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J Clin Invest. 1961;[40\(2\)](#):400-407. <https://doi.org/10.1172/JCI104267>.

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STUDIES ON PYROGENIC STEROIDS. I. SEPARATION, IDENTIFICATION, AND MEASUREMENT OF UNCONJUGATED DEHYDROEPIANDROSTERONE, ETIOCHOLANOLONE, AND ANDROSTERONE IN HUMAN PLASMA *

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(Submitted for publication June 13, 1960; accepted October 20, 1960)

Previous studies have shown that endotoxin (1-4), viruses (5-7), and pyrogenic substances from polymorphonuclear leukocytes (8-11) are capable of producing fever. A different group of pyrogenic substances was described by Kappas, Soybel, Fukushima and Gallagher (12), who reported that certain C₁₉ and C₂₁ steroids with a 3 α -hydroxy, 5 β configuration produce fever. The prototype compound is etiocholanolone¹ (13-16). Four to 8 hours after intramuscular or intravenous administration of this compound to volunteers, the temperature began to rise, reached a peak (103° to 105° F) at 12 hours, and generally subsided within 24 hours. Chills, anorexia, headache, malaise, myalgia, arthralgia, and leukocytosis accompanied the reaction.

Recently, Bondy, Cohn, Herrmann and Crispell (16) demonstrated unconjugated² etiocholanolone in the plasma of two patients during febrile attacks of etiocholanolone fever.³ When the patients were afebrile and asymptomatic, unconjugated plasma etiocholanolone was not detected.

* Supported by Grants 254 (C-7) and A-4222 from the National Institutes of Health, Bethesda, Md.

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¹ The following trivial names and abbreviations are used: etiocholanolone, ETIO (3 α -hydroxyetiocholane-17-one); androsterone, ANDRO (3 α -hydroxyandrostane-17-one); dehydroepiandrosterone, DHEA (3 β -hydroxy-5-androstene-17-one); etiocholanolone fever, EF; familial Mediterranean fever, FMF.

² The term *unconjugated* refers to those steroids extracted with an organic solvent (viz., chloroform) without previous hydrolytic procedures.

³ It was suggested that this type of fever be called etiocholanolone fever in order to distinguish this syndrome from familial Mediterranean fever on the one hand and the undifferentiated periodic fevers on the other (17).

The 24-hour urinary 17-ketosteroid patterns were normal when the patients were without symptoms. However, etiocholanolone was the only major 11-deoxy-17-ketosteroid isolated during the symptomatic interval.

Since the plasma clearance of 11-deoxy-17-ketosteroids, such as androsterone and etiocholanolone (18) is rapid (20 minute half-life), previous attempts to recover these steroids and dehydroepiandrosterone in an unconjugated form from plasma of normal subjects has been discouraging, since at most, only trace amounts were present (19, 20).

However, Savard (21) was able to isolate and identify (but not measure) unconjugated androsterone from 100 L of extracorporeal dialysis fluid obtained from a 22 year old patient with acute tubular necrosis and anuria. Moreover, Tamm, Beckmann and Voigt (22) analyzed 200 to 300 ml of normal human sera and found 17 to 61 μ g per 100 ml of total unconjugated 17-ketosteroids, measured by micro-Zimmermann reaction, after chloroform extraction, NaOH wash, and Girard separation. However, no separation of 11-oxy- and 11-deoxy-17-ketosteroid was attempted. On the other hand, Migeon (23) extracted 500 ml of normal human plasma and reported that "there was probably not more than 1 μ g per 100 ml each of unconjugated androsterone and dehydroepiandrosterone."

In the present study unconjugated plasma 17-ketosteroids were measured in normal subjects and in patients with fever of known origin, familial Mediterranean fever and etiocholanolone fever. Unconjugated plasma androsterone, dehydroepiandrosterone, and etiocholanolone were separated, measured and identified as 2,4-dinitrophenylhydrazones derivatives. An isotope dilution method

with tritium-labeled etiocholanolone, androsterone, and dehydroepiandrosterone was used to correct for losses during the procedures. The method is sufficiently sensitive to be useful in measuring unconjugated neutral 17-ketosteroids in plasma.

