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Non-polar extracts of serum from human males contain covert radioimmunoassayable testosterone

Addo, Samuel Boi, Ph.D.

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

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NON-POLAR EXTRACTS OF SERUM FROM HUMAN MALES CONTAIN COVERT RADIOIMMUNOASSAYABLE TESTOSTERONE

by

SAMUEL BOI ADDO

A dissertation submitted to the Graduate Faculty in Biology in partial fulfilment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1989

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May 22, 1989
date

Constance R. Martin
Chairman of Examining Committee
Dr. Constance Martin, Hunter College

June 8, 1989
date

Executive Officer

Linda M Mantel
Dr. Linda Mantel, City College

Edward Diamond
Dr. Edward Diamond, Queens College

Dr. Vincent Hollander, Mt. Sinai School of Medicine

Dr. Frederick Schatz, Mt. Sinai School of Medicine

Supervisory Committee

The City University of New York

Abstract

NON-POLAR EXTRACTS OF SERUM FROM HUMAN MALES CONTAIN COVERT RADIOIMMUNOASSAYABLE TESTOSTERONE

by

Samuel Boi Addo

Advisor: Dr. Vincent Hollander

A form of testosterone that cannot be detected by standard radioimmunoassay procedures was identified in the sera of healthy men. The sera were extracted with petroleum ether, subjected to mild alkali hydrolysis, purified by reverse phase high pressure liquid chromatography, and assayed by radioimmunoassay. Hydrolyzed non-polar serum extracts from ten adult male volunteers contained 2.0 ± 0.8 ng/ml testosterone. In contrast, no testosterone was found in hydrolyzed petroleum ether extracts of sera from eight women or in any of the unhydrolyzed samples. Two other women had values of 0.7 ng/ml and 0.8 ng/ml. Testosterone palmitate, used as a model compound, was hydrolyzable by the procedure and gave recoveries of more than 90%.

These findings reveal the presence of substantial quantities of novel naturally-occuring non-polar conjugates of testosterone (NPT) in sera of human males. The compounds may be analogous to long-chain fatty acid conjugates of estradiol identified in female sera. The lipoidal estrogens exert long-lasting biological actions attributed to slow release of free hormone. NPT may therefore act physiologically as hormone reservoirs when little or no androgen is released by the testis. They may also contribute to abnormal stimulation of androgen-responsive tissues. Some evidence for their presence in the sera of patients with prostatic cancer receiving androgen-supression therapy has been obtained.

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-To my parents, brothers and sisters for their steadfast love and support on which I could always count.

-Most of all to my beloved wife, Mary, for her patience and devotion. "... now abideth faith, hope and love but the greatest is love."

This thesis is dedicated to my sister-in -law, Jane, whose love and support I will always cherish.

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I. GENERAL BACKGROUND

A. Androgen function, chemistry and metabolism.

Androgens are 19-carbon steroid hormones secreted primarily by the testes, and by adrenal cortical cells of humans and other species. Small quantities are produced from other steroids in other organs, such as the liver. The ovaries synthesize androgens but only small amounts escape conversion to estrogens.

For a 19-carbon steroid to be an androgen, it should have a 17-oxygen function with either a 4-ene-3-oxo configuration, as in testosterone, or a 3-oxo group with a saturated A-ring as in dihydrotestosterone (DHT) (1). Androgenicity is totally lost if the oxygen function is absent, as in 4,16-androstadien-3-one. In addition, oxidation of a 17-oxosteroid drastically reduces androgenicity. Androgenicity of steroids can be measured in castrated mice or rats by the increases in weights of prostate glands that follow administration of known quantities of the compounds.

Androgens promote differentiation, growth and maturation of male primary and accessory reproductive organs and regulate their functions. They also invoke maturation of secondary sex characteristics and exert anabolic effects on skeletal muscle, bone marrow, skin,

liver,and kidney, and they regulate the functions of the pituitary gland and hypothalamus. Androgens are known to increase the activity of several enzymes such as acid phosphatases, arginase, amino acid oxidase and B-glucuronidase, but they decrease alkaline phosphatase activity in the prostate gland. Submaxillary gland epidermal growth factor (2,3), nerve growth factor (4), lethal growth factor (5,6), lymphoid tissue inhibitory growth factor (7) and protease levels are affected by steroids. Androgens also increase hair growth, although loss of scalp hair is said to be triggered by continued presence of androgens.

In males, testes are the major sites for biosynthesis of testosterone, much of which is normally converted to 5α-dihydrotestosterone (DHT) to interact with receptors in most target cells. Ofner et al.(8) showed that radioactive 5-αDHT is the major product formed when prostatic homogenates are incubated with [14-C]-testosterone. This conversion is brought about by the enzyme 5-αreductase, found in microsomal fractions. Some testosterone may also be converted to estradiol or androstanediol. There are however, species variations in patterns for testosterone metabolism.

Testosterone production is controlled mainly by luteinizing hormone (LH) secreted by adenohypophysial gonadotropes. Gonadotropin releasing hormone (GnRH) stimulates production and secretion of both LH and follicle stimulating hormone (FSH) by gonadotropes. LH stimulates the

secretion of testosterone from the Leydig cells which lie between the seminiferous tubules of the testes. Testosterone, in synergism with FSH, initiates and sustains spermatogenesis. In ovaries, LH stimulates synthesis of androgens by stromal and thecal cells, but very little androgen is secreted. The steroids are taken up by granulosa cells, in which FSH induces the synthesis of aromatase system enzymes that catalyze the conversion of androgens to estrogens. Androgens and estrogens exert negative feedback controls over GnRH and LH secretion. According to most observers, peptide hormones such as inhibin and related proteins are potent regulators of FSH synthesis and release (9,10,11), and other peptides affect the binding of FSH .

In men approximately 60% of circulating testosterone is bound to sex hormone binding globulin (SHBG), and an additional 38% is bound to albumins. Testosterone is also bound, to a slight extent, by cortisol binding globulin (CBG). Both free and albumin-bound testosterone are available for metabolism. It is widely believed that only the free steroid (2% of total) readily enters target cells. In women, circulating testoterone levels are much lower, with approximately 80% bound to SHBG and 19% to albumins. The blood levels of testosterone in men are in the range of 3-10 nanograms per milliliter of blood. It has recently been demonstrated that fatty acids, such as linoleic, oleic and

palmitic acids, at physiological concentrations, inhibit the binding of testosterone to albumin and SHBG (12). This finding suggests that fatty acids are important regulators of bioavailable testosterone.

Pathological conditions associated with abnormal androgen production include:
(a) premature secretion of adult levels of androgens in children, resulting in precocious puberty in boys and virilization in girls. (b) excessive secretion of androgens by tumors in men (adrenocortical and testicular tumors). (c) deficient testicular secretion in male hypogonadism. (d) testicular feminization (Androgen Insensitivity Syndrome).

B. Polar Steroid Conjugates.

Sulfokinases are enzymes involved in the conversion of certain steroids to their corresponding sulfates. They are found in soluble fractions of the testis, placenta, liver and the zona fasciculata of the adrenal gland. The best substrates are the 5-ene-3 -hydroxy steroids such as dehydroepiandrosterone (DHA). The enzymes require sulfate and magnesium ions for activity. The reactions involve :
(a) Sulfation of adenosine triphosphate (ATP) by SO_4 , catalyzed by ATP sulfurylase.
(b) Phosphorylation of adenosine-5- phosphate by adenosine phosphate kinase to yield phosphoadenosine-5-phosphosulfate.

