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Metabolism of testosterone by tissue enzymes

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Thesis

METABOLISM OF TESTOSTERONE
BY TISSUE ENZYMES

By

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INTRODUCTION

Until recently relatively little work had been done on the steroid biochemistry of the human skin. Most of the biochemical information now at hand has been obtained through gross and microscopic examination. Various staining methods have been used to study cytological details. Histochemical methods have also given much information on enzymes and their reactions. It has become increasingly evident, however, that only by using many different methods of investigation can we hope to obtain a true picture of the skin and its importance. The purpose of this study has been to elaborate further the metabolism of testosterone by human skin.

For future orientation a brief summary of some of the anatomical physiology of the skin is necessary. The skin is the largest and one of the more complicated organs in the body. Its functions are many and varied. It serves as a protection for the body, receives sensory impulses from the outside, secretes and excretes various substances, and acts as a temperature regulator.

The skin covers the surface of the body and consists of three portions. The outer portion of skin is called the epidermis and the underlying layer of dense connective tissue, the dermis. Beneath the dermis is the subcutaneous layer or looser connective tissue which in many places of

the body is transformed into the subcutaneous fatty tissue. The boundary between the epidermis and the dermis is easily defined. However, fibers pass back and forth from the dermis to the subcutaneous layer making sharp histological division impossible.

The epidermis is composed of stratified squamous epithelium and is fairly thin on most of the body except for the palms and soles. There are generally two portions; the external portion or stratum corneum, which is keratinized, and the inner portion or stratum Malpighii. The entire epidermis is devoid of blood vessels and is nourished by tissue fluids penetrating into the intercellular spaces of the Malpighian layer from the underlying connective tissue.

The dermis may also be divided into two portions. The surface or papillary layer projects into the epidermis, whereas the deeper dense portion of the dermis is known as the reticular layer. A division between the two layers can not be clearly defined since they are composed of interwinding elastic fibers and bundles of collagenous fibers which are condensed about the hair follicles and the sweat and sebaceous glands. The dermal appendages, i.e., hair follicles and various glands are found in the tissue under study and may be concerned with steroid metabolism.

The hair is formed by an invagination of the epidermis. The lower terminus of the hair root is expanded forming the hair bulb, which contains the generative matrix of the hair.

It has been suggested that the cyclic activity of the hair follicle may be modified by hormones. Also, that all hormones with androgenic activity regardless of their point of origin have a stimulating effect on the germinative epithelium (27).

The sebaceous or "oil" glands are scattered over the surface of the skin except in the palms and soles. Rounded sacs or alveoli are the secretory portions of the sebaceous glands. Generally several alveoli form a mass like a bunch of grapes and all of them open into a short excretory duct. Stratified squamous epithelium lines these excretory ducts of the sebaceous glands and is continuous with that of the hair follicle. Thus, the oily secretion is excreted either onto the hair or directly upon the surface of the external skin. These glands tend to increase in size under androgenic stimulation (27). The sebaceous gland is almost associated as a functionally integrated unit with a hair, the entire structure being called a pilo-sebaceous unit.

The eccrine sweat glands are distributed in the skin of almost the entire body. They are simple coiled tubular glands. There is the ductal portion composed of a double layered epithelial tube and a single layered tortuous secretory portion around which myoepithelial cells are present. The sweat delivered to the surface of the skin varies with the site and the general condition of the body.

The potentiality of human skin as a medium for the study of steroid metabolism was first indicated by Wotiz et al (54). From in vitro studies of the metabolism of testosterone by human tissue slices they concluded that the two tissues with outstanding ability to metabolize testosterone were breast cancer and normal skin. Breast cancer was found to metabolize from 6.0 to 30 uM of testosterone per mg. of DNA and normal skin, on an average, 9.0 uM. Δ^4 -androstene-3,17-dione was indicated as the major metabolic product of testosterone oxidation.

Further in vitro studies were carried out by Wotiz, Mescon and co-workers (55) on human skin. They found that adjacent biopsies contained similar amounts of DNA and exhibited similar rates of metabolism. Of the three sites studied: back, scalp, and axilla, the highest steroid metabolic activity was observed in the skin of the back and the highest DNA content in the skin of the scalp. The existence of about six other Zimmermann positive substances in addition to testosterone and Δ^4 -androstene-3,17-dione was demonstrated in this same group of experiments by means of paper chromatography and radio-autography.

In the original approach to steroid-enzyme relationships the investigators (16) postulated that steroid hormones exerted their action by influencing enzyme systems. It was felt that they might: 1) influence the concentration of enzyme systems, 2) function as components of enzyme

systems as in a protein steroid complex where the steroid acted as the prosthetic group, 3) act as accelerators or inhibitors of the enzyme reactions, directly or indirectly. At present it is felt that the steroids are important in themselves and, conversely, are acted upon by enzymes. When one looks at the problem of testosterone breakdown from both aspects it is impossible to tell whether, in the vast network of metabolic actions, it is of major or minor importance.

Primary studies of testosterone metabolism were carried out in vivo and the urine analyzed for breakdown products. The actual reactions which occur in intermediary testosterone metabolism are only imperfectly understood. Undoubtedly many of the intermediary compounds were absorbed into the bloodstream and tissues as steroid protein complexes. Others, unable to pass through in the glomerular filtrate until further catabolism occurred, can only be suggested.

In vitro experiments, where a known amount of testosterone was added to skin as substrate, may be felt to give a true picture of testosterone breakdown products per se. However, actual in vivo metabolism might be quite different. Due to the minute amount of steroid metabolized by skin actual quantitative measurement and identification of all but the major breakdown product has not been attempted. Their existence can only be postulated through paper chromatography and radio-autography. Also, if in the future testoe-

terone or other active androgenic compounds should be found to be synthesized by the skin, the quantities produced might be too minute to be detected by our present assay method.

HISTORY

The study of the steroid hormones is comparatively new. Much of the pioneer work on androgens was done by F.C. Koch (22) and his associates. In 1927 McGee (26) first clearly demonstrated androgenic material in lipid extract of bull testis.

The first pure crystalline androgen, androsterone, was obtained by Butenandt and Tscherning (7) from men's urine following the report by Loewe in 1928 of the presence of an androgenic material in this source (24). A second crystalline androgen was isolated from urine by Butenandt and Donnerbaum (5) in 1934, which became known as dehydro-epiandrosterone.

The primary site of androgen synthesis is believed to be the interstitial tissue of the testis. Androgens have also been reported present in the adrenal (34,35,36), ovary (10,30), placenta (13), anterior pituitary (32), and epididymis (19). Laqueur and his associates isolated the bull testis androgen in 1935 and named it testosterone (14). This compound was also isolated from stallion testis tissue (48), and was indicated in the boar testis (33). Several other androgens and related compounds were isolated from testicular tissue.

Immediately following its isolation, Ruzicka and Wettstein (38) and Butenandt and Hanisch (6) synthesized testosterone giving proof of its structure.

Early knowledge of the metabolism of androgens by tissues was obtained by analysis of urinary excretion products. Dorfman and Hamilton (17), Callow et al. (9), Schiller et al. (42), and Hoskins et al. (20) administered testosterone orally or intramuscularly and found that the increase in androgen content of the urine was directly proportional to the administered steroid.

In 1939 Callow (8) and Dorfman, Cook, and Hamilton (17) found through isolation procedures that the main portion of the administered testosterone was recovered as androsterone in the urine. Other metabolites of testosterone also isolated were the two stereoisomers etiocholan-3(α)-ol-17-one and epiandrosterone. In one case the androgen androstane-3(α),17(α)-diol was also isolated (42).

From the study of steroid compounds in the urine, epiandrosterone, etiocholan-3(α)-ol-17, androsterone, and possibly etiocholan-3(β)-ol-17-one, have been indicated as probably metabolic end products in testosterone metabolism (31).

Experiments performed by Block (2) indicated that cholesterol is a probable precursor of steroid hormones, and Brady (3) in 1951 showed further that tissue slices from hog, rabbit, and human testis can convert labelled acetate to testosterone. These latter experiments also showed that the labelling in testosterone was actually higher than that of cholesterol indicating that cholesterol

may not be the primary intermediate in the biosynthesis of testosterone. Lastly, Savard et al. (41) by perfusion studies of 1-C¹⁴-acetate through human testis showed the production of C¹⁴-labelled testosterone and Δ^4 -androstene-3,17-dione.

In 1947 it was observed by Samuels et al. (39) that the livers of rat, mouse, rabbit and human destroyed testosterone. Clark and Kochakian (11) were the first to isolate Δ^4 -androstene-3,17-dione and cis-testosterone, as well as to indicate the presence of small amounts of other unidentified steroids from the incubation of testosterone with rabbit liver slices. This work also showed that Δ^4 -androstene-3,17-dione could be reduced to testosterone, suggesting the reversibility of the reaction.

West and Samuels (51) in 1951 demonstrated catabolism of testosterone in kidney tissue from dog, rabbit, and guinea pig. The primary enzyme action was found to be that of oxidizing the hydroxyl group on C-17 to a ketone forming Δ^4 -androstene-3,17-dione. The co-enzyme, diphosphopyridinenucleotide (DPN) was active in both the dog and guinea pig systems.

Studies by Sweat et al. (46) suggested that DPN and citrate are involved in the metabolism of testosterone by liver. The products of the reaction in the presence of DPN are largely 17-ketosteroids, whereas those in the presence of citrate are not. Finally, in 1950 Sweat et al.(47)

succeeded in isolating an enzyme from steer liver which oxidizes testosterone to Δ^4 -androstene-3,17-dione. When the co-enzyme DPN was added to the rat liver since the amount of testosterone destruction and 17-ketosteroid formation increased. This catabolic picture was further accentuated by adding nicotinamide as a diphosphopyridine nucleotidase inhibitor. The addition of citrate to the mince increased testosterone destruction but without the appearance of 17-ketosteroids. Ofner (29) using rat liver and a number of micro procedures indicated a 20 per cent conversion of the substrate to Δ^4 -androstene-3,17-dione. It is of interest that from a species of *Pseudomonas* grown on testosterone Talalay and Dobson (49) demonstrated and purified a DPN-linked dehydrogenase which is capable of interconverting certain 3β - and 17β -hydroxysteroids and their respective ketosteroids.

Wotiz and Lemon (53) found a catabolic enzyme present in human prostatic tissue capable of metabolizing testosterone to Δ^4 -androstene-3,17-dione and eight other unidentified substances. Further studies by these authors (54) showed testosterone to be metabolized by normal human tissues, such as: prostate, endometrium, myometrium, intestine, smooth muscle, liver, skin, and breast, as well as malignant human tissues of the breast, prostate, bladder and cervix. The malignant tissue in each case showed greater metabolic activity of testosterone when

compared with its normal counterpart. Relatively high values for malignant breast tissue and normal skin were suggested. Some correlation was also shown between the disappearance of testosterone and the formation Δ^4 -androstene-3,17-dione. When prostatic tissue was incubated with testosterone there was an 11 per cent recovery of Δ^4 -androstene-3,17-dione. Conversely, incubation of prostatic tissue with Δ^4 -androstene-3,17-dione showed a 9 per cent recovery of testosterone, presenting evidence for the reversibility of the reaction.