MATERIALS AND METHODS

Subjects

1. *Afebrile subjects.* Five hundred ml of heparinized blood was collected between 8 and 10 a.m. from 4 healthy men and 2 healthy women, ranging in age from 23 to 45 years. The plasma was separated by centrifugation within 15 minutes of collection and frozen until analyzed. Ten determinations were performed on 110- to 120-ml portions of this plasma. In addition, 3,000 ml of fresh plasma, obtained from untreated patients with polycythemia vera, was used for isotopic and chemical recovery experiments.

2. *Febrile patients.* Unconjugated neutral 17-ketosteroids were measured on 12 hospital inpatients with fever (101° F or higher) of known origin. The clinical data are listed in Table I. Heparinized blood was obtained at the time of the indicated temperature elevation. The plasma was frozen until processed. Liver and kidney function tests and an endocrinological evaluation revealed no abnormalities. M.J. (ulcerative colitis, Table I) had received no steroid therapy for at least 3 months prior to entry to the hospital. None of the other patients had ever received steroids.

3. *Patients with etiocholanolone fever and familial Mediterranean fever.* The clinical data are listed in Table II. The patients with familial Mediterranean fever presented the classical family history, signs and symptoms described by Heller, Sohar and Sherf (24) and Lawrence and Mellinkoff (25). The patients with etiocholanolone fever were not of Mediterranean or Middle Eastern origin. Repeated cultures, skin tests, and pre-

cipitin and agglutination tests for a wide variety of infectious agents were unrevealing. Liver function tests, lymph node and liver biopsies were within normal limits. Total and differential white blood counts were normal except during acute febrile attacks, when a polymorphonuclear leukocytosis with a shift to the left (without eosinophilia) usually occurred.

Plasma samples were collected during the prodrome of an attack, while the temperature was rising (100° to 102° F). Plasma was also collected during an afebrile and asymptomatic interval when the patients were not receiving therapy. These plasma samples were also frozen until analyzed.

Preparation of radioactive 17-ketosteroids for isotope dilution

Two mg of etiocholanolone, mp 149° to 151° C, and 2.5 mg of androsterone,⁴ mp 181° to 183° C, were each exposed to 3 curies of tritium at 27° C and 0.39 atm for 14 days.⁵

The readily labile tritium was removed by a modification of a method described by Peterson (27) using 250 to 500 ml of methanol. After the methanol was removed by vacuum distillation, the dry impure steroid was dissolved in 20 ml of freshly distilled chloroform. One ml of 0.05 N NaOH was added, mixed, and the alkaline chloroform extract washed three times with 1 to 2 ml of distilled water. The aqueous phase was carefully removed by aspiration and the chloroform extract evaporated to dryness under nitrogen. The residue was dissolved in 1 ml of ethyl acetate:methanol (2:1) and applied as a streak adjacent to reference compounds of androsterone and etiocholanolone on washed (28) Whatman no. 1 chromatographic papers in the heptane:propy-

⁴ Etiocholanolone and androsterone were generously donated by Dr. Seymour Lieberman, Dept. of Biochemistry, Columbia University, New York, N. Y.

⁵ The Wilzbach procedure (26) was performed by the New England Nuclear Corp., Boston, Mass.

TABLE I
Clinical data in patients with fever of known origin

Patient	Sex	Age	Diagnosis	Rectal temp.	Vol. of plasma
		yr		° F*	ml
M.S.	F	22	Viral pneumonia	105	28
B.B.	F	28	Acute follicular tonsillitis	106	63
L.C.	M	29	Acute cholecystitis	103	41
H.J.	F	33	Viral pneumonia	102.5	40
M.R.	M	36	Acute gastroenteritis	104	55
E.N.	F	44	Malignant melanoma	101.2	45
M.J.	F	45	Ulcerative colitis	101	65
E.W.	M	51	Bronchopneumonia	103	45
S.G.	F	61	Lung abscess	102	50
A.C.	M	64	Acute appendicitis	103	55
M.I.	M	68	Chronic bronchitis; emphysema	101	47
M.Y.	F	73	Bronchopneumonia	103	33

* Plasma samples were obtained at the time of the indicated temperature. No sample contained as much as 0.5 µg etiocholanolone per 100 ml of plasma.