(c) Reaction of 'active' sulfate with the hydroxyl group of the steroid.

(a) SO +ATP ——————→ Adenosine-phosphosulfate (APS) + ADP

(b) APS +ATP——→Phosphoadenosine phosphosulfate (PAPS) +ADP

(c) PAPS + Steroid (R-OH)——→ R-O-SO_3 + 3,5-phosphoadenosine (PAP) + H^+.

Glucuronyl transferases catalyze the conjugation of steroids with glucuronic acid. The process involves transfer of the glucuronide moiety as uridine diphosphoglucuronic acid (UDPGA)

UDPGA + Steroid-OH ——————→Steroid glucuronide + uridine diphosphate. These polar steroids are excreted into either the urine or bile (13,14).

C. Non-polar Steroid Conjugates.

Apart from these known steroid conjugates, Hochberg et al. (15) clearly demonstrated the existence of an unusual nonpolar metabolite of estradiol, referred to as lipoidal derivative of estradiol (LE2). Hochberg et al. showed that ether extracts of blood from females treated with human menopausal gonadotropin (HMG) produced more immunoassayable estradiol after mild alkaline hydrolysis than in unhydrolyzed extracts. This was demonstrated by measuring estradiol by radioimmunoassay (RIA) using an

antibody specific for estradiol which did not cross-react with estradiol esters such as estradiol stearate and estradiol arachidonate. LE2 is very different from the previously known conjugates of estradiol (the sulfate and glucuronide) which can neither be extracted by ether nor hydrolyzed by a weak base. LE2, however, displayed chromatographic properties similar to those fatty acid esters of estradiol, esterified at the C-17 position.

In another experiment by Schatz et al. (16), slices of uteri and 7,12-dimethylbenzanthracene (DMBA)- induced mammary tumors were incubated separately with [3-H]-estradiol at 37 ^{o}C. After 5 hours of incubation, a labelled non polar product was obtained from both tissues. This product, when hydrolyzed, showed radioactivity that co-migrated with estradiol. They further demonstrated that estradiol but not estrone gave rise to LE2, and speculated that conjugation must occur at the C-17 position. This was shown by oxidizing [3-H]-LE2 and some carrier estradiol with pyridine chromium trioxide (16). The reaction mixture was extracted, hydrolyzed, and chromatographed on a high pressure liquid chromatography (HPLC) column. More than 90% of the carrier estradiol was converted to estrone, but the radioactivity chromatographed with estradiol. They reasoned that the fatty acid moiety at the C-17 position protected estradiol from oxidation. Acetylation of the phenolic hydroxyl group at the C-3 position of LE2 yielded a product

which was less polar than LE2 . On HPLC, LE2 was found to be less polar than estradiol acetate. It was therefore suggested that the free hydroxyl group for acetylation was probably the phenolic hydroxyl at the C-3 position.

Mellon-Nussbaum et al.(17), using mass spectrometry and gas chromatography/mass spectroscopy, also showed that the non-polar derivative, LE2 synthesized by bovine endometrial tissue, comprises a family of polyunsaturated fatty acid esters of estradiol, the most abundant being the arachidonate ester. It was demonstrated that the estradiol was esterified exclusively at the C-17 position. A study by Larner et al. (18), also revealed that these naturally occuring C-17 esters of estradiol, similar to synthetic aryl or alkyl estradiol esters, were long-acting (19,20). When animals were given either [3-H]-estradiol or [3-H]-estradiol stearate, those receiving the unconjugated steroid displayed greater radioactivity in their uteri soon after the injection. In contrast, animals given [3-H]-estradiol stearate had more radioactivity at later times (>10 hours). The conjugated steroid was also observed to invoke premature puberty as assesed by timing of vaginal opening. The sustained activities of estradiol esters have been attributed to slow release of free steroid from hydrophobic reservoirs (21). In another experiment (22), it was shown that various alkyl esters of estradiol were not ligands for estrogen receptors. This clarifies earlier controversies

(23) concerning whether conjugates can interact directly with receptors. Thus, the estrogenic effects of [3-H]-estradiol stearate must be preceded by hydrolysis of the ester to release free estradiol. In this case, LE2 may (i) act physiologically to provide some estrogenic activity where there is insufficient ovarian output of estradiol, or (ii) contribute to abnormal stimulation of estrogen-responsive tissues.

Androsterone esters, which show parallels with the above-mentioned estradiol esters, have been identified in human breast cyst fluid. Their origin is unknown. It is speculated however, that the molecules are transferred from the blood to the cyst fluid, where accumulation results in high concentrations. Another suggestion is that the androsterone fatty acid esters are synthesized by the epithelial cell lining of the cyst sac. Human breast tissue is also known to convert androsterone into long chain fatty-acid esters containing stearate, oleate, arachidonate and palmitoleate moieties with the unsaturated esters accounting for more than 90% of the total.

Since androgenicity depends partly on the presence of a 17-oxygen function, it follows that esterification at the C-17 position can drastically affect biological activity. Androsterone, a rather weak androgen, is known to occur in the esterified form (24). It may be speculated that physiologic levels of potent androgens are maintained not

only by converting excesses to less potent androgens, but also via esterification as a means of transient inactivation.

II.INTRODUCTION TO THESIS RESEARCH.

There is evidence that non-polar esters of estradiol occur in several tissues (15,16,17). Acyl-CoA/Acyltransferases that catalyze conjugation of fatty acid moieties to the steroid nuclei have been isolated from microsomal fractions (25) and have been shown to be capable of using testosterone, estradiol and androstanediol as substrates (26). It has also been demonstrated that free fatty acids such as linoleic and palmitic inhibit the binding of testosterone to both albumin and sex-hormone-binding-globulin (12). It is therefore likely that fatty acid esters of testosterone do occur in blood. Hitherto, the major known conjugate of testosterone has been the glucuronide, which is polar and not hydrolyzable by a weak base. The purpose of this study was to determine whether non-polar esters of testosterone are present in human serum or plasma. Testosterone palmitate was used as a model compound to delineate optimum conditions for hydrolysis, recovery, and chromatographic (HPLC) behavior of non-polar testosterone esters. The methods were then applied to non-polar serum extracts. The isolation of non-polar derivatives of testosterone would reveal a novel group of compounds that could play important roles in the maintenance of physiologic levels of androgen.

III. MATERIALS AND METHODS.

Sera from human male volunteers at least 20 years old, and from normal female volunteers 25 years or older

Acetonitrile (HPLC grade), methanol, benzene, ethyl acetate purchased from Fisher Scientific.

Potassium Hydroxide (KOH) [Mallinckrodt].

Polygram SIL G/uv 254 precoated plastic TLC sheets (40x80 mm) obtained from Macherey-Nagel.

Testosterone, Testosterone propionate, Testosterone enanthate were purchased from Sigma; [3-H]-Testosterone, [14-C]-Testosterone purchased from New England Nuclear.

uBondapak CN Reverse Phase Column(7.8mm x 30cm), Acro LC35 Filter (0.2um) , HPLC system with automated gradient controller pumps and a Lamda Max LC Spectrophotometer (Model 481) purchased from Waters Inc. Glass-distilled water was used in all HPLC solvent systems.