In vitro studies by Wotiz, Richterich-van Baerle, and Lemon (56) on blood serum showed the presence of an active testosterone-metabolizing enzyme system. Boiling the serum prior to incubation led to inactivation of the enzyme. A decrease in testosterone recovery with an increase in serum concentration was also observed. These facts led them to assume that a steroid protein complex had been formed. Extraction of testosterone immediately after its addition to serum and before incubation yielded only a 60 per cent recovery of the steroid, giving further evidence that a great part of the testosterone loss was not due to enzyme activity. Two metabolic products of testosterone were observed on paper chromatograms of the serum extracts. One was an unknown compound intermediary in position to testosterone and Δ^4 -androstene-3,17-dione and the other was identified as Δ^4 -androstene-3,17-dione.

In the late eighteenth hundreds Schulze (43) isolated a substance from wool fat which he regarded as closely related to cholesterol and he called it "ischolesterol". In 1930 Windaus and Tschesche (52) isolated two substances from "pure" ischolesterol: (a) lanosterol, and (b) agnosterol. Lanosterol constitutes about 92 per cent and agnosterol about 8 per cent of ischolesterol. Extensive chemical work has now definitely established that lanosterol has the same ring structure as cholesterol (37).

In 1941 Dimiter (15) showed that squalene was produced by human skin, and recently the presence of squalene was demonstrated in normal skin surface fats of humans. Finally, evidence was presented by Langdon and Block (23) that squalene is converted to cholesterol by the animal organism. In fact, the two tissues which apparently are most concerned with cholesterol synthesis are the liver and the skin. It had previously been indicated by Block that cholesterol was a probable precursor of the steroid hormones. However, no active androgenic compound has yet been demonstrated to be synthesized in the skin.

EXPERIMENTAL

A. METHODS

(1) Biopsy Taking

Skin biopsies were taken from the backs of normal male volunteers. A sterile 5 or 7 mm. electric punch was used to excise the tissue rapidly. No anesthesia was employed.

(2) Incubation

The tissues were scored with a razor to allow a maximum surface for the reaction. They were incubated immediately in 20. ml. of Krebs-Ringer phosphate buffer at pH 7.4 made up as follows:

4.5% NaCl	50 ml.
5.75% KCl	2 ml.
6.1% CaCl ₂	0.5 ml.
19.1% MgSO ₄	0.5 ml.
distilled H ₂ O	202.0 ml.
Na ₂ HPO ₄ buffer	52.5 ml.
(brought to pH 7.4 with 0.1 N HCl)	
Glucose	0.317 gm.
Nicotinamide	0.600 gm.

Five mg. of diphosphopyridine nucleotide, 20,000 units of penicillin-G and 1 mg. of testosterone dissolved in 0.1 ml. of absolute ethanol also were added to each incubation flask. For radio-autograms $4C^{14}$ -testosterone was used.

Incubation times varied from three to eighteen hours. After removal from the incubator the mixture was extracted immediately or deep frozen for later extraction.

(3) Extraction

The tissue was extracted three times with 20 ml. of acetone and the supernatant three times with 20 ml. of ether. The ether and acetone extracts were combined, evaporated to dryness, and taken up in 50 ml. of chloroform. The chloroform was washed twice with 20 ml. of dilute 0.2 N acetic acid (diluted 1-4 with distilled water), once with 20 ml. of 1 per cent sodium bicarbonate at pH 8, and twice with 20 ml. of distilled water (the resulting solution was at pH 7). The chloroform was then evaporated to dryness and the steroid recovered overnight in 50 ml. of 70 per cent methanol.

The 50 ml. of methanol were partitioned three times with 20 ml. of n-hexane to remove lipids. The n-hexane fractions were combined and back extracted three times with 20 ml. of 70 per cent methanol. The methanol fractions were combined and taken to dryness. The residue was kept in a dry state until it was chromatographed.

(4) Chromatography

Paper chromatography was carried out according to the method of Savard (40). Whatman #1 filter paper previously refluxed with alcohol and benzene was used. The paper was cut into 1 x 45 and 3 x 45 mm. strips allowing paper for a

common attachment at the top. The strips were dipped in a 50 per cent mixture of propylene glycol and methanol and the excess removed by blotting.

The steroid extract dissolved in methanol was applied to the top of the strips already impregnated with the stationary phase (propylene glycol) and then the steroid was blown dry with nitrogen. Generally 0.1 ml. of the extract was applied to each 1 cm. strip; however, the amount varied with the concentration of the steroid to be chromatographed and eluted.

The common base of the paper was placed between two glass plates. These plates were placed in a tray containing ligroin (the moving phase) saturated with propylene glycol vapors. The strips were removed from the tank and quickly examined under ultraviolet light for the presence of the darker appearing α, β -unsaturated ketosteroid zones against the fluorescing paper background. After drying in an oven the strips were stained (40) by immersing them in a 2.5 N solution of potassium hydroxide in ethanol and then were blotted between filter paper to remove the excess reagent. Following this, the strips were reimmersed in an ethanolic solution of *m*-dinitrobenzene, again blotted and heated at 75°C for 1-2 minutes to develop the color. Testosterone (a C-3-ketone) gave a blue zone on the chromatograms whereas Δ^4 -androstene-3,17-dione (a C-3,17-diketone) exhibited a pinkish violet color.

(5) Quantitative Determination

All steroid samples were chromatographed in triplicate on 1 cm. strips. One of the strips was stained by a modification of the Zimmermann reaction to ascertain the position of testosterone, its main metabolite, Δ^4 -androstene-3,17-dione, and other ketonic catabolites. Only testosterone and Δ^4 -androstene-3,17-dione were eluted from the strips. The eluates were quantitatively determined in a Beckman DU spectrophotometer at 240 m μ . A reference testosterone solution was established for each experiment and used to calculate the steroid concentrations.

(6) Radioautography

The radioactive testosterone obtained from New England Nuclear Corp. was characterized as follows: 101 Testosterone-4-C¹⁴, Lot. No. 10-67-1. Radioactivity, 10 μ c; weight, 2.42 mg.; specific activity, 4.13 μ c/mg. or 9.1×10^6 cpm/mg. The radioactive testosterone was carefully dissolved in a 10 ml. volumetric flask with benzene. To each experimental flask was added 1.0 ml. of a steroid solution containing 1.00 mg. of testosterone and 0.01 mg. of radioactive testosterone.

The procedure outlined under section 5 was repeated using 3 cm. paper strips. Instead of eluting the steroid the dried paper chromatographs were placed in an X-ray cassette and covered with a sheet of Kodak No-screen X-ray film. The film was exposed to the chromatogram for

2 and 5 week periods before development.

(7) Thiosemicarbazide Determination (4).

In identification of Δ^4 -androstene-3,17-dione as a metabolite of testosterone the chromatographically isolated steroid was reacted with thiosemicarbazide. The eluted steroid samples were first dissolved in 0.1 ml. of 95 Per cent ethanol and then 0.1 ml. of 95 per cent ethanol was placed in the reagent blank tubes. One-tenth of a ml. of reagent (5 mg. recrystallized thiosemicarbazide per ml. from 5 per cent acetic acid in methanol, which had been distilled from 2,4-dinitrophenylhydrazine) was added to the tubes and heated for 20 minutes in a steam bath. Five ml. of chloroform and 5 ml. of 0.1 M hydrochloric acid were added and the tube was shaken. The hydrochloric acid layer was removed from the top by pipetting and the chloroform again was extracted once with 5 ml. of 0.1 N hydrochloric acid and twice with 5 ml. of distilled water. The chloroform layer was evaporated to dryness in vacuo and redissolved in methanol. A spectrophotometric curve of the eluted sample was determined and compared with that of a known sample of the authentic steroid.

(8) Sulfuric Acid Test

For further identification of the testosterone metabolite steroid extracts from several experiments showing evidence of Δ^4 -androstene-3,17-dione production were pooled

and chromatographed. After elution from the paper the diketone was evaporated to dryness. Five ml. of sulfuric acid was added to the dried eluate and a curve from the degradation of the steroid was obtained every 5 minutes for one half hour on a DK 2 spectrophotometer. Curves from an authentic sample of Δ^4 -androstene-3,17-dione and the eluate were similar. The maximum and minimum values for Δ^4 -androstene-3,17-dione with this method had been reported to be 294 and 105 respectively (1).

(9) Adaptation of the "Thunberg Technique for Estimation of Dehydrogenase Activity" (50)

Using a Warburg apparatus a simple closed air system was established and the rate of O_2 uptake was measured instead of the reduction of methylene blue. The flask itself contained 0.3 ml. of 0.0027 M methylene blue, which acted as the hydrogen acceptor, 0.3 ml. of 0.5 per cent diphosphopyridine nucleotide, 0.3 ml. of 0.05 M NaCN to inhibit the cytochrome system, varying amounts of 10 per cent rat liver homogenate in M/15 pyrophosphate buffer at pH 8.2, and distilled water. The center well contained 0.2 ml. of 20 per cent KOH on a piece of folded filter paper to absorb any CO_2 produced. Lastly, the side arm contained 0.2 ml. of testosterone solution (1 mg./ml. absolute ethanol) or 0.2 ml. of absolute ethanol as control.

The flasks were closed and allowed to come to equi-

librium at 37.5°C for about 15 minutes. After the manometers were read, the contents of the side arm were carefully tipped into the flasks and the readings were recorded every ten minutes.

Calculations for the oxygen uptake found in tissue metabolism of testosterone by the modified "Thunberg Technique".

Calibration of Flasks and Manometers

Flask #	Wt. Hg.	Total wt.	T C	Density Hg.	$\frac{W}{D} = V$	Total Volume
13 M	10.369		24	13.5364	0.766	
" F	256.7450	267.1140	23	13.5389	18.963	19.7291
1 M	11.3400		24	13.5364	0.838	
" F	235.2670	246.6070	29	13.5242	17.396	18.234
8 M	9.0578		24	13.5364	0.669	
" F	246.0062	255.0640	26	13.5315	18.180	18.849
16 M	10.3320		24	13.5364	0.763	
" F	252.6086	262.9406	26	13.5315	18.668	19.431
39 M	11.0220		24	13.5364	0.814	
" F	226.6626	237.6846	24	13.5364	16.744	17.558
23 M	10.8444		24	13.5364	0.801	
(9)F	223.9448	234.7892	24	13.5364	16.544	17.345
82 M	9.6856		25	13.5340	0.716	
" F	250.3016	259.9872	27	13.5291	18.501	19.217
34 M	9.8633		25	13.5340	0.729	
" F	217.3354	227.1987	27	13.5291	16.064	16.793
10 M	10.1880		24	13.5364	0.752	
10 F	247.9986	258.1866	24	13.5364	18.322	19.074
19 M	10.8818		25	13.5340	0.803	

The calculations for the oxygen uptake by the contents of each experimental flask are described on the following page.

Calculation of Oxygen Uptake

The results for each experimental flask were calculated as follows:

change in mm. of manometer reading - thermo-barometer correction = actual change in mm. x flask constant = μ l. O₂ uptake

Determination of flask constant.

$$k = \text{flask constant} = \frac{Vg \frac{273}{T} + Vf\alpha}{P_o}$$

The values for flask (F) and manometer (M) #8 are presented.

