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THE *IN VIVO* GLUCURONIDE CONJUGATION OF RADIOACTIVE ETIOCHOLANOLONE AND ANDROSTERONE BY THE DOG KIDNEY *

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It is recognized that the liver readily conjugates reduced steroids (1) so rapidly that, under normal conditions, reduced steroids are not recovered in an unconjugated form in plasma but rather occur as sulfate (2–4), glucuronide (5), and probably phosphate (6) conjugates. Although Weichselbaum and Margraf reported the presence of small quantities of unconjugated tetrahydrocortisone [1] in 10 L of normal pooled plasma (7), confirming a previous observation in plasma from patients with advanced cirrhosis (8), this has not been the experience of others analyzing 50 to 100 ml of normal plasma. Moreover, most investigators have been unable to isolate significant amounts of unconjugated reduced androgen metabolites, viz., androsterone and etiocholanolone, in normal plasma [2] (*2, 3, 9*).

Recently, however, Bondy, Cohn, Herrmann and Crispell (10) demonstrated unconjugated etiocholanolone in the plasma of two patients with symptomatic etiocholanolone fever.[3] Conjugated etiocholanolone (12 to 15 mg per day) was present in the urine during the same symptomatic episode. When the patients were afebrile and without symptoms, the 24-hour urinary 17-ketosteroid patterns were normal, and unconjugated etiocholanolone was not detected in the plasma. It was postulated that an attack of this type of fever might be associated with *1*) transient overproduction of etiocholanolone and/or *2*) an intermittent inability to conjugate. The presence of conjugated etiocholanolone in urine and its absence in plasma during an attack of periodic fever is difficult to reconcile unless another organ in addition to the liver is involved in conjugation.

The rapid clearance of etiocholanolone and androsterone, after intravenous injection (12) and oral and intravenous administration of testosterone (13–15), suggests the possibility that these compounds may be conjugated by another organ besides the liver. The reports of Dutton (16), and Lipschitz and Bueding (17), that kidney slices synthesize glucuronide conjugates *in vitro* stimulated an investigation to determine whether dog kidney could conjugate radioactive etiocholanolone and androsterone *in vivo*.

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[1] Compounds referred to are etiocholanolone (3α-hydroxy-etiocholane-17-one); androsterone (3α-hydroxy-androstane-17-one); tetrahydrocortisol (3α, 11β, 17α, 21-tetrahydroxy-pregnane-20-one); tetrahydrocortisone (3α, 17α, 21-trihydroxy-pregnane-11, 20-dione); allo-tetrahydrocortisol (3α, 11β, 17α, 21-tetrahydroxy-allopregnane-20-one); allo-tetrahydrocortisone (3α, 17α, 21-trihydroxy-allopregnane-11, 20-dione).

[2] Dubrulle and Claeys (9) measured unconjugated plasma 17-ketosteroids in 16 normal adolescents and found 7 to 28 μg per 100 ml.

[3] Recently, this clinical entity was named etiocholanolone fever in order to differentiate it from the familial Mediterranean fever on one hand, and the many periodic fevers on the other (11).

MATERIALS AND METHODS

Six apparently healthy mongrel dogs (4 males and 2 females), weighing 8 to 17 kg, were used in these experiments. The dogs were anesthetized with Nembutal, 30 mg per kg. In order to insure an adequate diuresis, 5 per cent mannitol in hypertonic saline (166 mEq per L) was infused at a rate of 1.5 ml per minute through a cannula placed into the superior vena cava via the internal jugular vein. Through a mid-line incision the left kidney pedicle was exposed, the left ureter cannulated with polyethylene tubing (PE 205, Clay-Adams, New York, N. Y.) and the urine collected. After the renal vein was dissected free from the renal pedicle to the inferior vena cava, a polyethylene tubing (PE 160) with side holes cut near the tip was inserted into the renal vein at its junction with the spermatic (or ovarian) vein. At intervals this cannula was flushed with 10 ml