A liquid Scintillation Counter (Beckman LS3801) equipped with a data reduction system for linear regression plot and

a Beckman Spectrophotometer (Model 25) were used all radioactivity counts.

Testosterone Radioimmunoassay Kits were purchased from Wien Laboratories Inc.

Radioactive samples were counted in 3 ml of Aqueous Counting Scintillant (ACS) purchased from Amersham.

1.Extraction of testosterone by methylene chloride

To determine the effectiveness of methylene as a solvent for the extraction of testosterone, 50 microliters of [3-H]-testosterone (1.58×10^5 dpm) were added to 10 ml of distilled water, and then extracted with 3 ml of methylene chloride. One milliliter and 200ul aliquots of the methylene chloride extract were counted on a Beckman Liquid Scintillation Counter.

2.Extraction of testosterone with petroleum ether

To determine a suitable solvent which would extract less than 10% of added testosterone, 100 microliters [3-H]-Testosterone(7.5×10^5 dpm) were added to 10 ml of serum and extracted with 10 ml of petroleum ether or ethyl ether. Both ether fractions were then counted using a Beckman scintillation counter.

3.Preparation of Testosterone palmitate (T-pal).

A modification of the method described by Mellon-Nussbaum et al. (17) was used. Ten milligrams of testosterone was refluxed with an excess of palmitic anhydride (30mg) in one milliliter of dry, freshly distilled pyridine for three hours. Twenty milliliters of 10% hydrochloric acid was added and the mixture then extracted with petroleum ether. The petroleum ether was washed with 20 ml of 10% sodium bicarbonate and distilled water, followed by two washes with 50% aqueous methanol to remove unreacted testosterone. The petroleum ether was evaporated and the residue purified by high pressure liquid chromatography (HPLC), using solvent system I described below. The purified sample was chromatographed on thin layer chromatography (TLC) plates and developed in the solvent system described below. The absorption spectrum of the purified T-pal was determined with a Beckman spectrophotometer.

[14-C]-Testosterone acetate was prepared by refluxing acetic anhydride with some [14-C]-testosterone as described for T-pal .

4. Extraction of [14-C]-T-pal with petroleum ether.

To estimate the amount of non-polar testosterone ester extracted by petroleum ether, [14-C]-T-pal was prepared as

decribed above, and then purified by alumina adsorption chromatography. Samples were loaded on a column then eluted with benzene:ethyl acetate (3:1). About $1.1x10^3$ counts of the [14-C]-T-pal peak was added to 4 ml of serum and extracted three times with equal volumes of petroleum ether. One milliliter aliquots were counted after each extraction.

5. Chromatography

Thin layer chromatography (TLC) was used to determine the chromatographic behavior of testosterone and some testosterone esters. Microgram quantities of each sample were spotted on polygram SIL G/UV 254 precoated plates and then developed in benzene:ethyl acetate [2:1]

Reversed phase high-pressure liquid chromatography (RP-HPLC) was used to purify the synthesized testosterone palmitate. The solvent system was methanol: acetonitrile: water (2:1:1) [systemI]. Another solvent system(II), [acetonitrile:water (1:1)] was used to determine the elution times for testosterone and testosterone esters. Samples were loaded on a uBondapak CN reverse phase column and then isocratically eluted with solvent system II at a flow rate of 2 ml/min. The chromatographic separation was monitored with a UV spectrophotometer set at 240 nm.

6. Hydrolysis of samples.

A modification of the method described by Schatz et

al. (16) was used. It was chosen after several trials to determine the lowest and yet the most effective concentration of methanolic KOH that would saponify testosterone esters such as the propionate and palmitate.

An equal volume of 2% methanolic potassium hydroxide was added to an aliquot of the compound to be hydrolyzed and nitrogen was bubbled through the mixture. After incubation at 45 C for three hours, 8 ml of distilled water was added to the samples in polypropylene tubes and the mixtures extracted with 3 ml methylene chloride. The methylene chloride was evaporated and the residue taken up in 0.5 ml acetonitrile:water (1:1) for HPLC purification. Fractions 6 & 7; 8 & 9;10 & 11, corresponding to pre-testosterone, testosterone and post-testosterone peaks (A, B, and C respectively) were assayed for testosterone by radioimmunoassay. For each sample hydrolyzed, an equal amount was mock hydrolyzed with methanol under similar conditions.

7. Recovery experiments.

To determine the recovery of added T-pal from non polar serum extracts, the following experiments were performed:

(i)3.0 ng of testosterone was hydrolyzed or mock hydrolyzed and then purified by HPLC as described above. The pre-T, T and post-T fractions were assayed for testosterone by radioimmunoassay.

(ii) 3.9 ng of spectrophotometrically-quantitated T-pal was added to 200ul of a non-polar serum extract (equivalent to 0.8 ml of serum), then hydrolyzed or mock hydrolyzed. Another 3.9 ng of T-pal was separately hydrolyzed or mock hydrolyzed. A 200 ul aliquot of the non-polar serum extract was also separately hydrolyzed or mock hydrolyzed. After hydrolysis, each of the samples was purified by HPLC. Duplicates of the appropriate fractions were radioimmunoassayed for testosterone at two levels.

8. Radioimmunoassay of known amounts of testosterone added to fraction B.

To determine whether substances eluting with fraction B of hydrolyzed non-polar serum extracts affected the ligand-antibody binding in the RIA, 200 pg of authentic testosterone was added to evaporated aliquots of fraction B and assayed by radioimmunoassay.

9. Non-polar serum extracts.

Five to ten milliliters of freshly obtained serum samples were extracted with 10-20 ml petroleum ether. The extracts were evaporated and the residues taken up in 2.0 ml methanol. One-half milliliter aliquots were hydrolyzed or mock hydrolyzed, purified by HPLC, and assayed for testosterone by radioimmunoassay as described.

10. [14-C]-T-pal as an internal standard.

Aliquots equivalent to approximately 10 counts of [14-C]-T-pal were added to 5 ml of serum and the mixture was extracted twice with equal volumes of petroleum ether. Eight milliliters of the ether was evaporated and taken up in 1.0 ml methanol. 400 ul aliquots were hydrolyzed or mock hydrolyzed, extracted, and purified by HPLC as described. Aliquots of the elution fractions were counted. In addition, fractions A, B and C were assayed for testosterone by RIA.

11. Radioimmunoassay (RIA).

The method described by Wien laboratories was used to assay fractions A, B, and C at two levels. The antibody, which is specific for testosterone and dihydrotestosterone, was produced in rabbits in response to testosterone-3-oxime albumin (27,28). Each 100 ul of the reconstituted antibody binds 40-50% of 200 pg of [3-H]-testosterone (100 Ci/mmol) but does not cross-react with either testosterone esters such as the propionate, enanthate, or palmitate, or free steroids such as estradiol, cortisol, progesterone and pregnenolone. Samples to be assayed were evaporated in 12 x 75 mm borosilicate culture tubes, and the residue, reconstituted in 1 ml phosphate saline buffer (pH 7.4) containing 0.06% BSA. Samples were then incubated with 50 ul [3-H]-testosterone (1.5 uCi/ml) and 100 ul of the antibody

at 4 °C for 1 h. Dextran coated charcoal was used to separate bound and free steroid. Radioactivity in standards and samples was measured with a Beckman scintillation counter equipped with a data reduction system. A linear regression plot of Bound/Max Bound versus Dose (pg/tube) over a range of 0-1000 pg was used. Control samples containing 0, 200 and 400 pg testosterone were assayed with each run. The intra-assay %CVs at 200 pg and 400 pg levels were 7% and 9%, respectively (n=6). The inter-assay %CVs at the above-mentioned levels were 5.2% and 5% respectively (n=6).