Vf = volume of fluid in vessel = 3.35 ml. = 3,350 μ l

α = solubility in liquid of O₂ (gas/ml. liquid, Brodies fluid sp.gr. 1.034, at 760 mm. Hg and at temperature T, 25 = 0.028

Vg = volume of gas phase in flask = total volume (18.849) - fluid volume (3.35)

T = 273 - 25 = 298

P_o = 760 mm. Hg expressed in terms of manometer fluid = 760 x 13.60 (sp.gr. of Hg) / sp.gr. of manometer fluid

k for flask (F) and manometer (M) #8 = 1.448

The oxygen uptake from duplicate flasks was averaged and reported as single values in the following tables (1 - 5).

B. RESULTS

(1) Modified Thunberg TechniqueA. The effect of variations in testosterone concentration

In the first set of experiments (Table 1) the 10 per cent rat liver homogenate (enzyme) concentration was varied from 1.0 ml. through 0.5 ml. to 0.25 ml.

Large male rats, weighing about 200 grams, were killed instantly by a blow on the head. The liver was immediately excised and weighed. Sufficient M/15 pyrophosphate buffer at pH 8.2 was added to make a 10 per cent (weight/volume) solution and the mixture was homogenized in a pre-cooled Waring blender for 5 minutes.

Equal volumes of testosterone solution (0.2 ml.) were added to each flask. The substrate was varied as follows:

- I,IV Twenty-five mg. testosterone dissolved in 25 ml. of absolute ethanol.
- II Five mg. testosterone in 10 ml. of distilled water exposed to a 10 KC ultra sonic vibrator for 30 minutes.
- III Five mg. testosterone in 10 ml. of distilled water exposed to a 10 KC ultra sonic vibrator for 1 hour.

Table 1

ml. homo- genate	$\mu\text{l O}_2$ Uptake			
	I	II	III	IV
1.0	434	6.9	no change	74
0.5	268	10.6	" "	137
0.25	154	11.3	" "	64
control 0.5	168	8.4	" "	115

It was felt that ethanol itself might affect the metabolism; therefore, an additional experiment was done using an alcohol control. A general rise in metabolism with increasing amounts of tissue was seen for the concentration from 0.5 ml. to 1.0 ml. but the concentration of 0.25 ml. shows the greatest metabolism of all (Table 2).

Table 2

ml. homo- genate	$\mu\text{l. O}_2$ Uptake		
	Total change - Control = T.R. (tissue respiration)		
1.0	101	65	36
0.75	100	65	35
0.50	108	78	30
0.25	117	75	45

B. The effect of co-enzyme variation on testosterone metabolism

The first experiments were carried out with the co-enzyme diphosphopyridine nucleotide (DPN), giving inconclusive results. Therefore, it seemed advisable to run simultaneous experiments with DPN and triphosphopyridine nucleotide. With DPN the lowest tissue concentration, 0.1 ml., gave a negative reaction, and the rest - 0.2, 0.3, and 0.4 ml. - showed an O_2 uptake which decreased with increasing tissue concentration. TPN showed no O_2 uptake (Table 3).

Table 3

ml. homogenate	0.1	0.2	0.3	0.4
μ l. O ₂ uptake with DPN	-29	+21	+9	+7
μ l. O ₂ uptake with TPN	-3	-3	-4	0

C. The influence of various solvents on testosterone metabolism

1. bovine albumin solution

Another method was sought for bringing the testosterone into solution to prevent obscuring of the tissue respiration by alcohol dehydrogenase activity.

A dilute solution of bovine albumin was prepared by diluting 3 ml. of a 35 per cent standard solution to 100 ml. with distilled water. Fifty mg. of testosterone were dissolved in 50 ml. of this dilute albumin solution. The solution was again diluted 1:50 with distilled water and its concentration was determined in the Beckman spectrophotometer. A second steroid albumin solution was prepared similarly but it was also shaken at 37.5°C overnight in the Warburg apparatus. The results were recorded (Table 4).

Table 4

Albumin solution	ml. homogenate								
	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	
μ l. O ₂ uptake 1.	-11	-15	+3	-17	+18	+3	+115	-1	
μ l. O ₂ uptake 2.	-5	+4	+2	-3	-14	+1	+9	+5	

2. Use of Tween 80 and Triton

Solutions of 1 per cent Tween 80 and 1 per cent Triton were incubated with testosterone at 37.5°C for about 20 hours. From spectrophotometric readings the concentration of the Tween 80 solution was determined to be 100 µg. testosterone per ml. and the Triton, 135 µg. per ml. Slight but not significant evidence of O₂ uptake was found in the two lowest tissue concentrations with Tween 80. All other flasks showed no change.

3. Utilization of a water soluble steroid preparation

Lastly, 10 mg. of testosterone-β-D-glucopyronuronoside (57) were dissolved in 10 ml. of hot distilled water. Again inconclusive results were obtained (Table 5).

Table 5

ml. homogenate	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8
µl. O ₂ uptake	+4.8	-9.2	+7.7	-3.9	-2.2	+1.3	+6.1	+4.6

(2) In Vitro Incubation Method

A. Verification of the method

As the manometric experiments gave variable results in relation to enzyme concentrations, it was decided to return to the original method of experimentation. For a check of the method a 10 per cent rat liver homogenate was used as the enzyme source. The homogenate was prepared as previously described for the "Thunber Technique" with

the exception of the buffer (Krebs-ringer phosphate buffer at pH 7.4).

A solution of testosterone was prepared by dissolving 50 mg. of testosterone in 50 ml. of absolute ethanol. One ml. of this solution was used in each flask. On doubling the tissue concentration consecutively the loss of substrate appeared to increase directly with increasing enzyme concentration (Table 6).

The tissue homogenate was incubated and extracted as previously described under Methods. One-tenth of the extract (100 micrograms of steroid) was chromatographed, in triplicate, on 1 cm. strips. Results of elution and quantitative determination on the Beckman spectrophotometer of two strips are seen in Table 6. The third chromatographic strip of each group was stained with the Zimmermann reagents. Reproductions of these stained strips were drawn and photographed (Figure 6).

B. A General experiment with human skin

A group of experiments were undertaken to check the previously reported testosterone metabolism of the skin (55).

Tissue samples were removed from the subject's back with a 5 mm. biopsy punch. Controls and experiments were carried out in duplicate. The control tissues were boiled in buffer for 20 minutes before adding steroid and DPN. One group of the control tissues was incubated for 3 hours

and the other deep frozen until ready for extraction. The results can be seen in Table 7. Corresponding chromatograph strips stained by the Zimmermann reagents are found in Figure 7.

The loss of testosterone by elution from paper was computed and found to be 19.8 per cent. Other preliminary experiments had shown about an average recovery of 85-90 per cent of the original steroid when eluted from paper. In this experiment, as in those following, the value of the eluted substrate was taken as 100 per cent recovery of testosterone.

Testosterone was incubated without tissue and there was a 9 per cent loss. The presence of an additional less polar compound was indicated by the appearance of a purple Zimmermann stain. It is probable that this was Δ^4 -androstene-3,17-dione. On incubation of tissue with substrate a 24.9 per cent loss of testosterone was observed. When the tissue was boiled 20 minutes before incubation a 12.7 per cent loss was found, giving a 3.7 per cent difference from the steroid incubated without tissue. This difference was believed to be due to incomplete destruction of the enzyme by insufficient boiling. However, when the tissue was boiled but not incubated after addition of testosterone a 28.4 per cent loss was encountered. It was felt that this represents an error in experimentation.

C. Variation in metabolism due to change in surface area

In this experiment, set up in the same manner as the preceding one, 7 mm. biopsies were used. Its purpose was to test for variation in enzyme activity due to change in surface area, and to increase the boiling time for the controls. The first set of flasks contained whole tissue; the second set of flasks, tissue scored through the epidermis with a scalpel; the third set of flasks, tissue which had been frozen and shaved very fine with a scalpel (partly homogenized). The control tissue was boiled for one hour and then cooled to room temperature before the testosterone and DPN were added. Results are recorded in Table 8.

On the chromatographic strips stained with the Zimmermann reagent (Figure 8) the same compounds appeared to be present in all of the incubated tissues: testosterone, polar compounds in small quantities, a compound with a travel rate of Δ^4 -androstene-3,17-dione, and an unknown compound running between this and testosterone. These compounds are also found to a much lesser degree in the testosterone incubated without tissue. Only in the unincubated testosterone control are they absent.

The quantity of tissue was doubled to 14 mm. and the incubation time increased to 4 hours using the same method of experimentation; all flasks were incubated and then immediately deep frozen. After processing and quantitative determination the results shown in Table 9 were obtained.

It would seem that no metabolism has occurred. As in the previous experiment, only testosterone appears on the stained chromatographed strips of the homogenized tissue (Figure 9). With incubated tissue the same picture was found: weak blue polar compounds, testosterone, an unknown violet staining compound, and a purple staining compound (probably Δ^4 -androstene-3,17-dione) running in order of decreasing polarity.

D. Effect of increasing amounts of tissue

Incubations were performed as described in the previous experiments and 7, 14 mm. biopsies of cut tissue were used. A fresh solution of DPN was prepared, as usual, just before incubation. Both testosterone and its major metabolite, the compound believed to be Δ^4 -androstene-3,17-dione, were eluted and quantitated by the spectrophotometer. The results are shown in Table 10.

It was seen that although the tissue concentration was doubled from 7 to 14 mm. little difference in substrate loss could be observed. At the same time, however, the metabolism of testosterone to Δ^4 -androstene-3,17-dione appeared to double.

Corresponding chromatographic strips obtained in the usual manner and stained by the Zimmermann reagents are found in Figure 10.

E. The determination of the catabolic products of radioactive testosterone

An experiment to check the metabolites of testos-

terone observed in previous studies with the Zimmermann stain was performed with radioactive testosterone. The steroid residue after extraction was evaporated to dryness and dissolved in 1 ml. of absolute ethanol. One-half of the ethanolic steroid solution was applied to 3 cm. chromatographic strips and developed for 20 hours. Upon drying the strips were placed in an X-ray cassette with a sheet of No-screen X-ray film. After an exposure period of five weeks the film was removed and developed. A photograph of the film is found in Figure 11A. At least 8 compounds, as reported by other authors (55), were seen.

For further determination of the breakdown products of testosterone an experiment with radioactive testosterone was run simultaneously with, and under the same conditions as, the other experiments. Quantitative recoveries of testosterone and Δ^4 -androstene-3,17-dione were determined by spectrophotometric measurements.

In the experiment two solutions of testosterone were mixed together so that .150 ml. contained 1 mg. testosterone and 0.01 mg. of radioactive testosterone. The specific activity per flask was 0.046×10^6 cpm/mg. The steroid-tissue mixture was incubated for 20 hours and the results were recorded in Table 11.

The chromatograph strips for each incubation mixture can be observed in Figure 11B, and corresponding radiograms are found in Figure 11C.

The eluate believed to be Δ^4 -androstene-3,17-dione was reacted with thiosemicarbazide. Curves computed from spectrophotometric readings showed the same peaks with maximum values at 270 mu and 300 mu for a known sample of Δ^4 -androstene-3,17-dione and the eluted compound (Figure 11D).