TABLE II
Clinical data and unconjugated plasma 17-ketosteroid levels in patients with etiocholanolone fever and familial Mediterranean fever *

Patient	Sex	Age yrs	Diag.	Age at onset yrs	Freq. of attacks wks	Duration, attacks days	Temp.† ° F	Vol. of plasma ml	Radioactive recovery				Corrected plasma values			
									DHEA	ETIO	ANDRO	DHEA	ETIO	ANDRO	DHEA	ETIO
T.N.	M	10	EF	8	4-5	3-5	98.7 102.0	52	57.8	67.2	80.0	<0.5	1.0	2.0	<0.5	1.0
W.H.	M	28	EF	13	3-4	3-4	98.6 103.0	53	56.8	52.6	73.3	7.3	6.5	0.8	7.3	6.5
H.P.	M	30	EF	10	4-6	3-4	98.6 101.8	62	65.0	60.2	54.1	4.1	2.0	5.6	4.1	2.0
M.K.†	M	44	EF	18	3-4	3-5	98.5 102.0	30	26.1	87.6	61.0	28.3	6.7	1.6	28.3	6.7
V.E.	M	48	EF	34	4	3-4	98.8 102.5	23	59.8	70.6	83.0	7.2	7.3	1.1	7.2	7.3
W.S.	M	28	FMF	23	irreg.	2	98.6 103.0	50	61.4	77.9	62.2	10.4	4.1	8.7	10.4	4.1
A.N.	M	32	FMF	17	4-6	4-5	103.0	50	54.7	61.5	61.8	1.5	<0.5	2.3	1.5	<0.5
R.Q.	F	42	FMF	27	irreg.	2-3	102.0	100	51.2	48.2	50.9	0.8	0.8	1.5	0.8	0.8
									76.1	70.7	80.0	0.7	0.7	8.7	0.7	0.7

* For abbreviations in this and following tables, see footnote 1 to text.

† Plasma samples were collected during attacks at the time of the italicized temperature measurements.

‡ This patient was reported (16) and subsequently reinvestigated.

§ These samples were lost.

lene glycol system (29).⁶ The mobility of the radioactive peak, located by isotopic scanning, coincided with the mobility of the Zimmermann positive staining reference standards. Most of the radioactive impurities were more polar and easily separated from the tritium-labeled androsterone and etiocholanolone. The radioactive steroid area corresponding to the reference standard was eluted four times with 10-ml washes of the ethyl acetate: methanol (2:1) mixture, dried under nitrogen, and rechromatographed along with appropriate standards in the 1:1 heptane:96 per cent methanol system (30). The radioactive steroids were located and eluted as described. The isolated tritium-labeled steroids were purified by two additional paper chromatographic procedures in the same systems.

Dehydroepiandrosterone-7 α -H³-acetate⁷ was converted to the free steroid by hydrolysis with acetyl cholinesterase (Nutritional Biochemicals Corp.) by the method of Peterson and colleagues (31). The free steroid was washed, dried, extracted, and purified by paper chromatography as described.

Constant specific activity was observed following reverse isotope dilution of the free tritium-labeled steroids using paper chromatography, and column chromatography of the 2,4-dinitrophenylhydrazone derivatives (32) on 3.9 and 5.0 per cent alumina micro-columns as described below (Table III). The constant specific activities were interpreted as evidence for the radiochemical purity of the steroids.

Plasma 17-ketosteroids

Separation. Approximately 5,000 cpm (0.2 ml) of tritium-labeled etiocholanolone (5.2 μ c per μ g), androsterone (2.6 μ c per μ g), and dehydroepiandrosterone (4.4 μ c per μ g) were pipetted into a 250 ml centrifuge tube and evaporated to dryness. From 45 to 50 ml of plasma was added and the tube was shaken for about 2 minutes to insure complete mixing of the tritium-labeled steroids and the plasma. 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 vacuo* and transferred quantitatively with chloroform to washed Whatman no. 1 filter paper in the heptane:96 per cent methanol system of Bush and Wiloughby (30). Twenty-five μ g each of reference compounds androsterone, etiocholanolone, and dehydroepiandrosterone were applied together in a single spot, on each side of the extract area. A sheet of filter paper 19 cm wide conveniently accommodated three reference area spots and two extract spots. The paper was equilibrated for 4 hours and the mobile phase allowed to de-

⁶ Paper chromatography is carried out in a room with constant temperature of 20° C and relative humidity of less than 50 per cent.