volumes of heparin-isotonic saline solution (0.2 mg per ml). The renal artery was dissected free and a ligature passed beneath it. At zero time the renal vein was cross-clamped at its junction with the inferior vena cava with a Potts bulldog (serrefine) clamp. Renal vein blood collection was begun and was manually controlled to prevent collapse or over-distention of the renal vein. Heparin-rinsed syringes were used for the blood collection. Systemic venous blood was collected via the jugular catheter during the same period and urine flow was also collected as a separate fraction. With the renal artery delivered up into the wound by traction on the ligature passed around it, thus serving to partially occlude the artery during the injection period, isotopically labeled steroid(s) (Table I), dissolved in 0.2 ml absolute ethanol and diluted to 2.0 ml with heparin-isotonic saline, were injected by direct needle puncture of the renal artery. The injection time varied from 15 to 30 seconds. It should be pointed out that the tip of the needle was positioned as closely as possible to the entrance of the renal artery into the kidney (Figure 1). In one experiment, a serrefine clamp instead of the sling ligature was used to constrict the renal artery at its origin. In order to eliminate any return of injected isotope by venous radicles in the renal capsule or by the lymphatics, the entire kidney as well as its pedicle was dissected from its bed and surrounded by a plastic envelope.

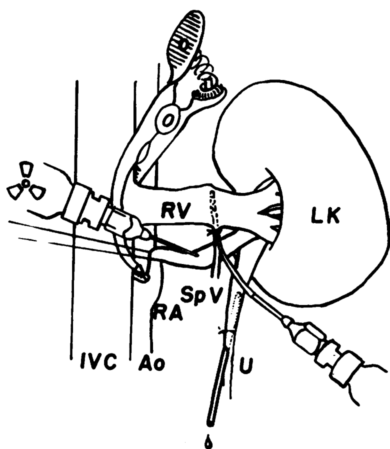

FIG. 1. A DIAGRAMMATIC REPRESENTATION OF THE DOG KIDNEY PREPARATION. The left ureter (U) is shown with the catheter in place. The spermatic vein (Sp V) is cannulated and a polyethylene catheter, with a side hole at its tip, is passed into the renal vein (RV). The renal vein is occluded with a serrefine bulldog clamp at its junction with the inferior vena cava (IVC) just as blood is withdrawn from the renal vein catheter. The rate of withdrawal is adjusted to prevent collapse or overdistention of the vein. As soon as the renal vein is occluded, isotopically labeled steroid(s) is injected rapidly by direct puncture of the renal artery (RA). A single ligature around the renal artery near its origin from the aorta (Ao) delivers the vessel into the wound and partially occludes it.

After injection of the isotope(s), full perfusion of the kidney was re-established by removal of the sling or the clamp. Up to 225 to 260 ml of renal vein blood was collected in 6 to 8 minutes. Hypotension occurred in only one dog. At the end of this collection time the pedicle was clamped and the renal pelvis washed three times with isotonic saline. These washings were added to the 4 to 6 ml of urine formed during the experimental period. Nephrectomy was performed at the conclusion of the experiment.

The plasma was separated from the cells by centrifugation within 15 minutes of collection (18) and frozen until analyzed. The urine sample and kidney were processed on the day of the experiment. The plastic bag and lymph contained in it, as well as the catheters, were analyzed for radioactivity.

Separation of plasma steroids

Free steroids. The plasma was thawed at room temperature. Two ml of 1 N NaOH per 40 ml of plasma was added, mixed, and the plasma extracted three times with 2 vol of freshly distilled chloroform. Emulsions were broken by centrifugation. The chloroform extract was dried in a rotating vacuum evaporator at 37° C and the residue dissolved in 10 to 15 ml of chloroform. Radioactivity (cpm) was determined with corrections for quenching. This extract (i.e., that portion of steroids extractable with chloroform without hydrolytic procedures) has been designated as the "free" (unconjugated) fraction. The redissolved residue was concentrated to dryness *in vacuo,* carefully dissolved in 1.0 ml of chloroform and applied as a 2.0 cm streak, along with appropriate reference standards, to washed (19) Whatman no. 1 filter paper in the heptane-propylene glycol system [4] (20). The chromatogram was scanned for radioactivity and the data from a count rate meter traced on an Esterline-Angus recorder. The pilot strip containing the reference standards and a 0.25 cm portion from the center of the "extract strip" were cut out and stained with Zimmermann reagent. There was no Zimmermann positive material detected on the "extract strip" except in the carbon^{14} experiment. However, there was very close agreement of R_f between the Zimmermann positive reference compounds and the location of the radioactive compound(s) by isotopic scanning. The appropriate area was cut out and eluted with 4.0 ml of absolute ethanol. An aliquot of this eluate was counted for radioactivity. The remainder was dried *in vacuo,* dissolved in chloroform and chromatographed in the 96 per cent methanol: heptane (1:1) system of Bush and Willoughby (21). The "free" compound(s) was located and counted as described.