F. The effect of incubation on testosterone

Due to the presence of breakdown products found in the controls (incubated testosterone) it was decided that an experiment should be carried out in the usual manner but without the addition of skin. DPN was not used because of its instability. Twenty thousand units of penicillin-G were added to two flasks, two flasks were autoclaved before incubation (10 lbs., 230°C., 10 min.), and two flasks were incubated as in previous experiments. All but the autoclaved flasks showed some degradation. The chromatographic strips stained with the Zimmermann reagent indicated the presence of Δ^4 -androstene-3,17-dione and other compounds, as well as testosterone. It seemed that bacteria might be causing the metabolism (Figure 12A).

G. Investigation of testosterone metabolism due to bacteria

Three sets of flasks with the same basic components as in previous experiments were set up in duplicate. All flasks contained the same amount of testosterone (1 mg.) and 20,000 units of penicillin. Half of each set of flasks were incubated and immediately deep frozen:

the other half were cultured for bacteria after incubation.

The first set of flasks (containing tissue) were incubated immediately. The second set of flasks (no tissue) were autoclaved then incubated, and, lastly, the third set of flasks (no tissue) were incubated.

Before culturing, the flasks were well shaken. One milliliter of the incubation mixture was used for a pour plate with beef infusion agar. Sterile technique was utilized for preparation of the pour plates and the bacterial count obtained for all samples was zero. The remaining contents of the flasks were centrifuged and the supernatant decanted. The precipitate was used to inoculate blood agar plates and broth tubes. The tissue itself was placed on the blood agar plate.

No growth could be observed on the blood plates inoculated from the autoclaved flasks. A gram stain of the broth revealed nothing significant.

There was one colony on the blood plate from the incubated testosterone and a gram stain of the broth showed yeast and a few gram negative rods or amorphous material (Table 12).

There were many non-hemolytic bacteria along the streak and around the tissue of one of the blood agar plates with tissue; the other had none. The gram stain of the broth smear showed the presence of yeast and gram negative rods, some of which seemed to contain gram positive granules.

A culture of the skin bacteria was transplanted and kept viable for later reference

The other paired flasks were removed from the deep freeze, thawed, extracted, and the steroid recovery computed. Results of the entire experiment are summarized in Table 12 and the corresponding chromatographs are seen in Figure 12B.

A further experiment was carried out with the isolated skin bacteria alone. Three sets of incubation flasks containing testosterone were autoclaved for one-half hour at 10 pounds pressure and at 227°C. The first set of flasks were incubated immediately upon removal from the autoclave. Approximately one-fourth of a 5 mm. loopful of the skin microorganism Pseudomonas pyocyaneus was added to the second set of flasks before autoclaving and to the third set of flasks after autoclaving. All were incubated for 22 hours and produced the results found in Table 13.

These studies all presented evidence for bacterial metabolism of testosterone. Only where tissue was used in the incubation was one able to detect a stain for Δ^4 -androstene-3,17-dione by the Zimmermann reaction, and this was only slight. The absence of Δ^4 -androstene-3,17-dione might have been due to the absence of DPN and glucose from the experiment. Since the quantitative loss of substrate was apparent, some sort of further degradation of the steroid might have occurred. These possibilities might well account for some,

if not all of the loss in the controls.

The culture of the skin bacteria was flourishing, and the following tests were performed in an attempt to identify it:

Blood agar plate - The microorganisms appeared in irregular, mucoid, grayish green colonies with a slight silver or metallic sheen on the edges. A strong sweet odor was very apparent.

Eosin methylene blue plate - The microorganisms with the same characteristic odor formed rough pink colonies of the non-lactose fermentors.

Glucose fermentation - The reaction was positive.

The above reactions presented strong evidence that the organism was Pseudomonas pyocyaneus.

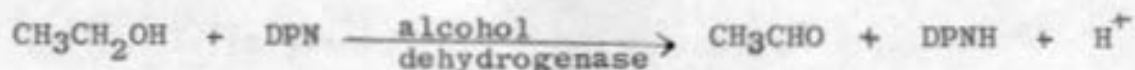
Previously Talalay (49) has shown that a species of Pseudomonas was capable of oxidizing testosterone to Δ^4 -androstene-3,17-dione. This species of bacteria was known to be resistant to penicillin. Sensitivity studies with antibiotics were carried out and it was found that the most effective bacteriostatic agent was streptomycin.

DISCUSSION

To determine the metabolism of testosterone by human skin an accurate analytical method was necessary. The method utilized by Wotiz, Mescon and coworkers (55) in the previous studies with skin, while quantitatively adequate, had been quite time consuming. A more direct means for the measurement of metabolic activity was sought. It was hoped that the use of manometry would give a direct measurement of enzyme activity. Sodium cyanide was added to inhibit the cytochrome system and in this reconstructed metabolic system methylene blue was used as the hydrogen acceptor. The effect of varying the concentration of the tissue enzyme and substrate was investigated. It appeared that the enzyme and testosterone concentrations had no effect on the oxygen consumption. The results varied only with the amount of ethanol. No direct increase of oxygen consumption with tissue concentration was observed. The solution of testosterone in absolute ethanol or its suspension in distilled water had no effect on the lack of uniformity in the results. However, the reaction was greatly intensified when ethanol was used as the steroid solvent, most likely due to alcohol dehydrogenase activity (28). The results of the experiments in Table 1, showing the effect of various testosterone solutions on the oxygen uptake by a 10 per cent rat liver homogenate, suggested

that the ethanol was causing the oxygen absorption.

Alcohol dehydrogenase an enzyme present in tissue can transfer a hydrogen atom from alcohol to the co-enzyme diphosphopyridine nucleotide (DPN). The reaction is as follows:



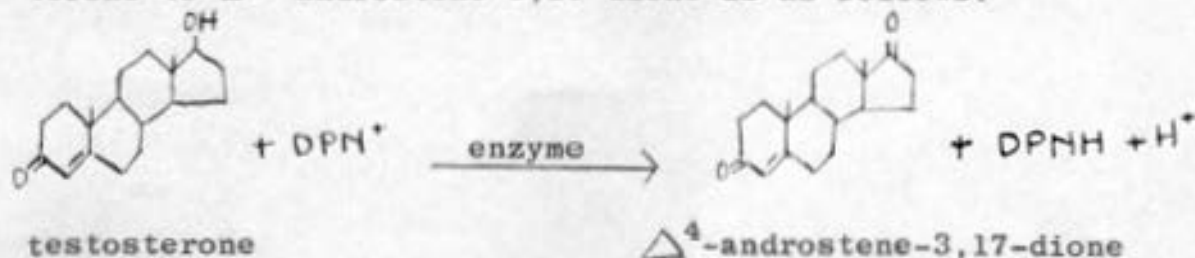
The existence of alcohol dehydrogenase activity was further borne out by the results shown in Table 1. It can be seen that only where ethanol was utilized as the substrate solvent was there an appreciable uptake of oxygen. However, since the ethanol volume was equal (1 ml.) in all flasks the variations in oxygen uptake could not be accounted for by this reaction alone. In experiment I (Table 1) the oxygen uptake appeared to vary directly with tissue concentration. The results were not duplicated in experiment IV (Table 1) indicating an error in the experimental technique.

In order to ascertain whether the entire oxygen consumption was due to alcohol dehydrogenase activity the experiment was repeated using ethanol as the steroid solvent. A control with ethanol and no steroid was incubated simultaneously. The controls were found to utilize oxygen at a fairly constant rate, and accounted for two thirds of the oxygen consumption. Because of these findings the remaining oxygen consumption was considered insignificant.

Diphosphopyridine nucleotide (DPN) was used as a co-enzyme in this study. In earlier work Sweat (46) and co-workers found DPN to be a necessary co-enzyme for the conversion of testosterone to Δ^4 -androstene-3,17-dione by tissue enzymes. Furthermore it was found that crude tissue extracts fortified with DPN increased the destruction of the α,β -unsaturated ketone groups, while the more purified extracts acted only on the hydroxyl group at carbon 17. Their work indicated that the favored route of testosterone metabolism was through Δ^4 -androstene-3,17-dione which was formed by the DPN-activated enzyme. The primary point of interest in this study has been this DPN-activated enzyme in human skin.

Using liver slices unfortified with DPN Clark and co-workers (11, 12) obtained only a small amount of the diketone Δ^4 -androstene-3,17-dione after incubation with testosterone. They were also able to obtain evidence for the reversibility of the reaction as shown by the appearance of epi-testosterone.

The general equation for the metabolism of testosterone to Δ^4 -androstene-3,17-dione is as follows:



In our study nicotinamide was utilized with the co-enzyme DPN as a nucleotidase inhibitor. Because of its structural similarity to the nucleotide the nicotinamide is considered to be a competitive inhibitor for the enzyme.

Since the experimental results with the adapted "Thunberg technique" had proven unsatisfactory triphosphopyridine nucleotide (TPN) was utilized in simultaneous experiments with DPN.

In recent experiments with enzymes from animal tissues and Pseudomonas fluorescens Kaplan (21) had indicated a relationship between DPN and TPN. According to this author, if such a reaction were to occur in the system

$$- \text{DPN} + \text{TPN} \xrightarrow{\text{transhydrogenase}} \text{DPN}^+ + \text{TPNH} - \text{an increase}$$

in TPN would tend to inhibit this secondary reaction.

With DPN the lowest enzyme concentration showed some oxygen uptake, however the uptake decreased with increasing enzyme concentration. No oxygen uptake was found with TPN. Perhaps as Kaplan suggested, TPN combined with the enzyme completely inactivating it.

Since the change in the co-enzyme had proved inefficient in correcting the defect in the method the problem remained unsolved.

Smith and Kochakian (44) had also obtained irregular tissue respiration when they incubated rabbit liver slices with testosterone and ethyl alcohol.

As another means of overcoming the blocking out

effect of the oxygen absorption by alcohol dehydrogenase the substrate (testosterone) was dissolved in a dilute solution of bovine serum albumin. Two solutions were prepared. The steroid was dissolved by shaking; (a) at room temperature by hand, and (b) at 37°C overnight in the Warburg apparatus. As in previous experiments it can be seen from Table 4 that the results were erratic and did not show any apparent relation to tissue (enzyme) concentration. Of the two albumin solutions the one which had been incubated overnight at 37°C showed the least activity. This might have been due to the formation of a steroid protein complex resulting in a lower concentration of steroid available for reaction.

As a further means of introducing the substrate to the enzyme without the use of alcohol the steroid was suspended in a 1 per cent solutions of the wetting agents "Tween 80" and "Triton". The two lowest tissue (enzyme) concentrations (0.5 ml. and 0.6 ml.) with the substrate in "Tween 80" resulted in the same oxygen uptake, and the other flasks showed no uptake. These negative results were probably caused by denaturation of the proteins in the presence of these wetting agents.

Finally an aqueous solution of testosterone- β -D-glucopyronuronoside (57) was used as a tissue substrate. Further irregular results were obtained. The conjugation of testosterone with glucuronic acid did not have a stabil-

izing effect on the reaction.

All these results showed that regardless of the steroid preparation utilized, variation in components and their concentrations, or change in incubation time, no valid enzyme concentration curve could be established. Therefore, this enzyme system could not be studied by manometric techniques in an unpurified preparation.

The method used by Wotiz, Mescon, Doppel and Lemon (55) for the study of this enzyme system, although more time consuming, still appeared to be the best approach to the problem. For verification of the method a 10 per cent rat liver homogenate was used as the enzyme source. The loss of testosterone increased directly with increasing tissue concentration (Table 6). Thus the method was satisfactory for the purpose of studying the quantitative aspects of the enzymatic reaction.