⁷ Dehydroepiandrosterone-7 α -H³-acetate, SA 2.46 mc per mg, lot no. 15-258-9, from New England Nuclear Corp.

TABLE III
Specific activities of standard tritium-labeled 17-ketosteroids following reverse isotope dilution

Method	Solvent system	Specific activity		
		DHEA	ETIO	ANDRO
		<i>cpm/μg</i>		
	Free compounds*			
Paper chromatography	Heptane: 96% methanol (1:1)	57,781	74,475	78,024
	2,4-Dinitrophenylhydrazone derivatives			
Column chromatography				
3.9% Alumina	Benzene: chloroform (4:1)	58,685	77,369	82,450
5.0% Alumina	Benzene: chloroform (4:1)	59,700	77,694	83,390

* The radioactive steroids were separated by paper chromatography after the addition of 10 μg of each reference standard. The tritium-labeled free steroids were converted to the 2,4-dinitrophenylhydrazones and measured spectrophotometrically. Radioactivity was determined with corrections for quenching. The (formed)steroid 2,4-dinitrophenylhydrazones were rechromatographed on columns and specific activity determined as described.

velop for 12 to 14 hours, after which the paper was air-dried. The three standard strips were cut off and the reference compounds located by staining with Zimmermann reagent, according to the technique of Migeon and Plager (20). Appropriate areas corresponding to the reference standards and paper blanks were cut out, placed in glass-stoppered test tubes, and eluted with 4 ml of absolute ethanol for 45 minutes at 37° C. Paper blanks were usually obtained below the androsterone (least polar compound) area. However, if the androsterone migrated to the bottom of the paper, a paper blank was taken either above the dehydroepiandrosterone (most polar compound) area, well away from any ultraviolet-absorbing material or below the etiocholanolone area.

Measurement. Two-ml portions of the eluates were pipetted into acid-cleaned 15 × 85 mm Pyrex tubes and dried *in vacuo* for the 2,4-dinitrophenylhydrazine reaction (32). The samples were dissolved in 0.5 ml of absolute ethanol and 0.04 ml of freshly prepared 2,4-dinitrophenylhydrazine reagent (2.0 mg per ml of absolute ethanol acidified with 0.05 ml 12 N HCl) was added. The mixture was allowed to stand overnight at room temperature. The following day, 0.5 ml distilled water and 0.5 ml Benedict's reagent were added. The tubes were heated for 15 minutes in a vigorously boiling water bath, and, after cooling, 0.5 ml of freshly distilled chloroform was added. The tubes were stoppered quickly to prevent evaporation of the chloroform. Transfer of the steroid-dinitrophenylhydrazone to the chloroform layer was facilitated by vibrating the test tube containing the Benedict reagent: chloroform mixture for 1 minute with a Dremel hand drill, model 2 (Dremel Mfg. Co., Racine, Wis.), which was fitted with a one-eighth inch rod-like bit, bent at a 20° to 25° angle. The tubes were allowed to stand at room temperature for 1 hour. The aqueous layer was carefully removed by aspiration. The chloroform layer (0.5 ml) was transferred to 0.8 ml microcuvets with 1.0 cm light path, and absorbance was measured at 368 $m\mu$ in a Beckman model DU spectrophotometer. A standard curve was constructed on each occasion with etiocholanolone-2,4-dinitrophenylhydrazone. Since androsterone and

dehydroepiandrosterone-2,4-dinitrophenylhydrazones have the same absorbance maxima and extinction coefficients at 368 $m\mu$ (32), these compounds may be substituted in constructing the standard curve. The absorbance followed Beers' law from 0.3 μg ($A = 0.030$) to 3.0 μg ($A = 0.300$). The optical density of the samples was at least 2.5 to 4 times that of the paper blanks.⁸

A 0.5 ml portion of the ethanol eluate 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 13 to 15 per cent for tritium. Since aliquots of the eluates from the paper blanks had only background radioactivity, no radioactive correction for paper blanks was necessary. The final value was calculated, taking into account the absorbance of the blank, the dilution factor, and the isotope recovery (28).

