make the production of plasma T even smaller. However, these calculations are very sensitive to small changes in Δ production rate; e.g., an increase of estimated Δ production of M.L. from 3 to 3.5 mg per day would entirely eliminate T secretion.

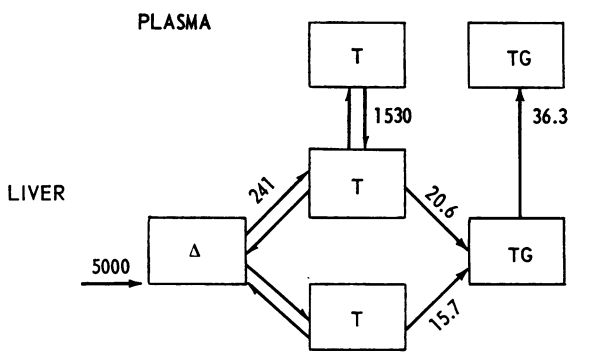

FIG. 3. C.T. TESTOSTERONE SECRETION AND PRODUCTION RATES ASSUMING A PRODUCTION OF ANDROSTENEDIONE OF 5,000 μG PER DAY. All rates are in micrograms per day.

2) The disposal of exogenous Δ-H^3 is the same as that of endogenous Δ. Although this assumption would not have been questioned in the past, it is no longer certain that a labeled steroid mixes completely with the steroid being produced in the liver. Since most of Δ is probably derived from D, quite possibly a portion of this Δ, synthesized in the liver, may undergo further metabolism before entering the plasma. In this case, only a fraction of Δ production would be available for conversion to T, a situation exactly analogous to that which we have described for T. This would have the effect of reducing the percentage of conversion of Δ to T to less than that found by administration of exogenous Δ-H^3, thereby decreasing the significance of the Δ → TG pathway.

3) It has been assumed that TG is an end product and is not further metabolized. The fate of steroid glucuronosides has been rigorously examined only for androsterone glucuronoside (10), which did not undergo further metabolism.

4) The site of conversion of Δ-H^3 to T and TG has been assumed to be peripheral, probably in the liver and not in the adrenals or gonads. In an extensive literature dealing with the administration of labeled steroid precursors, no evidence has been presented that a measurable amount of hormone containing the label is synthesized by the glands. The results obtained here make it clear that wherever the site of conversion of Δ to T, conjugation of some of this T occurs at the same site.

It is apparent from the uncertainties connected with these assumptions and the variability of the Δ → TG pathway among subjects that measuring the production rate of plasma T is extremely difficult. Even if similar detailed analyses were performed in each subject, there may be no way of determining the production rate of Δ available for synthesis of T.

To what extent do these considerations decrease the usefulness of a TPR based on the specific activity of urinary TG? Since urinary TG can never be assumed to be a unique metabolite of plasma T, the TPR cannot be accurate. However, when the secretion rate of T is high, the relative contribution of Δ → TG pathway to urinary TG becomes smaller, and the TPR approaches the plasma T production. Thus, in young men the TPR is probably not greatly in error and may be useful in physiological studies. We have, for instance, shown that the TPR increases when human chorionic gonadotropin is given (1) and decreases under the influence of androgen (11).

In women, however, T secretion may be very low so that the TPR substantially overestimates the production of T as in Figure 2. Significant increases in T secretion or production could be easily masked by a smaller contribution of the Δ → TG pathway to urinary TG.

Our demonstration of the Δ → TG pathway has a parallel in studies of the production rate of progesterone described by Tait (12). The secretion rate of progesterone was determined both by the metabolic clearance method and from the specific activity of urinary pregnanediol. It was postulated that intrahepatic conversion of steroid precursors to progesterone occurred and that some of this progesterone was metabolized to pregnanediol before entry into the plasma. Thus, in this case pregnanediol was not a unique metabolite of plasma progesterone. A similar situation could exist in the measurement of estrogen production rates in men and ovariectomized women, since most of the estrogen may be synthesized peripherally from T or Δ.

This peripheral synthesis and immediate conjugation of a steroid is of physiologic significance in those instances when the steroid is a hormone. In such cases, the determination of the plasma levels of the hormone will provide a better measure of the hormonal state than the measurement of the production rate. Since a glucuronoside of a steroid hormone can no longer be assumed a unique metabolite of the plasma pool of that hormone, the use of such metabolites for production rate measurements must be experimentally validated. Under conditions in which peripheral interconvertibility of steroids may be demonstrated, evaluation of side pathways may profitably be undertaken with the experimental design herein described. Furthermore, our demonstration of at least two T compartments emphasizes the need for caution in the acceptance of new metabolic pathways that may be demonstrated by isotope dilution when a single compartment is assumed.

Summary

Since androstenedione is converted to testosterone peripherally, studies were performed to test the assumption that urinary testosterone glucuronoside is a unique metabolite of plasma testosterone. It was shown that some testosterone derived from androstenedione peripherally is conjugated to the glucuronoside without ever entering the plasma as testosterone. Thus, urinary testosterone glucuronoside is not a unique metabolite of plasma testosterone.

A consequence of this is that testosterone production rates measured by dilution of labeled testosterone in urinary testosterone glucuronoside overestimate the testosterone production to a variable extent. The demonstration of multiple metabolic steps before mixing in the plasma pool is of general importance in the evaluation of production rates and in the consideration of new metabolic pathways.

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