This previous work with human skin showed a testosterone loss averaging 50 per cent. Controls for testosterone concentrations were not repeated in individual experiments but were computed from a standard chart. Also about 15 per cent of the steroid may have been lost in elution from the paper and should not have been considered as metabolic loss. With both of these facts taken into consideration actual metabolism appeared to be about 60 per cent of that originally indicated.

When skin was incubated with testosterone (Table 7) there was a 25 per cent loss of the steroid, while boiled

tissue showed a 13 per cent loss and testosterone incubated without tissue gave a 9 per cent loss. Therefore, there is an enzyme system in human skin which is able to metabolize testosterone. Furthermore, there is an indication of some other cause of testosterone breakdown as loss occurs when testosterone is incubated without tissue.

There is no significant variation in enzyme activity with a change in surface area (Table 8). An 81 per cent recovery of testosterone from whole tissue, a 79 per cent recovery from scored tissue, and a 77 per cent recovery from homogenized tissue was found. Either the tissue is quite permeable to the steroid, or the major enzyme activity occurs in the outer layers of the skin. In a preliminary experiment (Simpson, unpublished data) where autopsied skin was sectioned parallel to the surface and its metabolism of testosterone determined a differential site of enzyme activity was indicated. The greatest activity appeared in the outermost layers of the skin. Perhaps changing the surface area of the skin, in this study, does not alter enzyme activity because of steroid permeability and the surface action of the enzyme.

In this experiment (Table 8) all tissue incubations showed greater metabolic activity than the controls (boiled tissue and incubated testosterone). The enzyme or enzyme system converting testosterone to Δ^4 -androstene-3,17-dione is quite stable and resistant to heat since

extensive boiling of the tissue did not completely destroy it. The possibility that the enzyme was activated by heat (45) was ruled out as the testosterone was not added until the tissue and buffer solution had been cooled to room temperature. A slight degradation of the steroid was found in the incubated testosterone control.

When the amount of tissue was doubled (Table 9) a very slight loss of the substrate occurred. This loss varied between 2 and 6 per cent for tissue incubated with testosterone, and higher values were found for the controls. It is possible that the steroid became bound to the increased tissue protein, and thus was not free to react with the enzyme. This may have been due to the condition of the skin at the time of biopsy. Since some metabolites were observed on the paper chromatographic strips stained with the Zimmermann reagent the apparent lack of testosterone disappearance could not be explained by an absence of the enzyme, but may be because of experimental errors in the quantitative estimation.

The first quantitative recovery of the compound believed to be Δ^4 -androstene-3,17-dione is shown in Table 10. Doubling the tissue concentration appeared to have little effect on the amount of recovered testosterone, but the recovery of its major metabolite was doubled. The percentage of Δ^4 -androstene-3,17-dione eluted from the chromatographs was so low, however, that more extensive

experimentation is required to establish a valid quantitative relationship.

It was suggested from the experiment described in Table 10 that Δ^4 -androstene-3,17-dione is a major metabolite of testosterone, but that it account for only a small part of the total metabolism. There was also an indication that the production of this diketone was proportional to the enzyme concentration. Again it was observed that the enzymatic reaction was not completely destroyed by boiling.

An incubation with skin and 4-C¹⁴-testosterone was carried out in the manner described previously and the extracts chromatographed on 3 cm. paper strips. After a two week exposure to X-ray film followed by development 8 compounds could be seen (Figure 11 A) confirming the previous findings of Wotiz and co-workers (55).

Some quantitative differences in testosterone metabolism are evident from the radioautographic studies (Figure 11 A). Tissue incubated with testosterone (strips 1 and 2) showed more metabolism than all controls. Boiled tissue with testosterone (strips 3 and 4) showed less formation of "polar compounds", indicating the existence of several enzyme systems, some of which are more heat stable than others. Only a very slight decrease is notes in the amount of the compound occupying the position of Δ^4 -androstene-3,17-dione. Testosterone incubated without tissue (strips 7

and 8) gave evidence of some degradation when compared with the un-incubated testosterone (strips 13 and 14). There also appeared to be a slight impurity present in the original testosterone. Perhaps some Δ^4 -androstene-3,17-dione is to be found in the non incubated testosterone, nevertheless, only the polar compounds seem to increase when testosterone is incubated without tissue. Therefore, any additional Δ^4 -androstene-3,17-dione observed with tissue incubation must be of enzymatic origin.

Two experiments were performed simultaneously; one with 4-C¹⁴-testosterone and the other with non radioactive testosterone. Twenty five per cent of the radioactive testosterone was recovered as Δ^4 -androstene-3,17-dione, as against 3 per cent of the non radioactive steroid. This quantitative difference resulting from identical experimental conditions might be due to insufficient elution from paper or some other obscure error in methodology.

These experiments (Table 11) confirmed the earlier hypothesis that Δ^4 -androstene-3,17-dione was a major metabolic product of testosterone and that this diketone accounts for only a fraction of the testosterone breakdown. The total loss of testosterone ranged from 12 to 43 per cent. This metabolite was confirmed to be Δ^4 -androstene-3,17-dione by thiosemicarbazone formation (Figure 11 D) and by infra-red and ultra-violet spectro-

photometry. From radioautograms (Figure 11 C) it can be seen that at least ten other degradation products of testosterone have been formed. The steroid nucleus may also have been degraded into other products not detected by the Zimmermann reagent.

The most outstanding characteristic of the enzyme responsible for the degradation of testosterone in human skin is its remarkable heat stability. There was relatively little effect produced by heat on the enzymatic activity in question as evidenced by the controls with boiled human skin. Possibly, with heating, the enzyme may be protected by forming a complex with other proteins or lipids in the tissue. This complex may be easily precipitated by heat, forming a protective covering around the enzyme, or they may perform their protective action in some other more complex manner.

A further characteristic of this enzymatic reaction in human skin is its great variability. There are several possible explanations for this variability.

It may be due more to differences in the skin from individual subjects than to the experimental conditions. The metabolism ranges from less than 10 per cent with one subject to over 50 per cent with another. The steroid loss in the average incubation ran between 20 and 25 per cent.

The variability in testosterone metabolism might also

be explained by competitive inhibition. The degradation of the steroid may depend upon the amount of enzyme available in the skin. The enzyme concentration in turn may depend on the physiological conditions of the body at the time of biopsy.

Overnight incubation of the substrate without the addition of skin produced a small amount of Δ^4 -androstene-3,17-dione (Figure 12 A). Only the autoclaved and non incubated testosterone failed to show this compound after staining with the Zimmermann reagent. Accordingly it was thought that this metabolism may have been brought about by bacterial action.

Therefore, the incubation solutions and the human skin were tested for the presence of microorganisms. In Figure 12 the results of simultaneous duplicate experiments for qualitative and quantitative determination of bacterial metabolism of testosterone can be observed. Where microorganisms were present a quantitative loss of the steroid was seen, but no production of Δ^4 -androstene-3,17-dione was observed. (Figure 12 B) Both yeast and gram negative microorganisms were found in the unautoclaved flasks (Table 12). The bacteria alone (Pseudomonas pyocyaneus) were tested for their reaction on the substrate and a 22 per cent loss of testosterone occurred (Table 13).

In the studies by Wotiz et al. (55) the biopsy sight

had been "sterilized" with ethanol. This was later abandoned for fear of alcohol dehydrogenase activity. It has been demonstrated by some authors (25) that most of the bacteria are deep within the openings of the cutaneous appendages where they can not be readily removed or killed. Thus in these previous experiments with or without ethanol "sterilization" bacteria might have accounted for some testosterone degradation. Wotiz, Richterich-van Baerle and Lemon (56) used gram stains on sediment smears from their serum incubation mixtures and detected no bacterial contamination. Serum and most of the other tissues utilized in the earlier studies of testosterone metabolism by Wotiz et al. (53,54) exist naturally in sterile conditions. The fact that skin nearly always contains some bacteria, as opposed to other sterile tissues, may account for some of the variability observed in this study.

In the present investigation of skin bacteria, the gram negative organism was identified as Pseudomonas pyocyaneus an organism almost completely resistant to penicillin.

As shown in Table 13 when the eluted testosterone which had been autoclaved and incubated was taken as a 100 per cent recovery of the steroid, a 93 per cent recovery was found with autoclaved bacteria. This slight loss of testosterone may be due to some catabolism of the steroid hastened by increase in heat during the initial period of

autoclaving. Seventy eight per cent of the incubated substrate was recovered when viable bacteria were added to the autoclaved testosterone. However, no Δ^4 -androstene-3,17-dione was observed on the Zimmermann stained strips (Figure 13). The absence of this compound may be attributed to the fact that DPN and its nucleotidase inhibitor, nicotinamide, were absent from this experiment, or that the steroid metabolite was immediately degraded if it was produced.

Talalay (49) has shown that a species of Pseudomonas could be adapted to grow on testosterone. He isolated the metabolic product from the incubation of testosterone with this organism and this substance displayed the chromatographic characteristics of Δ^4 -androstene-3,17-dione. Species of Pseudomonas are also known to contain enzymes which catalyze other reactions with DPN. One of these enzymes is transhydrogenase (21) which is responsible for electron transfer between the pyridine nucleotides.

Summary

The existence of an enzyme system in human skin which metabolizes testosterone to Δ^4 -androstene-3,17-dione has been established.

Twenty to twenty five per cent of the steroid was found to be metabolized with only a small fraction being recovered as Δ^4 -androstene-3,17-dione.

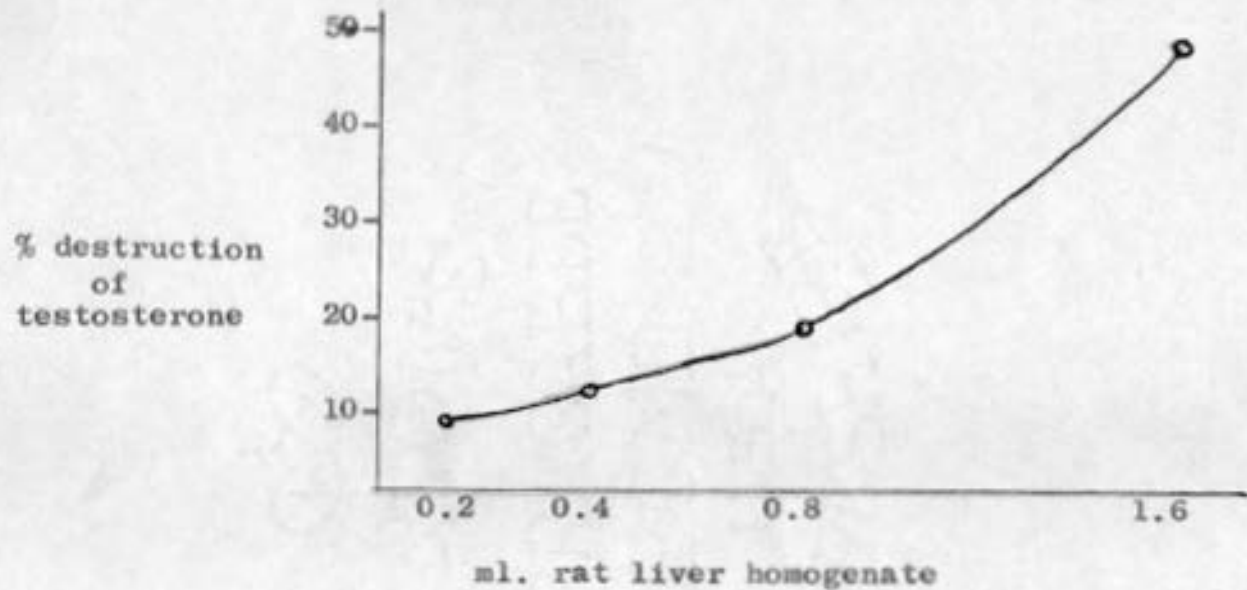
At least two enzyme systems seem to be involved in testosterone metabolism in the human skin; one or more in the production of unknown "polar compounds", and the other in the formation of Δ^4 -androstene-3,17-dione. The former of these systems are the more heat labile.