IV. RESULTS:

Preliminary experiments showed that the antibody did not cross-react with testosterone fatty acid esters such as the propionate and palmitate, even at levels of 2 ng per assay tube (equivalent to more than 20 ng/ml).

It was also observed that methylene chloride extracted more than 90% of [3-H]-testosterone added to water (Table 1). As shown in Table 2, petroleum ether, however, extracted less than 8% of the total [3-H]-testosterone added to serum.

T-pal studies.

Synthesized T-pal was partially purified by washing with 50% aqueous methanol to remove any unreacted testosterone.

As shown in Table 3 and Figure 1, synthesized T-pal shows a peak at 240 nm, similar to the absorption spectrum of testosterone.

Testosterone and testosterone esters have different Rf values that range from 0.3 to 0.8 (Table 3b). The more non-polar forms showed greater Rf values.

Mock hydrolyzed and hydrolyzed T-pal had Rf values of 0.8 and 0.3, respectively, when chromatographed on SIL/G uv plates and developed in the solvent system described above.

On HPLC reverse phase column, the elution times for testosterone, testosterone acetate (T-ac), testosterone propionate (T-pr), and testosterone enanthate (T-en) were 8.0, 11.5, 12.5 and 17.2 minutes, respectively (Fig.2). Mock hydrolyzed [14-C]-testosterone acetate eluted at approximately the same time as the unhydrolyzed ester (11min) whereas hydrolyzed [14-C]T-ac showed a radioactive peak at 8 minutes, similar to that of free testosterone (Fig.3).

Recovery experiments.

Recoveries of 90% and 75% were obtained for "mock hydrolyzed" and "hydrolyzed" testosterone, respectively. Testosterone was detected in fraction B only (Table 4).

It was observed that more than 90% of the [14-C]T-pal added to serum was recovered after three equal-volume petroleum ether extractions (Table 4b and figure 4). Hydrolysis of purified T-pal showed detectable testosterone only in fraction B, with recoveries of 114% and 128%. When known amounts of T-pal were added to serum and hydrolyzed or mock hydrolyzed, recoveries of 109% and 115% were obtained in fraction B only (Table 5).

When [14-C]-T-pal was used as an internal standard, both the radioactivity peak and the detected testosterone occurred only in fraction B of hydrolyzed samples (Figure

5). No testosterone was found in hydrolyzed or mock hydrolyzed distilled water, mock hydrolyzed T-pal, or fractions A and C.

As shown in Table 6, 89% recovery was obtained by radioimmunoassay for known amounts of testosterone added to fraction B of hydrolyzed or mock hydrolyzed non-polar serum samples.

Table 7 shows the amounts of testosterone detected in fraction B of hydrolyzed and mock hydrolyzed non-polar serum samples. Of the ten samples from presumably healthy females analyzed, eight had undetectable levels whilst two contained less than 0.8 ng/ml. In contrast, values for all the males examined were in the range of 2.0 $\pm$ 0.8 ng/ml of testosterone.

Table 1. Percent of added [3-H]-Testosterone, extracted by methylene chloride

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About 1.6 x 10^5 dpm of [3-H]-Testosterone was added to 10 ml of water and extracted with 3 ml of methylene chloride. 1.0 ml and 200 ul aliquots from the 3 ml methylene chloride were counted in a Beckman scintillation counter.

* corrected for volume

Sample	Counts (dpm x 10^4)	% Recovery
50 ul [3-H]-T	15.8	
1.0 ml $MeCl_2$	14.7	93
200 ul $MeCl_2$	*15.0	95

Table 2. Quantity of [3-H]-Testosterone extracted by petroleum or ethyl ether.

About 7.5×10^5 dpm of [3-H]-Testosterone was added to 10 ml of serum then extracted with 10 ml of either petroleum ether (PE) or ethyl ether(EE). The ether fractions were counted

Sample	Counts (dpm x 10^5)	% Recovery	% Remaining after extraction
[3-H]-T	7.50 ± 0.06		
EE extract of serum + [3-H]T	7.40 ± 0.15	98	-
PE extract of serum + [3-H]T	0.55 ± 0.01	7.3	-
Counts in serum after EE extraction	1.5 ± 0.02	-	20
Counts in serum after PE extraction	6.6 ± 0.10	-	88

Table 3. Absorption spectrum of testosterone (T) and testosterone palmitate (T-pal).

The Absorbance of authentic T and synthesized T-pal was determined over a 220-270 nm range.

Wavelength (nm)	ABSORBANCE x 10^3		
	2.5ug T	25 ug T	T-pal
220	27	472	150
230	96	1020	178
235	136	1340	205
240	169	1650	225
242	173	1710	220
245	172	1730	215
250	154	1640	179
255	110	1300	123
260	-	-	67
270	-	-	10