Radioactivity was counted to a standard error of ± 2 per cent in a liquid-phosphor scintillation counter (Technical Measurements Co., model LP-2, New Haven, Conn.) which had a counting efficiency of 15 per cent for tritium and 65 per cent for carbon^{14}. When the

[4] All chromatograms were run in a room with constant temperature and humidity.

carbon scintillation counting eluates were low, they were quantitatively transferred to 2.0 sq cm areas on aluminum planchets at infinite thinness and counted in a specially constructed windowless gas-flow counter [5] which had a background of 1.5 to 2.0 cpm.

Conjugated steroids. After the chloroform extraction 2 volumes of absolute ethanol was added to the plasma. The precipitated protein was removed by centrifugation and further extracted three times with 1 volume of redistilled *n*-butanol saturated with water. The precipitate was discarded and the ethanol and butanol extracts were pooled and dried *in vacuo.* The residue was dissolved in 25 ml of 70 per cent methanol and washed three times with equal volumes of redistilled hexane. The hexane layers, which contained no significant radioactivity, were discarded and the washed methanol layer was taken to dryness *in vacuo.* The residue was redissolved in 1.0 ml of 70 per cent methanol. An aliquot was counted and the remainder streaked on Whatman no. 3 MM filter paper for electrophoresis. Reference standards of androsterone and etiocholanolone glucuronides and sulfates were applied adjacent to the streak. Paper electrophoresis was carried out as previously described (5). The conjugated compounds were located on the electropherogram by radioactive scanning and staining of the reference compound with Zimmermann reagent. The mobility of the Zimmermann positive standard coincided with the location of the radioactive peak.[6] No "free" androsterone or etiocholanolone was detected at the origin of the electropherogram by these techniques or after elution and counting (as described for tritium or carbon[14]). The sulfate area and remainder of the paper contained no measurable radioactivity after elution with 70 per cent methanol.

The radioactive conjugate area was cut out and eluted with 70 per cent methanol. An aliquot was counted and the remainder, along with adjacent reference standards, was chromatographed in the ethyl acetate : hexane : glacial acetic acid : water (120 : 80 : 60 : 140) system of Schneider and Lewbart (22). The steroid conjugate was located by radioactive scanning and staining with Zimmermann reagent, eluted, counted, and then rechromatographed in the *n*-butanol : butyl acetate : 10 per cent acetic acid (20 : 80 : 100) system (23). After radioactivity was measured, the eluate from the second chromatogram was hydrolyzed with β-glucuronidase [7] as previously described (5). The released "free" radioactive steroid was extracted with chloroform, counted, and chromatographed in the 96 per cent methanol : heptane system. The "free" compound was located, eluted, and radioactivity was determined as described above.

Separation of urinary steroids and steroids in lymph and plastic bag

The urine, lymph and plastic bag were each extracted twice with 100 ml of chloroform for "free" steroids. This was followed by extraction twice with 100 ml of *n*-butanol for conjugated steroids. The extracts were counted with allowances for quenching but were not processed further.

Separation of steroids from the kidney

The kidney was cut into small pieces, homogenized with 2 vol of 0.25 M sucrose and extracted three times with 100 ml chloroform washes. The extract was filtered through washed glass wool, concentrated to about 2.0 ml *in vacuo* and radioactivity measured.

The homogenate was extracted twice with 200 ml of *n*-butanol for conjugated steroids. This extract was filtered, concentrated and counted as described. The "free" and conjugated fractions were not characterized further.

RESULTS

Renal vein plasma. The total radioactivities of the "free" and conjugated fractions are listed in Table I. Percentages are corrected to 100 ml of plasma. These values are lower than were the actual radioactive recoveries (except experiment VI) since more than 100 ml of plasma was obtained. The uncorrected total "free" and conjugated radioactive steroid recoveries in the renal vein plasma represent 30 to 56 per cent of the original amount of radioactivty (cpm) injected. When tracer quantities of etiocholanolone-H^3 were injected, almost twice as much conjugated tritium-labeled etiocholanolone as "free" was found. There was twice as much "free" steroid as the conjugated form when tracer amounts of androsterone-H^3 were injected. After tracer amounts of androsterone-H^3 and etiocholanolone-H^3 were injected simultaneously, a greater amount of etiocholanolone glucuronide (16 per cent) than androsterone glucuronide was found (12 per cent of injected dose per 100 ml).