The enzyme in human skin responsible for the conversion of testosterone to Δ^4 -androstene-3,17-dione appears to be heat resistant. Its destruction by heat may be prevented by the formation of a tissue protein or lipid complex.

As many as twelve compounds have been clearly noted on the radiogram both with the incubated tissue and the boiled tissue controls (Figure 11 C).

One or more microorganisms may be responsible for the slight degradation occurring in the incubated testosterone without tissue. However, this action is not likely to be of any significance in the metabolism of testosterone since: a.) they do not produce Δ^4 -androstene-3,17-dione, b.) they were only isolated once, and c.) they are present only in very small concentrations.

Table 6



ml. homo- genate	% met.
0.2	9
0.4	12
0.8	19
1.6	49

Metabolism of Testosterone by Rat Liver Homogenate

Table 7

Flask #		240 m μ reading	% testosterone recovered	% testosterone lost
1,2	tissue boiled and incubated	0.390	87.3	12.7
4,5	tissue boiled not incubated	0.328	71.6	28.4
7,8	tissue incuba- ted, no tes- tosterone	0	0	0
10,11	tissue incuba- ted with tes- tosterone	0.344	75.1	24.9
13,14, 15	testosterone incubated, no tissue	0.417	91.0	9.0
	testosterone not incubated but eluted	0.458	100.0	0
	original tes- tosterone un- treated	0.571	--	--

Table 8

Flask #		240 m μ reading	% testosterone recovered	% testosterone lost
1,2	whole tissue	0.523	80.5	19.5
3,4	scored tissue	0.514	79.1	20.9
5,6	homogenized tissue	0.492	75.7	24.3
7,8	boiled tissue	0.540	83.1	16.9
9,10, 11	testosterone incubated, no tissue	0.626	96.3	3.7
12,13	testosterone not incubated but eluted	0.650	100.0	----

Table 9

Flask #		240 m μ reading	% testosterone recovered	% testosterone lost
1,2	tissue homogenized and incubated	0.422	97.9	2.1
3,4	tissue cut and incubated	0.405	94.0	6.0
5,6	tissue homogenized, boiled, and incu- bated	0.389	90.3	9.7
7,8	testosterone incubated	0.358	83.1	15.9
9,10	testosterone not incubated but eluted	0.431	100.0	---

Table 10

Flask #		Testosterone			Δ^4 -Androstene-3,17-dione		
		240 m μ reading	% recovered	% lost	240 m μ reading	% recovered	Unaccountable loss - %
1,2	7 mm. tissue cut and incubated	0.383	77.5	22.5	0.007	1.4	21.1
3,4	7 mm. tissue boiled 1 hr., incubated	0.317	64.1	35.9	0.011	2.2	33.7
5,6	14 mm. tissue cut and incubated	0.370	74.8	25.2	0.015	3.0	22.2
7,8	14 mm. tissue boiled 1 hr., incubated	0.395	79.9	20.1	0.000	0	20.1
9,10	testosterone incubated	0.449	91.1	8.9	---	---	---
11	testosterone not incubated but eluted	0.494	100.0	---	---	---	---

Table 11
Radioactive Testosterone

Flask #		Testosterone			Δ^4 -Androstene-3,17-dione		
		240 m μ reading	% recovered	% lost	240 m μ reading	% recovered	Unaccountable loss - %
1,2	tissue cut radioactive testosterone incubated	0.211	43.3	56.7	0.122	25.1	31.6
3,4	tissue cut boiled radioactive testosterone incubated	0.291	59.8	40.2	0.069	14.2	26.0
7,8	radioactive testosterone incubated	0.477	97.0	---	0	0	2.1
13	radioactive testosterone not incubated but eluted	0.487	100.0	---	0	0	---

Non-Radioactive Testosterone

5,6	tissue cut testosterone incubated	0.200	44.1	55.9	0.012	2.7	53.2
9,10	incubated testosterone	0.438	96.7	---	0	0	3.3
15	testosterone not incubated but eluted	0.453	100.0	---	0	0	---

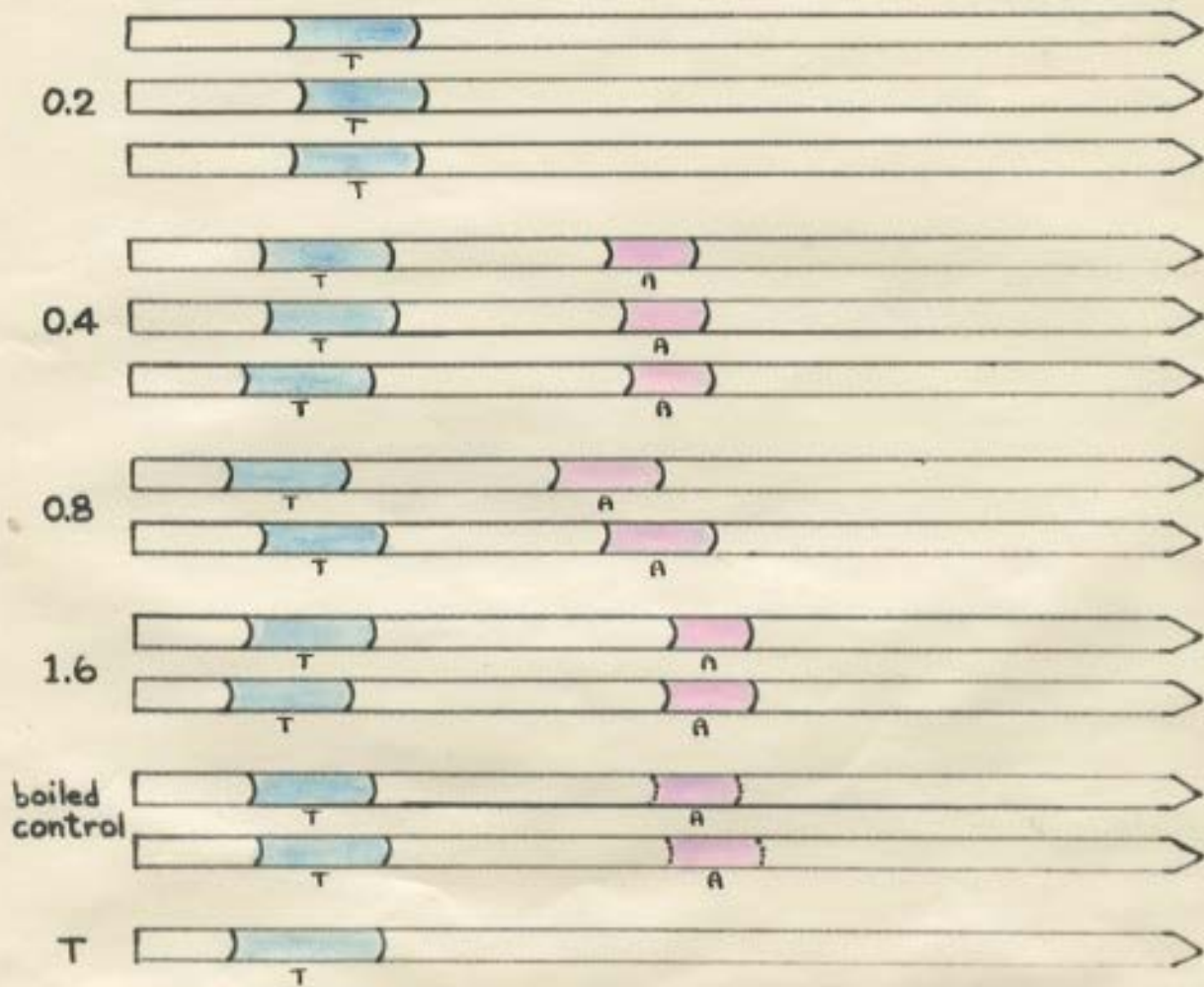
Table 12

Flask #		Pour plates	Blood plate	Broth	% Recovery testosterone
3,4	autoclaved testosterone	---	---	---	100
1,2	incubated testosterone	---	1 colony 1 colony	yeast and gram negative rods	80
9,10	incubated testosterone and tissue	---	many along streak and around tissue	" granules with gram positive or nothing	86

Table 13

Flask #		240 m μ reading	% testosterone recovered
5,6	testosterone autoclaved incubated	0.222	100
3,4	testosterone and bacteria autoclaved incubated	0.207	93
1,2	testosterone autoclaved incubated with viable bacteria	0.172	78

Figure 6



Metabolism of testosterone by a 10 per cent rat liver homogenate

T = testosterone

A = compound believed to be Δ^4 -androstene-3,17-dione

Figures: 0.2, 0.4, 0.8, and 1.6 indicate the milliliters of the tissue homogenate utilized.

The dotted lines designate a very light stain.

All strips are stained with the Zimmermann reagents.

Figure 7



Metabolism of testosterone by human skin (5 mm. biopsies)