RESULTS

Plasma determinations. Normal values and isotopic and chemical recoveries are listed in Table IV. Twelve patients with fever of known cause (Table I) had less than 0.5 μg per 100 ml unconjugated etiocholanolone, dehydroepiandrosterone and androsterone.

⁸ It has been observed that the steroid 2,4-dinitrophenylhydrazones can be further purified by the method of microalumina chromatography. In this way, the mean \pm standard deviation elution blank readings at 368 $m\mu$ are $A = 0.005 \pm 0.002$. After chemical measurements are performed, radioactive recovery is determined by direct plating at infinite thinness of the steroid-2,4-dinitrophenylhydrazones on planchets and counting the tritium in a windowless gas-flow counter (Automatic Sample Changer, model C 100 B, Nuclear-Chicago Corp., Des Plaines, Ill.). The efficiency for tritium is 28 to 30 per cent. This modification has increased the sensitivity from 0.5 μg to 0.2 μg per 100 ml.

TABLE IV

Normal unconjugated plasma 17-ketosteroid values; isotopic and chemical recoveries

	Range [10]*	Isotopic recovery [92]		Chemical recovery [12]†	
		Mean ± SD			
	μg/100 ml	%		%	
DHEA	<0.5‡ - 1.2	70	± 10.4	81.0	± 21.8
ETIO	<0.5 - 1.2	78	± 13.0	66.7	± 19.6
ANDRO	<0.5 - 0.8	67.6	± 18.6	57.5	± 21.8

* Figures in brackets represent the number of determinations performed. The normal range represents 10 measurements performed on the plasma of 6 volunteers.

† Twelve experiments were performed for each of the steroids, with the following amounts of reference compounds added to 100 to 200 ml of plasma: 1 μg [5]; 2 μg [3]; 5 μg [2]; 10 μg [1]; 20 μg [1]. The accuracy of the method is reproducible at the 1.0 μg per 100 ml level (mean ± SD = 1.0 ± 0.25).

‡ The sensitivity of the method permits 0.5 μg to be interpolated from the standard curve. However, absorbance of the samples corrected for paper blank absorbance corresponding to lower than 0.5 μg cannot be interpolated. Thus the actual lower limit of the normal range has not been defined.

In Table II are listed the unconjugated plasma 17-ketosteroid measurements in three patients with familial Mediterranean fever and in five patients with etiocholanolone fever. It was not possible to perform duplicate determinations because of the limited amount of plasma available. As can be seen, etiocholanolone was not detected in the plasma of the three febrile patients with familial Mediterranean fever or in the plasma of the patients with etiocholanolone fever when they were afebrile.

However, elevated amounts of plasma etiocholanolone were detected during the febrile episodes of etiocholanolone fever. Moreover, a significant increase of plasma dehydroepiandrosterone was also demonstrated during the same symptomatic attack. During symptomatic episodes, three of the patients (W.H., H.P. and V.E.) had a 2-, 3- and 13-fold increase, respectively, of unconjugated androsterone, compared with the afebrile period.

Identification of plasma 17-ketosteroids. The 2,4-dinitrophenylhydrazone derivatives of unconjugated etiocholanolone, androsterone, and dehydroepiandrosterone isolated from all of the samples of plasma analyzed (a total of 10 L) were pooled separately and chromatographed on 1,000 mg of neutral aluminum oxide (Woelm) columns (12.5 × 0.3 cm). A stationary phase of benzene: petroleum ether (1:1) was used for the adsorption chromatography on 3.9 and 5.0 per cent hydrated alumina columns. The individual com-

pounds were isolated by elution with benzene: chloroform, 4:1 (32). They were then reappplied to fresh 3.9 and 5.0 per cent alumina and eluted with 0.05 per cent ethanol in benzene (33). Identification of the steroid-dinitrophenylhydrazones was verified by characteristic position of the peaks on elution from the columns and maintenance of constant specific activities calculated on the basis of the absorbance of the eluted material at 368 mμ and the radioactivity, counted as described above, and corrected for quenching (Table V).