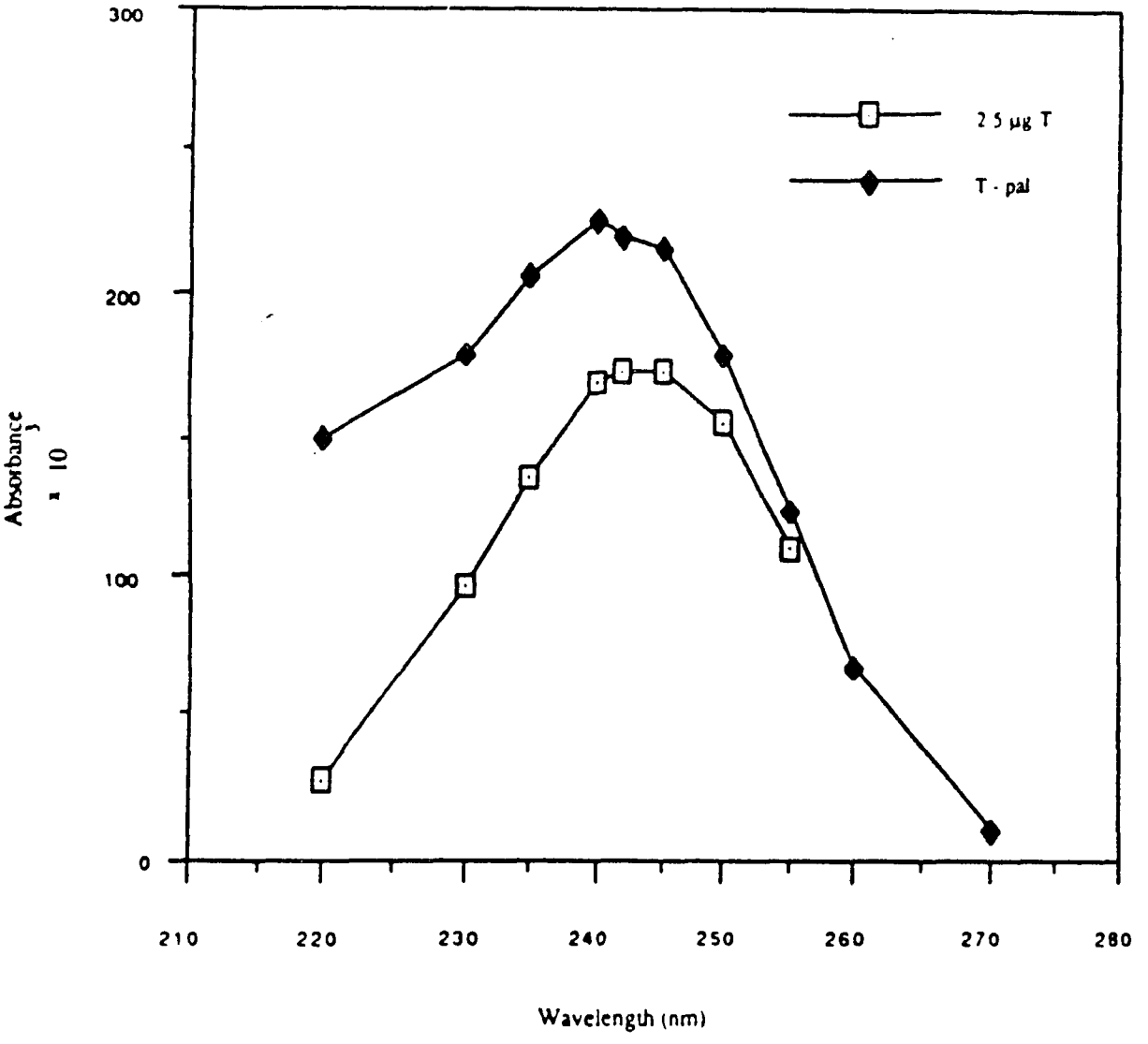

FIGURE 1. Absorption spectrum of authentic testosterone and synthesized testosterone palmitate.

Table 3b. Thin-layer chromatography (TLC) of testosterone and some testosterone fatty acid esters.

Compound	Rf
Testosterone (T)	0.3
T. acetate	0.6
T. propionate	0.7
T. enanthate	0.77
T. palmitate	0.84

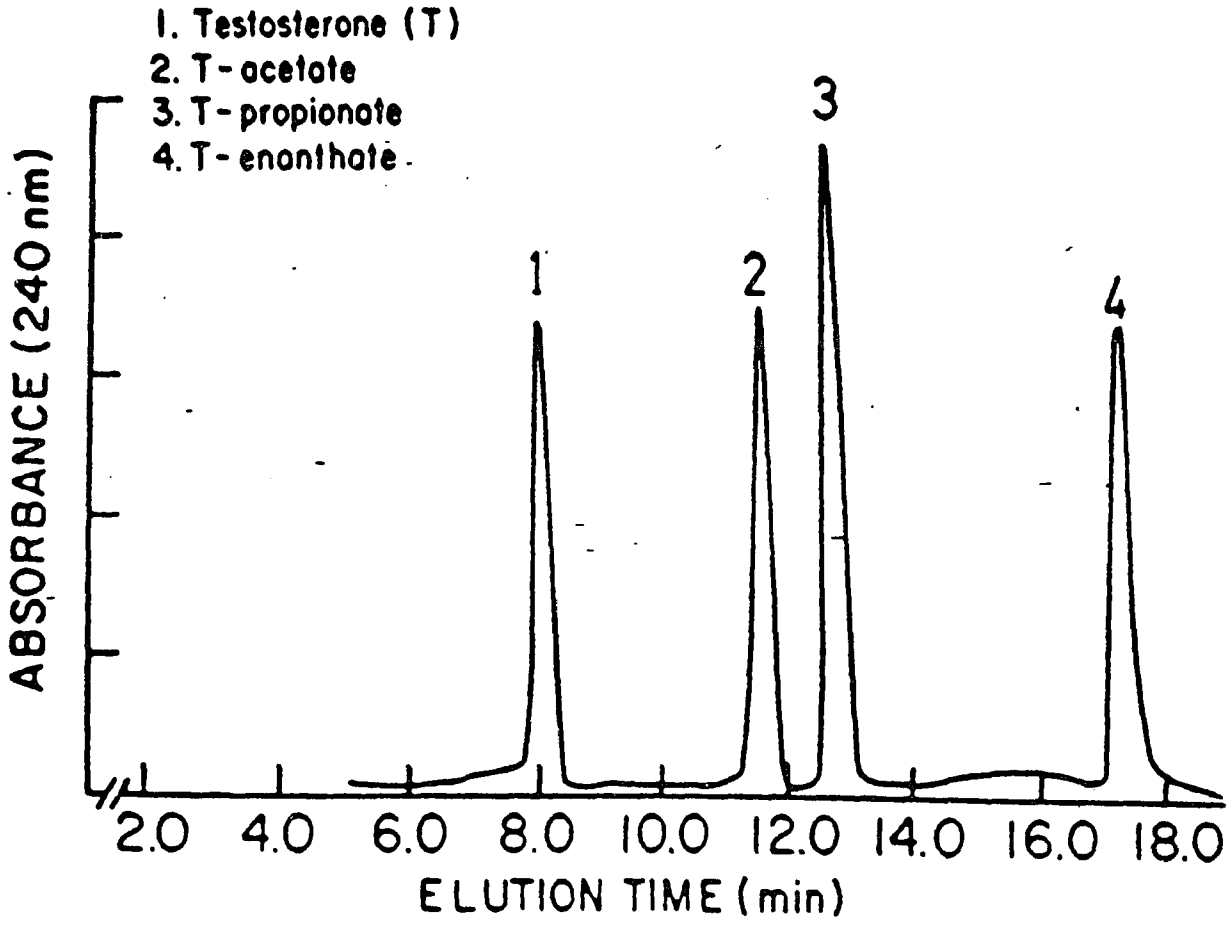

FIGURE 2. Reversed phase HPLC of testosterone and some testosterone esters. Samples were isocratically eluted with acetonitrile : water (1:1) at 2 ml per minute. Absorbance was monitored at 240 nm.

Table 4. Effect of "hydrolysis" on Testosterone.

Approximately 3 ng testosterone was "hydrolyzed" or "mock hydrolyzed", and purified by RP-HPLC . Fraction B was assayed by RIA. No testosterone was found fractions A and C. The values given are the Mean ± SD.

Sample	Observed pg/tube	Expected pg/tube	%Recovery
Mock Hydr.	180 ± 7	200	90
Hydrolyzed	150 ± 4	200	75
Dist.Water	-	-	-