T = testosterone

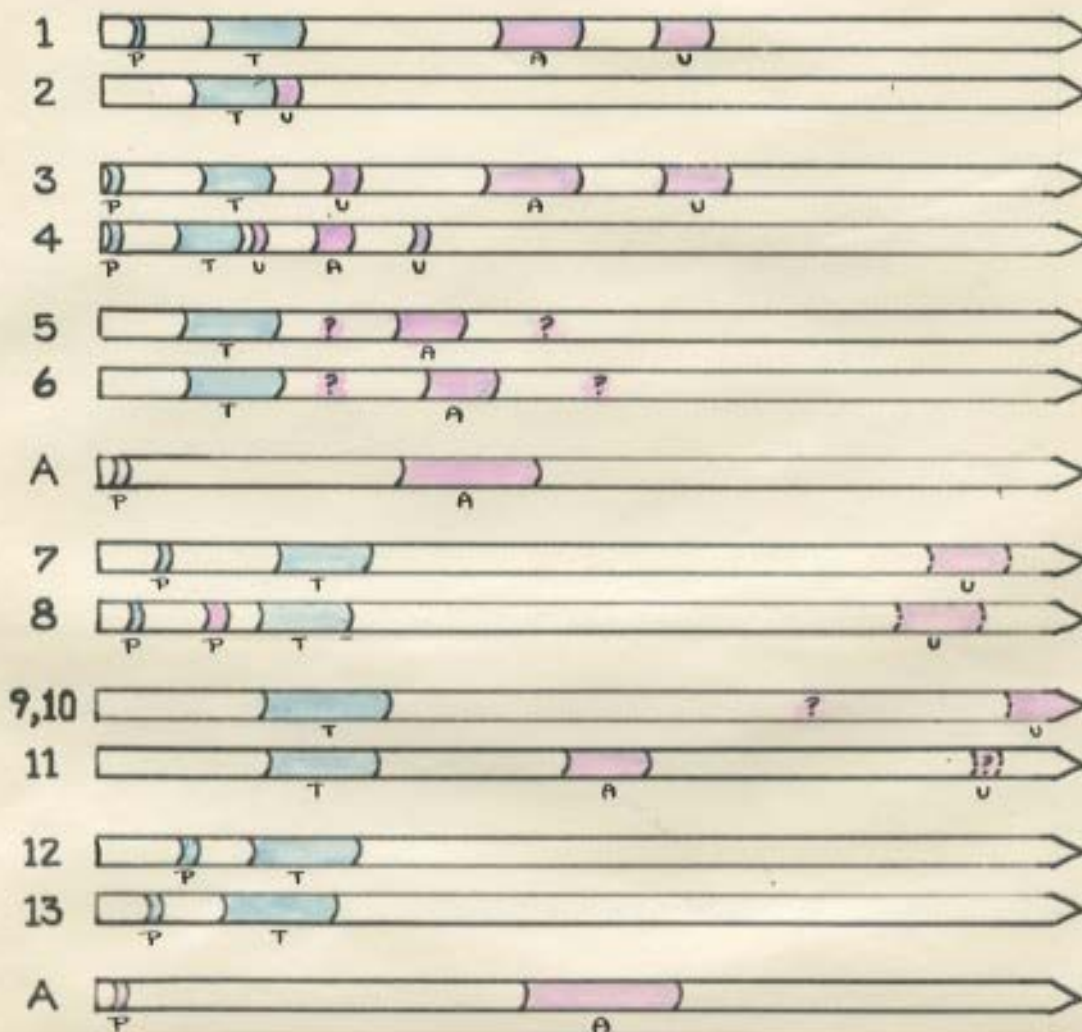
A = compound believed to be Δ^4 -androstene-3,17-dione

P = polar compounds (unknown)

U = unknown ketonic compound

The numbers of the strips correspond to the flask numbers in table 7.

Figure 8



The effect of variation in surface area on the metabolism of testosterone by human skin (7 mm. biopsies)

T = testosterone

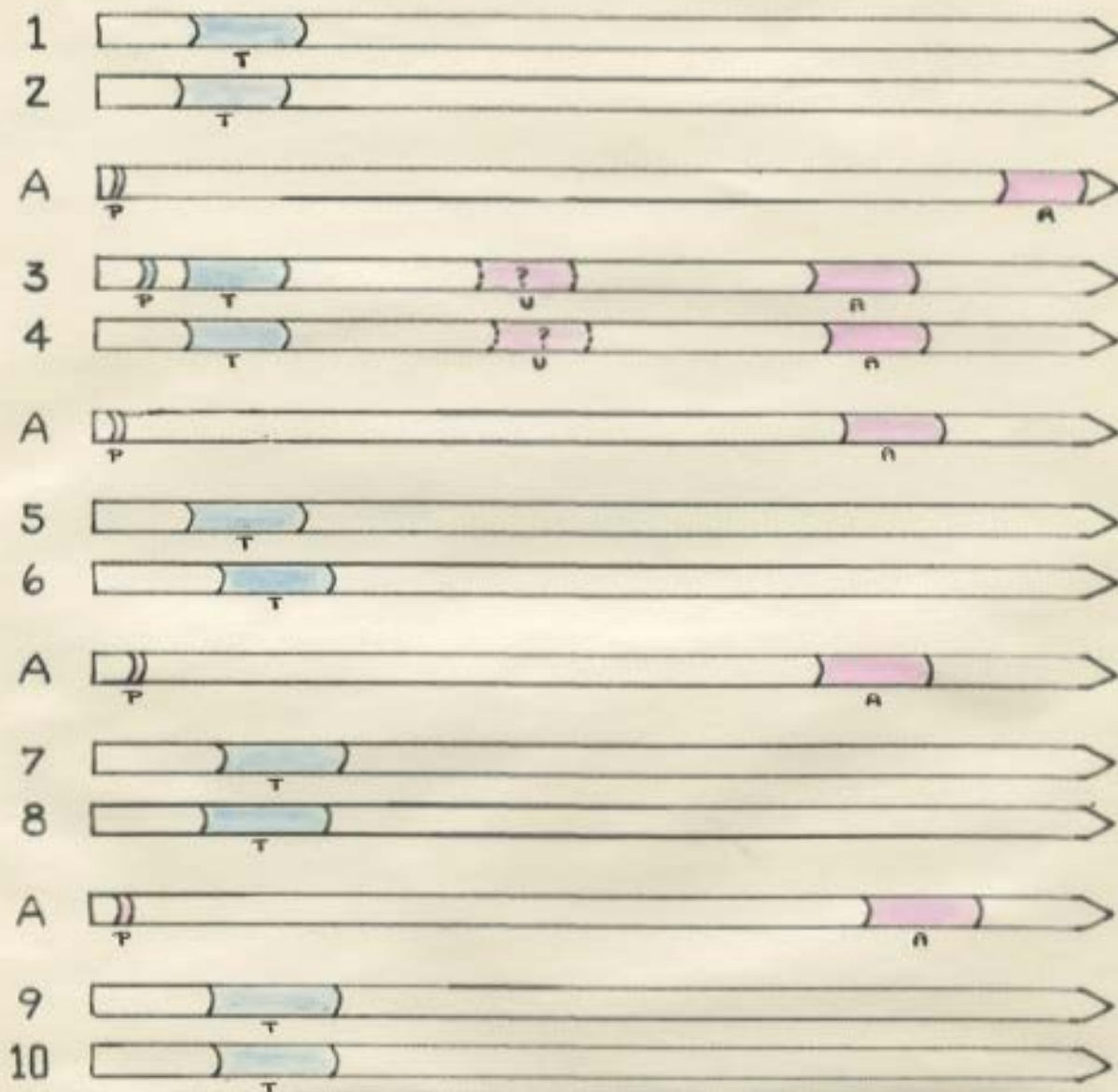
A = compound believed to be Δ^4 -androstene-3,17-dione

P = polar compounds (unknown)

U = unknown ketonic compounds

The corresponding quantitative results are expressed in table 8.

Figure 9



Testosterone metabolism by human skin (14 mm, biopsies)

T = testosterone

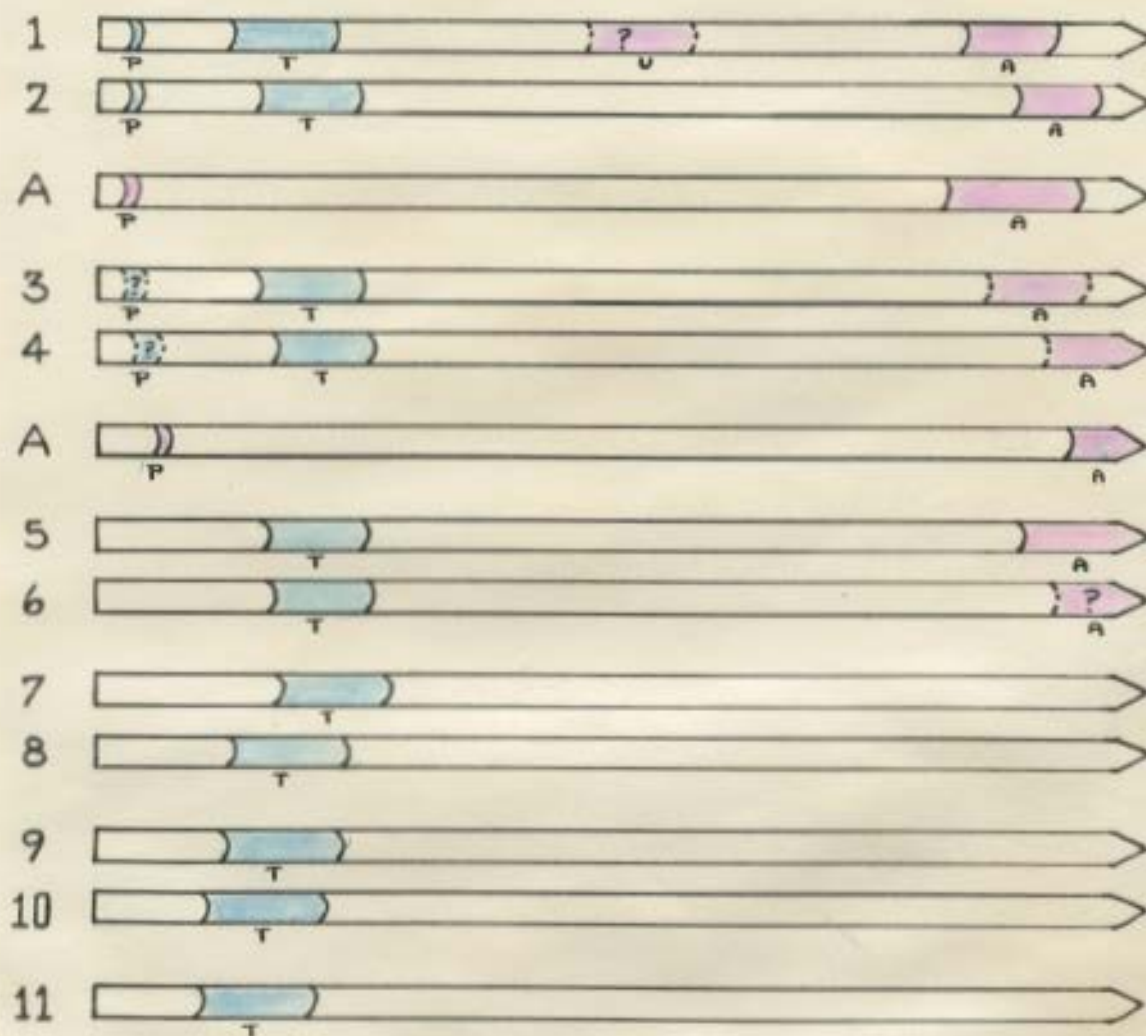
A = compound believed to be Δ^4 -androstene-3,17,dione

P = polar compound (unknown)