DISCUSSION

It is well established that isotopic labeling can be used to measure and to correct for losses of small amounts of steroids in analyzing plasma. The use of radioactive tracer(s) for correction is justified provided that certain conditions are met. First, the radioactive steroids must be as free as possible from contamination. This is especially important when the steroids are made radioactive by exposure to tritium gas. Second, radiochemical purity must be established and maintained. Third, the manipulations of extraction, paper chromatography, elution, and measurement of plasma steroids must affect equally both endogenous and added steroids. These conditions were strictly adhered to in the course of the development of the method described, and they facilitated an accurate chemical measurement.

Nevertheless, the sensitivity of the microchemical method prevented measurement below 0.5 μg per 100 ml of plasma since, if less than this amount of plasma 17-ketosteroid were present, it would produce an optical density reading indistinguish-

TABLE V

Specific activities of pooled unconjugated 17-ketosteroid 2,4-dinitrophenylhydrazones

Alumina % hydration*	Solvent system	Specific activity†		
		DHEA	ETIO	ANDRO
		cpm/μg		
3.9	Benzene:CHCl ₃ (4:1)	4,820	5,244	7,530
5.0	Benzene:CHCl ₃ (4:1)	4,792	5,120	7,400
3.9	0.05 % Ethanol in benzene	4,780	5,100	7,320
5.0	0.05 % Ethanol in benzene	4,768	5,086	7,220

* The per cent hydrated alumina and solvent systems were employed in the order listed.

† Before column chromatography the specific activities of the pooled (10 L of analyzed plasma) tritium-labeled 17-ketosteroid 2,4-dinitrophenylhydrazones were: DHEA, 4,760; ETIO, 5,208; ANDRO, 7,480. The 2,4-dinitrophenylhydrazones were measured at 368 mμ in a spectrophotometer as previously described. Corrections for quenching were performed.

able from that of the paper blanks. Since the actual lower limit of the normal range has not been defined, statistical analysis of the data was not undertaken. However, the method did afford, by its sensitivity and reproducibility, an opportunity to investigate possible aberrations of androgen metabolism during fever.

Recently it was shown that the administration of certain steroids produces fever in human subjects. Kappas and co-workers (34) reported that the febrile response to steroids with a 3α -hydroxyl, 5β configuration differed from that produced by bacterial pyrogens (35). Steroid pyrogens, unlike bacterial pyrogens, were associated with a much longer latent period (4 to 6 hours) and did not produce tolerance after repeated administration (13). Moreover, these steroids did not produce fever in certain experimental animals (e.g., the dog or rabbit) which commonly respond to bacterial pyrogens (1, 2, 36). According to recent observations of Kappas and associates (34), the pyrogenic response to steroids was not due to the release of endogenous pyrogen.

This report describes five patients with acute attacks of etiocholanolone fever who had elevated unconjugated plasma etiocholanolone levels during the prodrome of chills, anorexia, abdominal and/or chest pain, myalgia, arthralgia, headache, and fever. Normal amounts of unconjugated etiocholanolone were detected in the plasma during the afebrile intervals. These observations support the thesis that the presence of the pyrogenic steroid may in some way be responsible for the development of this type of fever. However, the actual mechanism is still obscure. Unconjugated etiocholanolone was not found in the plasma of patients with fever of known origin or in three patients with familial Mediterranean fever. Therefore it seems unlikely that fever itself causes the presence of the steroid.

The plasma concentrations of dehydroepiandrosterone, an adrenal androgen (37), and (in three instances) androsterone were also increased in the febrile patients with etiocholanolone fever.