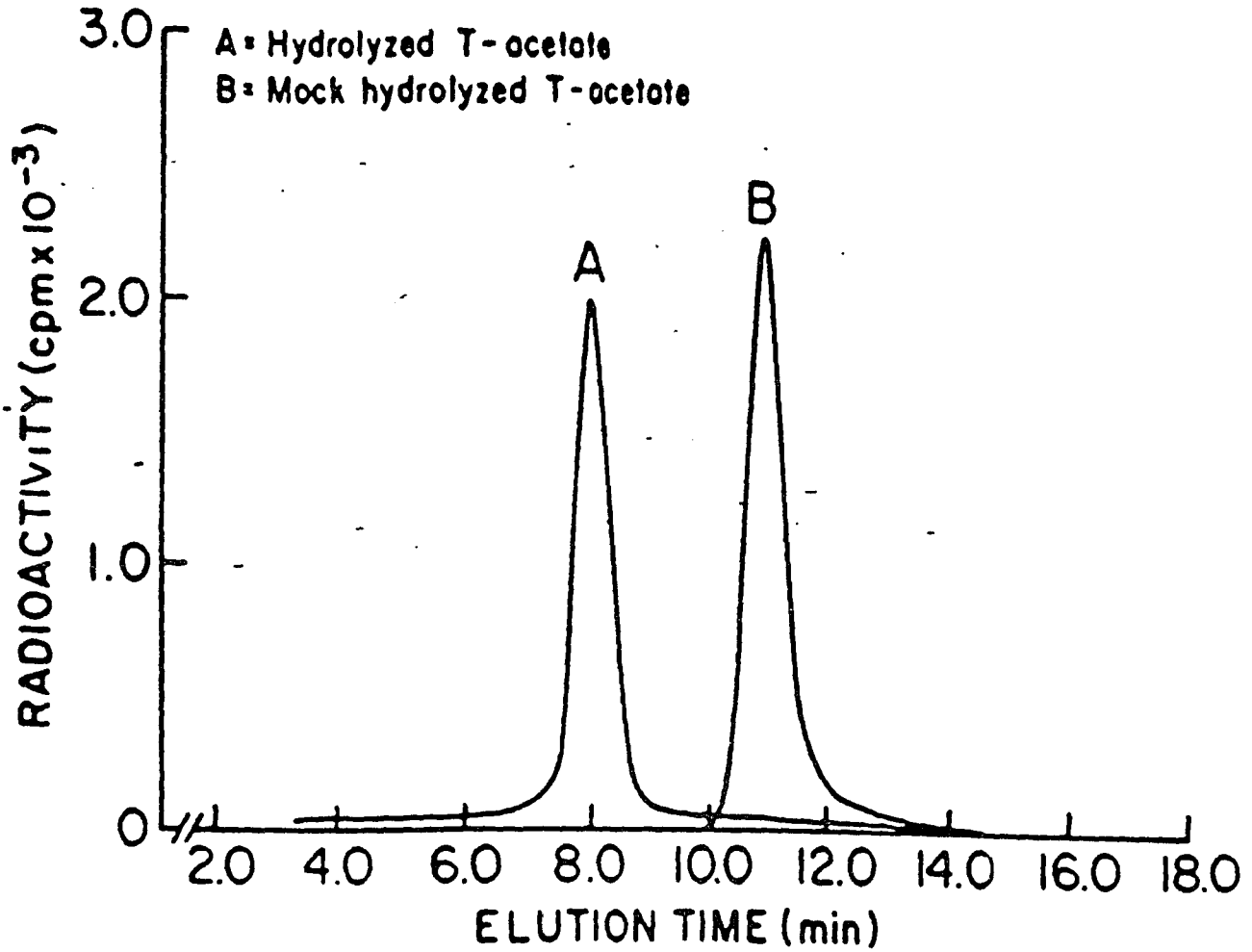

FIGURE 3. Reversed phase HPLC of hydrolyzed and mock hydrolyzed [14-C]-testosterone acetate. Samples were eluted with acetonitrile : water (1:1).

Table 4b. Extraction of [14-C]T-pal from serum.

About 1000 cpm [14-C]T-pal were added to 4 ml of serum and extracted 3x with an equal volume of petroleum ether. After each extraction, 1 ml aliquots were counted in duplicate. The values given are Mean ± SD.

Extraction #	Counts (cpm)	% Recovery (cumulative)
1	504 ± 8	44.5
2	362 ± 14	76.4
3	218 ± 11	95.6

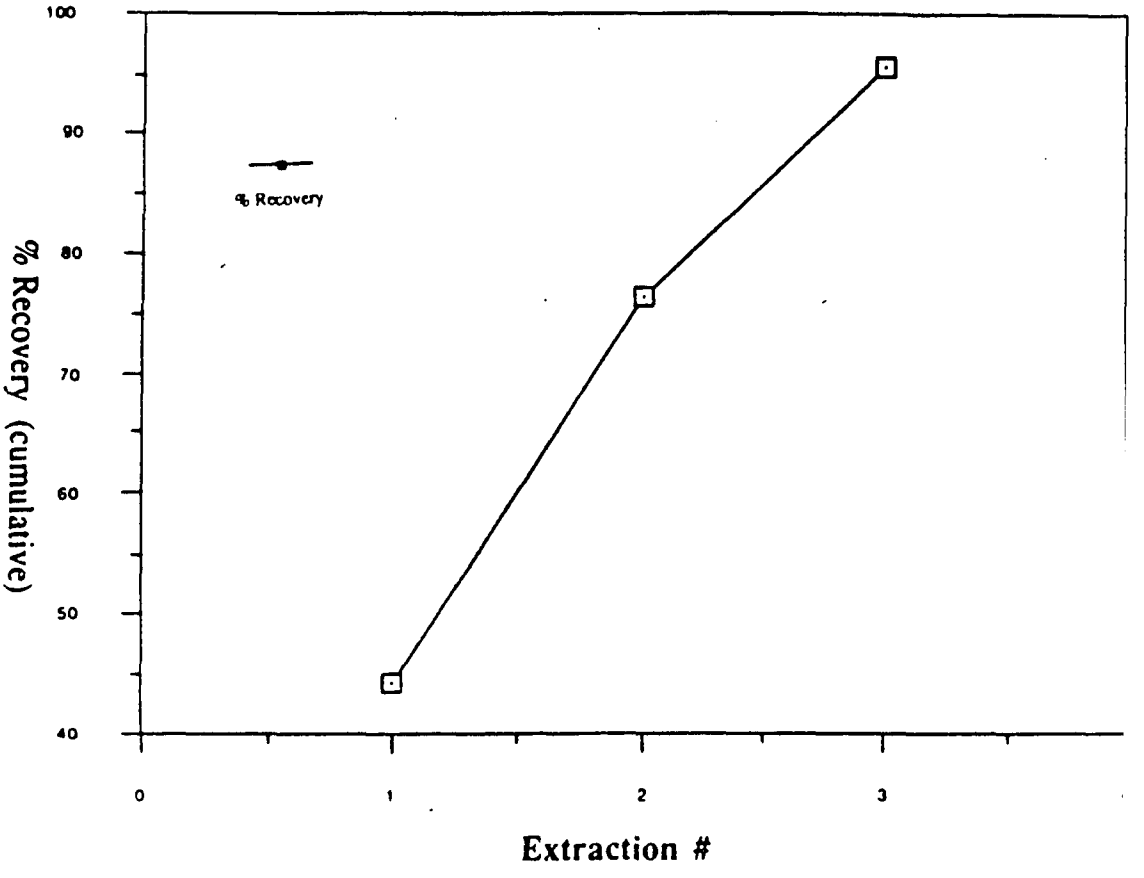

FIGURE 4. Extraction of [14-C]-T-pal with petroleum ether. Radioactive ester was added to 4 ml of serum then extracted three times with equal volumes of petroleum ether. Aliquots of each extract were counted on a Beckman liquid scintillation counter.

Table 5. Hydrolysis and Recovery of T-pal.