Jugular vein plasma, urine, lymph, plastic bag and kidney. No significant radioactivity was found in either the "free" or conjugated urinary fractions, tubings, lymph or plastic bag. There was 0.08 to 0.4 per cent of the dose "free," and 0 to 0.1 per cent of the dose radioactive conjugated fractions per 100 ml in the internal jugular vein plasma. After homogenization and extraction of

[5] Dr. Melvin V. Simpson of the Department of Biochemistry, Yale University, generously allowed us to use this specially constructed counter.

[6] In experiment VI (Table I) the androsterone glucuronide and sulfate areas were also located directly on 0.25 cm of the extract strips.

[7] Beta-glucuronidase was obtained from Warner-Chilcott Laboratories, Warner-Lambert Pharmaceutical Co., New York, N. Y.

TABLE I

"Free" and conjugated steroids in renal vein plasma

Experiment	Steroid*	Specific activity	Amount administered†	Dose recovered "Free" steroid	Dose recovered Conj. steroid	Volume renal vein plasma
		μc/μg	*cpm*	*%/100 ml renal vein plasma*		*ml*
I	Androsterone-4-C^{14}	0.0035	2.22×10^4	15.5	10.5	142
II	Etiocholanolone-H^3	5.2	4×10^5	8	16	125
III	Androsterone-H^3	2.6	5.7×10^5	28	14	115
IV	Etiocholanolone-H^3	5.2	6.2×10^5	15	3‡	120
	Androsterone-H^3	2.6	6.1×10^5	20		
V	Etiocholanolone-H^3	5.2	6.4×10^5	8	16.5	122
	Androsterone-H^3	2.6	6.15×10^5	34	12	
VI§	Androsterone-H^3	2.6	2.75×10^5	18.2	13.3	90

* Androsterone-4-C^{14} was prepared biosynthetically from testosterone-4-C^{14} (New England Nuclear Corp., Boston, Mass.). The compound was isolated by gradient elution chromatography (24) after enzymatic hydrolysis and extraction of urine. Its identity was verified by infrared analysis. Androsterone (mp 181 to 183° C) and etiocholanolone (mp 149 to 151° C; donated by Dr. Seymour Lieberman, Columbia University, New York, N. Y.), were exposed to tritium gas (Wilzbach procedure) by the New England Nuclear Corp. The tritium-labeled steroids were thoroughly washed, purified, identified and characterized to constant specific activity by paper and column chromatography. The details of the method will be reported in a separate publication.

† The dosage of steroid represents the difference between the amount administered and the quantity extractable from the syringe and needle after injection.

‡ This animal was profoundly hypotensive 4 minutes after the injection of steroid. Because of the low percentage, conjugated steroids were not further separated.

§ Carrier androsterone glucuronide and sulfate (500 μg of each) were added to the renal vein blood at the conclusion of this experiment.

the renal tissue, 36 per cent "free" and 0 to 1 per cent conjugated, of the injected isotopic dose, were detected. A typical isotope recovery analysis is summarized in Table II.

Identification of androsterone and etiocholanolone glucuronides. The isolated radioactive steroid conjugates were identified as glucuronides of androsterone and etiocholanolone by virtue of their mobilities on paper by electrophoresis followed by chromatography in two systems. After separation of the compounds by these techniques, β-glucuronidase hydrolysis resulted in the release of the "free" steroid which was identified by paper chromatography.[8] Significant radioactivity was detected throughout the isolation and identification procedures.

DISCUSSION

These studies demonstrate that the dog kidney formed carbon[14] and tritium-labeled androsterone and etiocholanolone glucuronide *in vivo*. Although the glucuronide portion of the steroid conjugate was not identified, it is unlikely that any other conjugate would behave in the manner described. Because of their negative charge, glucuronides of either the C_{21} or C_{19} steroids migrate as a class and at a slower rate than do the sulfates toward the anode on paper electrophoresis (5). Thus it is impossible to separate the C_{21} and C_{19} glucuronides by this technique. However, using Schneider and Lewbart paper chromatographic systems (22, 23), labeled etiocholanolone and androsterone glucuronides were easily isolated.

An insignificant amount of radioactivity was found in the peripheral plasma, indicating that the *in vivo* technique excluded the liver (1, 25) and duodenum (26) from this conjugation. The effi-

[8] In experiment VI carrier androsterone glucuronide and sulfate (Table I) were added to the renal vein plasma. After processing and purification by the methods described, the androsterone glucuronide fraction maintained constant specific activity. There was no detectable radioactivity in the androsterone sulfate fraction.