Although liver enlargement was present in the patients with etiocholanolone fever, liver function tests and biopsies were normal. Nevertheless, our data suggest that one hepatic defect occurs in this syndrome, i.e., a change in the pathway of androgen metabolism toward excessive etiocho-

lanolone production. Tomkins (38) described two separate and specific steroid reductases which catalyze the reduction of the double bond on the A ring of steroids, to produce, in one case, 5β -steroids and, in the other, 5α -steroids such as androsterone. The acute attacks of etiocholanolone fever may be associated in some way with a decrease of the activity of the 5α -steroid reductases and concomitant increase of 5β -reductases. The analogous situation of increased etiocholanolone excretion, as opposed to androsterone excretion, has been described by Bradlow, Hellman, Zumoff and Gallagher (39) and Hellman and associates (40) in hypothyroidism. The administration of triiodothyronine reversed the clinical status and restored the androsterone, etiocholanolone ($5\alpha/5\beta$) ratio to normal. Thyroid function measured by protein-bound iodine or butanol-extractable iodine and radioactive iodine uptake was within the normal range in the five patients with etiocholanolone fever. It would thus seem in the present study that this defect in C_{19} metabolism is unrelated to thyroid function. The precipitating causes and pathogenesis of etiocholanolone fever remain to be elucidated.

It has also been suggested (17) that an intermittent defect in conjugation might occur concomitantly with increased etiocholanolone production at the time of an attack of etiocholanolone fever. Kappas and colleagues (12) reported that substitution of an acetate radical on the third carbon atom of etiocholanolone inhibits pyrogenic activity. Kappas' observations suggest that etiocholanolone in plasma must be unconjugated if it is to account for the pyrogenic activity during attacks of etiocholanolone fever. Since the data indicate that unconjugated plasma etiocholanolone is associated with attacks, a possible aberration in conjugation has been hypothesized. In fact, preliminary data suggest that an attack is associated with decreased glucuronide conjugation of plasma tetrahydrocortisone and tetrahydrocortisol without increased β -glucuronidase activity.

Although the present studies have failed to find an increase in the unconjugated plasma etiocholanolone in patients with fever of known cause, only 12 such patients were studied. It is still too early to be certain that abnormalities of steroid metabolism do not occur in association with types of fever other than those studied in this brief series.

SUMMARY

A method is presented for the separation of unconjugated plasma 17-ketosteroids and micro-measurement of their 2,4-dinitrophenylhydrazone derivatives. An isotopic radiochemical dilution technique with pure tritium-labeled etiocholanolone, dehydroepiandrosterone, and androsterone is applied to correct for losses during the procedure. The normal plasma ranges are: etiocholanolone < 0.5 to $1.2 \mu\text{g}$ per 100 ml; androsterone, < 0.5 to $0.8 \mu\text{g}$ per 100 ml; dehydroepiandrosterone, < 0.5 to $1.2 \mu\text{g}$ per 100 ml. The total mean \pm SD isotopic and chemical recoveries are 72 ± 14 and 67 ± 21 per cent, respectively.

Each of the measured 2,4-dinitrophenylhydrazone derivatives of unconjugated etiocholanolone, dehydroepiandrosterone, and androsterone was identified by characteristic elution mobility and maintenance of constant specific activity after four separate column chromatographic procedures.

Elevation of unconjugated plasma etiocholanolone (a known pyrogenic steroid) and dehydroepiandrosterone was demonstrated in five patients during attacks of etiocholanolone fever. In addition, three of the five patients had elevated unconjugated plasma androsterone levels. Dehydroepiandrosterone and etiocholanolone were within the normal range during the afebrile intervals. Unconjugated etiocholanolone was not detectable ($< 0.5 \mu\text{g}$ per 100 ml) in the plasma of 12 patients with fever of known cause and 3 febrile patients with familial Mediterranean fever.

It has been suggested that in acute attacks of etiocholanolone fever, an intermittent decrease of hepatic 5α -reductases may be associated with a simultaneous increase in 5β -reductases, resulting in the production of excessive amounts of etiocholanolone at the expense of androsterone. Conclusive evidence of a defect in conjugation of 17-ketosteroids in etiocholanolone fever remains to be determined.

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