Known amounts of T-pal, with or without non-polar serum extracts, were hydrolyzed, purified by HPLC and fraction B assayed for testosterone by RIA. Fractions A and C showed no radioimmunoassayable testosterone. The values given are Mean ± SD. T-pal = Testosterone palmitate. NP = non-polar

	Hydrolyzed pg/tube	Expected pg/tube	% Recovery
NP. Extract	70 ± 3	-	
T-pal	284 ± 12	248	114
	159 ± 6	124	128
NP.+ T-pal	349 ± 16	318	109
	183 ± 11	159	115
Dist. Water	-	-	-

Table 6. Authentic testosterone added to fraction B of hydrolyzed non-polar serum extracts.

To assess whether a substance eluting in fraction B was causing artifactual increases in radioimmunoassayable testosterone, 200 pg of authentic T was added to aliquots of fraction B and assayed by RIA. NP= non-polar; T= testosterone

Sample	Mean T pg/tube	Expected T pg/tube	%Recovery.
NP.extract	385 ± 5	-	-
NP. + T	522 ± 16	585	89
Dist.H_2O	60 ± 30	-	-
H_2O + T	290 ± 31	260	111

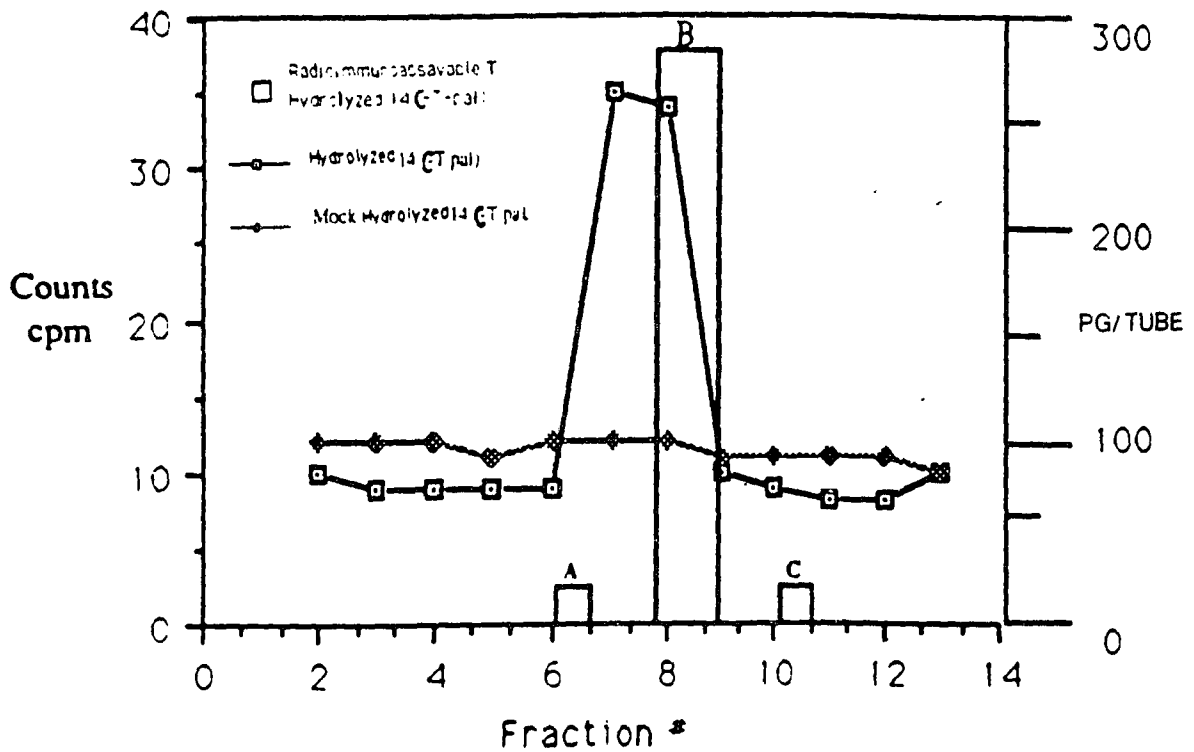

FIGURE 5. Hydrolysis of [14-C]-T-pal. After hydrolysis or mock hydrolysis, samples were purified by HPLC and the elution fractions were counted. Fractions A, B, and C were also assayed by RIA.

Table 7. Radioimmunoassayable testosterone in non-polar serum extracts from ten males and ten females. #1-10 - Males # 11-20 -Females

Subject #	Mean Testosterone ng/ml ± SD
1	1.8 ± 0.3
2	3.6 ± 0.8
3	1.5 ± 0.1
4	1.9 ± 0.5
5	1.6 ± 0.1
6	1.4 ± 0.2
7	1.4 ± 0.1
8	1.1 ± 0.2
9	3.5 ± 0.5
10	1.8 ± 0.4
11	0.6 ± 0.4
12	0.8 ± 0.1
13- 20	No detection

V. DISCUSSION

It has been known for many years that blood plasma contains sulfate and glucuronate androgen conjugates. Such molecules are neither extractable with petroleum ether nor hydrolyzable with mild alkali. In this study, non-polar testosterone derivatives were extracted with petroleum ether and subjected to mild alkaline hydrolysis. Appropriate fractions of the hydrolysates were then purified with HPLC, and an antibody specific for testosterone was used for the radioimmunoassay. Preliminary studies revealed that the antibody did not detect testosterone esters even at levels of 2.0 ng per assay tube. Petroleum ether extracted less than 8% of added radioactive testosterone from serum, thus eliminating the interference from free testosterone.

Approximately 90 % of added [14-C]-T-pal was recovered when the serum was extracted three times with equal volumes of petroleum ether. A better recovery assessment can be expected when the exact nature of the non-polar form of testosterone which yields increased radioimmunoassayable testosterone on alkaline hydrolysis is determined by mass spectrometry.

In very early experiments, non-polar serum extracts were hydrolyzed and assayed by RIA without prior HPLC purification. A high "blank" was obtained for mock

hydrolyzed samples. I therefore reasoned that introduction of HPLC purification could eliminate substances that would otherwise interfere with the RIA.

Hydrolysis of T-pal and/or non-polar serum extract.

T-pal was used as the model non-polar form of testosterone. It was synthesized by reacting palmitic anhydride with testosterone. The yield was about 50%, and unreacted testosterone was removed by washing with 50% aqueous methanol. Further resolutive separation was achieved by reverse-phase HPLC. After HPLC purification, T-pal migrated as a single spot with an Rf of 0.8 on polygram SIL G/uv 254 TLC plates, as described in the Materials and Methods section. Its molar extinction coefficient was assumed to be the same as that for testosterone (T), since both T and T-pal showed absorption spectra with peaks at around 240 nm. These wavelengths are characteristic for compounds with alpha/beta keto unsaturation in the A-ring of the steroid nucleus. The fatty acid moiety did not have any apparent effect on the absorption spectrum.

When T-pal was hydrolyzed alone, more than 90% was recovered as testosterone in the predetermined testosterone elution fraction. Adjacent fractions did not show any detectable testosterone. More than 95% of known amounts of T-pal added to non-polar serum extracts were similarly recoverable in the appropriate elution fraction (fraction B). Mock

hydrolyzed samples however, did not show any detectable testosterone in fraction B. Recoveries above 100% are attributed to small errors in the estimation of expected quantities from spectrophotometric determinations.