TABLE II

Percent isotope recoveries after the administraion of androsterone-H^3 into the renal artery

	Per cent of total dose
Total renal vein plasma (per 100 ml)	31.5
Peripheral venous plasma (per 100 ml)	0.08
Kidney	37.0
Plastic bag and lymph	1.0
Polyethylene tubings	0
Urine	3.0
Total	72.58

ciency of the dog kidney glucuronide conjugation system was 3 to 16.5 per cent of the injected isotopic dose per 100 ml per 6 to 8 minutes (Table I).

Because there are conformational differences between androsterone and etiocholanolone at the third carbon atom, speculation arises concerning the relative ease of conjugation of these compounds. The α-hydroxyl group at the third carbon atom in the 5β compound, etiocholanolone, is *equatorial* to the A ring. On the other hand, the hydroxyl group on this carbon atom is *axial* to the A ring and more sterically hindered in the 5α compound, androsterone. Since it is known that an esterifying reaction proceeds more rapidly when the acceptor group is *equatorial* rather than *axial* (27, 28), it is reasonable to predict that under similar conditions more etiocholanolone than androsterone will be conjugated. Of course, this will be true only if the enzyme systems, cofactors, substrates and so forth are available to effect glucuronide synthesis.

The data in Table I favor this hypothesis since, under similar experimental conditions, a higher percentage of etiocholanolone glucuronide than of androsterone glucuronide was found. Slaunwhite and Sandberg (12) injected carbon^{14}-labeled androsterone and etiocholanolone into three women and found that the etiocholanolone glucuronides were cleared more rapidly than were the glucuronides of androsterone. Two of the three women had slightly higher levels of etiocholanolone glucuronides than of androsterone glucuronides, 15 minutes after injection. Thus, the more rapid conjugation of etiocholanolone as compared with androsterone may be explained by the conformational differences between the 3α-hydroxy groups of the two compounds. It follows, if this hypothesis is correct, that under similar conditions tetrahydrocortisol and tetrahydrocortisone may be conjugated more rapidly than are their allo derivatives, since the 3α-hydroxy group is *equatorial* to the A ring in the former, and *axial* in the latter compounds.

After conjugation, C_{19} glucuronides and sulfates are rapidly excreted by the kidneys (12–15). However, the actual physiological mechanism is not completely resolved. Bongiovanni and Eberlein demonstrated in a human volunteer that androsterone glucuronide was cleared from the kidney by tubular secretion (29). Kellie and Smith (30), on the other hand, reported clearance values for androsterone and etiocholanolone glucuronides comparable with those of inulin and suggested that these compounds may be excreted by glomerular filtration alone. In our experiments we were able to detect no more than 3 per cent of the injected radioactivity in the urine despite the large amount of free isotope "trapped" in the kidney. The significance of these findings is not understood and warrants further investigation.

Although it was demonstrated that the dog kidney synthesized steroid glucuronides *in vivo,* the site of renal conjugation and the eluicidation of this process in man remain to be determined.

SUMMARY

1. The conjugation of radioactive etiocholanolone and androsterone to their respective glucuronides was demonstrated (*in vivo*) in a dog kidney preparation. These glucuronides, isolated from the renal vein plasma, were characterized by their mobilities on paper electrophoresis, paper chromatography, hydrolysis with β-glucuronidase, and paper chromatography of the resultant "free" compound. Significant radioactivity persisted throughout the isolation and identification procedures. In one experiment androsterone glucuronide was characterized by purification to constant specific activity. No significant amounts of radioactive glucuronide conjugates were found in the urine or internal jugular vein plasma. Highly significant amounts of free isotope were "trapped" in the kidney parenchyma.

2. Three to 16.5 per cent of the injected dose of the labeled steroid per 100 ml of plasma was converted to the steroid glucuronide during the 6- to 8-minute perfusion of the kidney.

3. A hypothesis is presented which may explain the higher yields of etiocholanolone glucuronide than of androsterone glucuronide formed by this technique. The lesser steric hindrance of the *equatorial* α-hydroxyl group on the third carbon atom of etiocholanolone may facilitate rapid glucuronide conjugation. Although androsterone is conjugated readily, the rate of the reaction may be slightly less because of steric hindrance of the *axially* placed α-hydroxyl group on the third carbon atom.

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