"Hydrolysis" of Testosterone

To ascertain whether the experimental procedures caused artifactual increases in radioimmunoassayable testosterone, known amounts of authentic testosterone were "hydrolyzed" or "mock hydrolyzed". Recoveries of 90% and 75% obtained for "mock hydrolyzed" and "hydrolyzed" samples, respectively, demonstrate that the testosterone detected in fraction B was not due to an artifact. Alkali is known to cause enolization of the alpha-, beta- keto unsaturation in the A ring of the steroid nucleus. This might explain the lower recovery of "hydrolyzed" testosterone. All samples were therefore hydrolyzed or mock hydrolyzed in an atmosphere of nitrogen.

Radioimmunoassay of known amounts of testosterone added to fraction B .

To show that hydrolysis of non-polar serum extracts or subsequent purification did not produce substances eluting in the fraction of interest (B) which could affect the antibody and thereby lead to overestimation of radioimmunoassayable testosterone, known amounts of authentic testosterone were added to fraction B of

hydrolyzed samples. Recoveries of about 95% establish the authenticity of measured radioimmunoassayable testosterone in hydrolyzed non-polar serum extracts.

Non-polar serum extracts.

Hochberg et al. (15) showed that mild alkaline hydrolysis released free steroids from the estradiol fatty acid conjugate. I therefore reasoned that if testosterone fatty acid conjugates are present in human serum, they should be extractable with a non-polar solvent, and hydrolyzable by mild alkali to release the free steroid.

It was observed that petroleum ether extracted more than 70% of [14-C]-T-pal added to serum after two equal-volume extractions. Petroleum ether was therefore a suitable solvent for the procedure.

The HPLC system used clearly separated testosterone from its fatty acid esters. It was reasoned that if the pre-testosterone, testosterone, and post-testosterone elution fractions were assayed for testosterone by RIA, only the testosterone fraction should show detectable testosterone. The observation that only the testosterone elution fraction of hydrolyzed non-polar serum extracts reacted with the specific antibody, supports the assertion that non-polar form(s) of testosterone occur in serum from human males. More than 90% of authentic testosterone added to fraction B from hydrolyzed non-polar serum extracts was recoverable.

Recoveries much greater than 100% would have indicated that the measured testosterone was probably due to an artifact.

The results obtained indicated a mean of 2.0 $\pm$ 0.8 (SD) ng/ml of testosterone, released after alkaline hydrolysis, in male volunteers 20-40 years old, whereas 8 out of 10 female samples had no detectable testosterone. Two females showed levels much lower than the mean for males. It is likely that levels in males and females vary from one individual to another.

Polar steroids have been known to occur naturally in the blood of humans, and their levels have often been used to assess endocrine status. It is only recently that non-polar steroid conjugates, which occur naturally in human plasma and other tissues, have been described (15). Hochberg et al showed that estradiol fatty acid esters occur in the blood of some women treated with human menopausal gonadotropin (HMG). Estradiol fatty acid esters have since been characterized, and it was determined that conjugation of the fatty acid moiety occurs exclusively at the C-17 position (16,17).

Similar to synthetic estradiol esters, the naturally occurring forms were found to be long-acting (18) and to exert their estrogenic effects only after endogenous hydrolysis by enzymes (22). Solo et al.(23), however, proposed the possibility that the esters interact directly with receptors.

The enzymes, Acyl CoA/acyl transferases, which enhance conjugation of fatty acid moieties to steroids, are also known to occur in microsomal fractions of cell homogenates (29). The possible reaction steps would be:

a. Reaction of a fatty acid with ATP to form an acyl adenylate derivative and pyro-phosphate (PPi).

b. Fatty acyl adenylate reaction with coenzyme A to form fatty acyl-CoA and adenosine monophosphate (AMP) Both a and b are catalyzed by a thiokinase.

c. Reaction of the fatty acyl-CoA with the steroid (R"-OH) to form the fatty acid conjugate of the steroid and coenzyme A, the reaction being catalyzed by an acyl transferase.

a. $R\text{-}COOH + ATP \longrightarrow RCO\text{-}AMP + PPi$

b. $RCO\text{-}AMP + HS\text{-}CoA \longrightarrow RCO\text{-}S\text{-}CoA + AMP$

c. $RCO\text{-}S\text{-}CoA + R"OH \longrightarrow RCO\text{-}OR" + HS\text{-}CoA.$

Mooradian et al. (12) have recently reported that physiologic levels of fatty acids such as palmitic and linoleic inhibit the binding of testosterone to sex hormone

binding globulin (SHBG) and albumins. In males, about 70% of available testosterone is bound to SHBG, which has high affinity not only for testosterone, but also for estradiol and other steroids with 17-B-OH groups. Since the OH-group at the C-17 position of the steroid is essential for both binding to SHBG and conjugation to fatty acid moieties (16,17), it can be speculated that the observation by Mooradian et al. is probably explained by the formation of fatty acid conjugates of testosterone. Martyn et al. have reported on the properties of fatty acyl CoA /estradiol-17 acyltransferase in bovine placenta (26). They asserted that testosterone, androstenediol, and dehydroepiandrosterone are also substrates for the enzyme.

Long-chain fatty acid conjugates of ecdysone have been isolated from invertebrates such as the cattle tick, Boophilus microplus (30), the adult female cricket Gryllus bimaculatus (31), and the house cricket Acheta domestica (32). They may also exist in other classes of arthropods (33). Endogenous conjugation of long-chain fatty acids to steroids may thus be more widespread than hitherto suspected.

NPT may (i) act physiologically to provide some androgenic activity where there is diminished of gonadal output or (ii) contribute to abnormal stimulation of androgen-responsive tissues. If this hypothesis is valid, NPT could have clinical implications, especially for hormone

therapy of prostatic cancer. In a study by Baulieu et al. (34) on the metabolism of testosterone and the action of metabolites on rat prostate glands grown in organ culture, release of large amounts of DHT and androstanediol in the medium are described. In the nuclei however, they detected DHT and an unidentified non-polar component. If this unidentified non-polar component is the same as the NPT herein described, there are at least two implications: (a) the prostate is able to make NPT. (b) NPT may be capable of acting directly in the nucleus, without prior hydrolysis. NPT may therefore affect prostate gland functions.

Prostatic cancer is very common in males 50 years of age and older. Unfortunately, it is often detected after metastasis has occurred. Some preliminary evidence that NPT is present in prostatic cancer patients under androgen-suppression therapy has been obtained. Six patients show higher levels of NPT than normal males. A study of NPT levels in several age-groups of men may provide information regarding mechanisms involved in the onset of prostatic cancer. NPT may also contribute to relapses in treated patients. Prostatic cancer usually responds to hormonal therapy for about 2 years, and then relapses. The level of NPT detected in six prostatic cancer patients (2.9 ng/ml) far exceeds DHT levels (0.5 ng/ml). NPT may therefore play some covert role in prostatic cancer relapses. It is possible that endogenous esterases hydrolyze NPT to release

free testosterone. The free testosterone could then be reduced to DHT by 5-αreductase. Hitherto, long-acting GnRH analogs and anti-androgens such as flutamide have been used in combination to achieve castrate levels of testosterone. The GnRH analogs cause eventual desensitization and down-regulation of testicular receptors for luteinizing hormone (35), whereas anti-androgens annul the effects of remaining free testosterone. I speculate that controlling NPT levels during prostatic cancer treatment could be beneficial.

This dissertation describes the existence of a nonpolar form of testosterone (NPT) in the serum of males. More information on the characterization, distribution in men and other species, biosynthesis, and physiology of NPT is needed.

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