U = unknown ketonic compound

? = stain very light and questionable

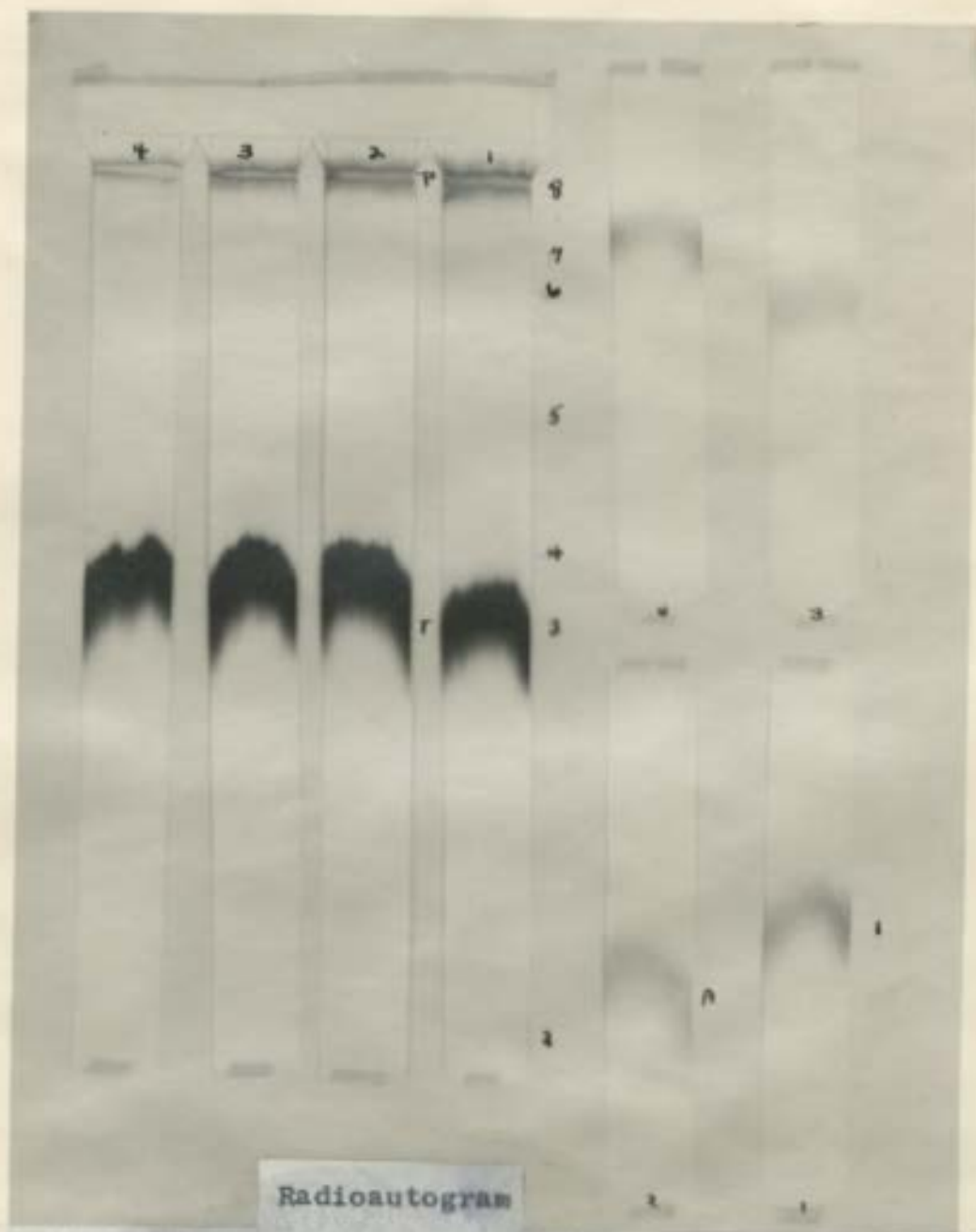
Figure 10



The effect of tissue concentration on testosterone metabolism (7 and 14 mm. biopsies)

T = testosterone
 A = compound believed to be Δ^4 -androstene-3,17-dione
 P = polar compound (unknown)
 U = unknown ketonic compound
 ? = stain very light and questionable

Figure 11 A (1)



Radioautogram

Metabolism of testosterone by human skin

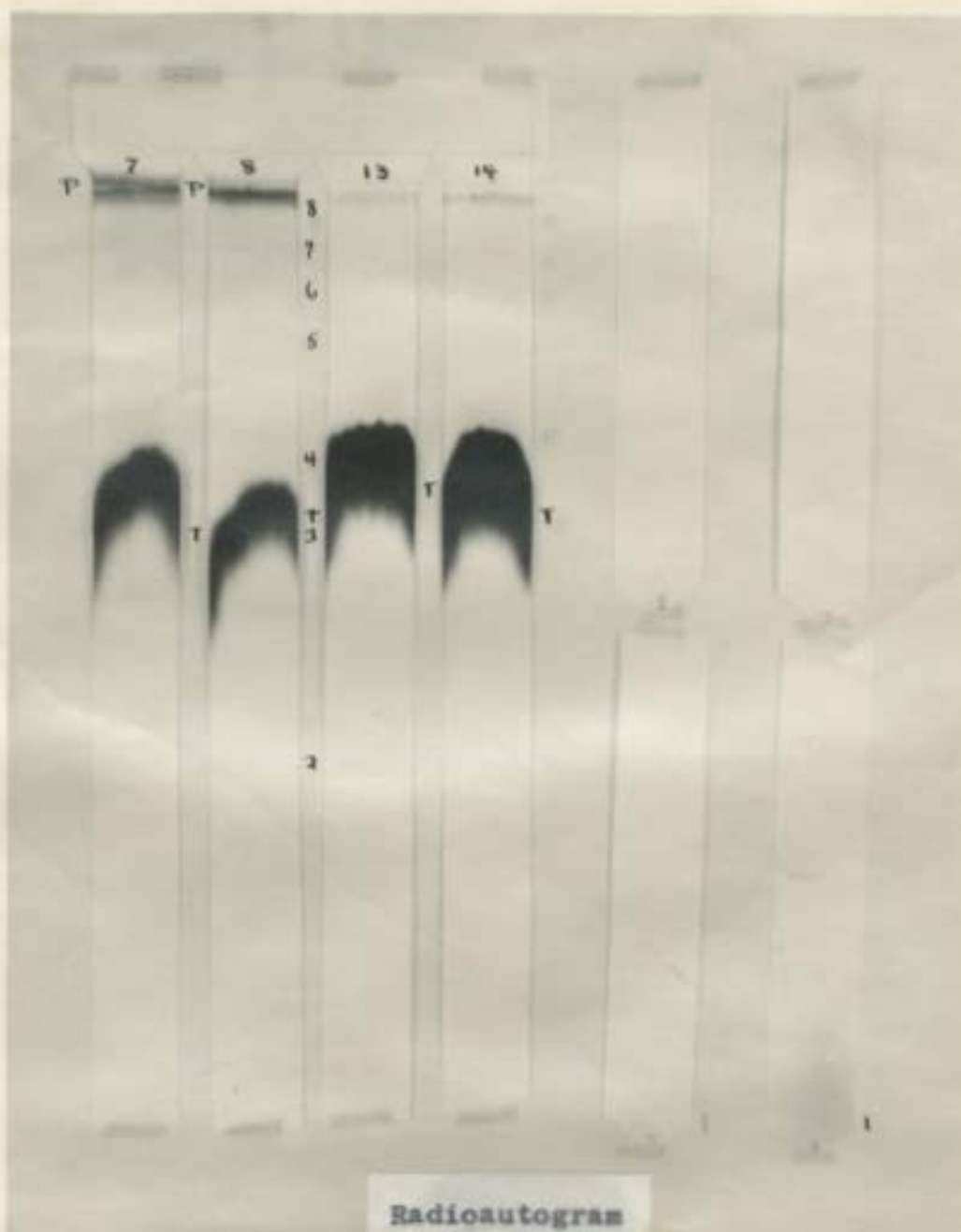
T = testosterone

A = compound believed to be Δ^4 -androstene-3,17-dione

P = polar compound

Eight compounds can be observed

Figure 11 A (2)



Incubated and non-incubated testosterone

T = testosterone
P = polar compound

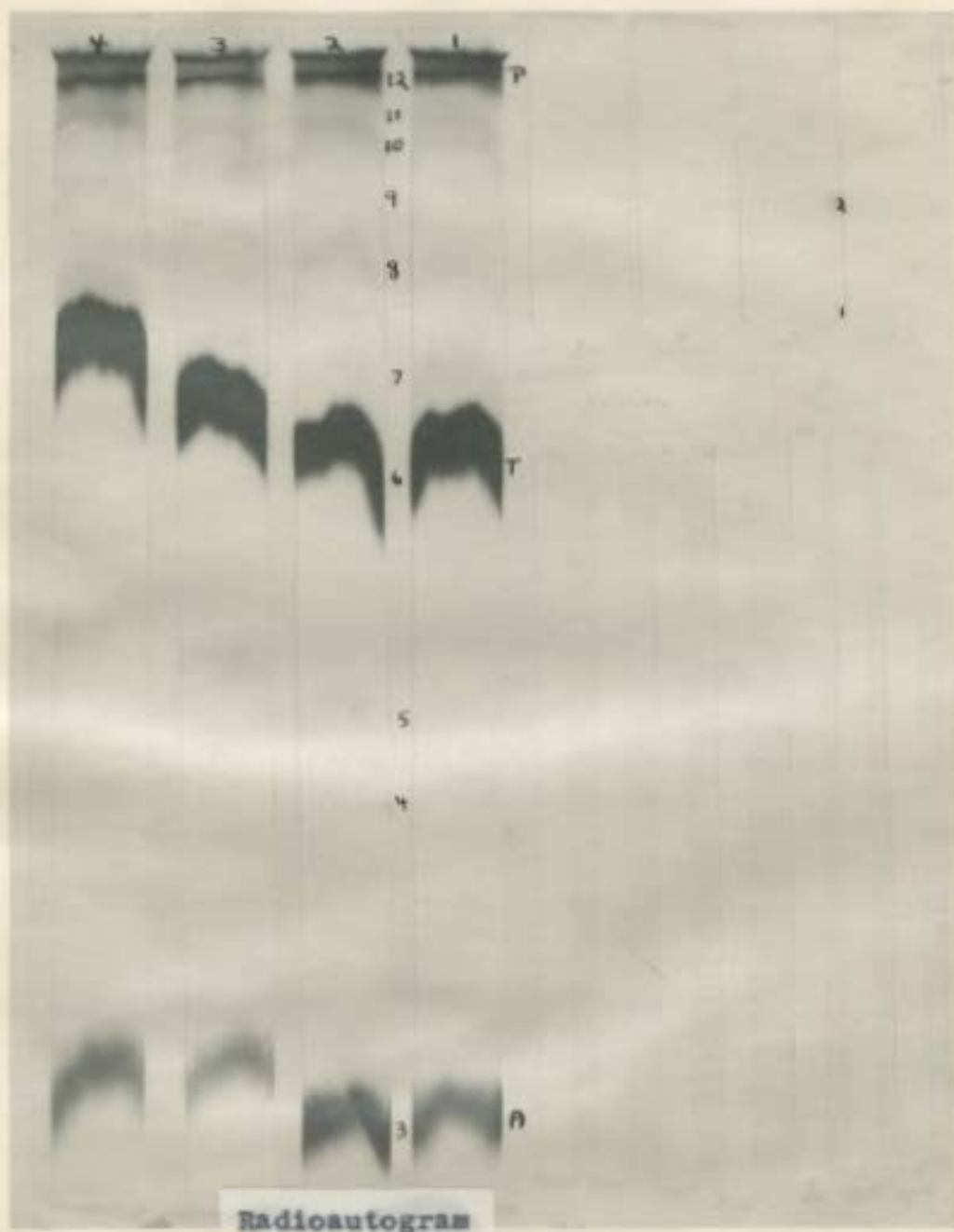
Figure 11 B



Metabolism of radioactive testosterone by human skin

- T = testosterone
- A = compound believed to be Δ^4 -androstene-3,17-dione
- P = polar compound
- U = unknown ketonic compound
- ? = stain very light and questionable

Figure 11 C



Radioautogram

Metabolism of testosterone by human skin

T = testosterone

A = compound believed to be Δ^4 -androstene-3,17-dione

P = polar compound

Twelve compounds can be observed

Figure 11 D

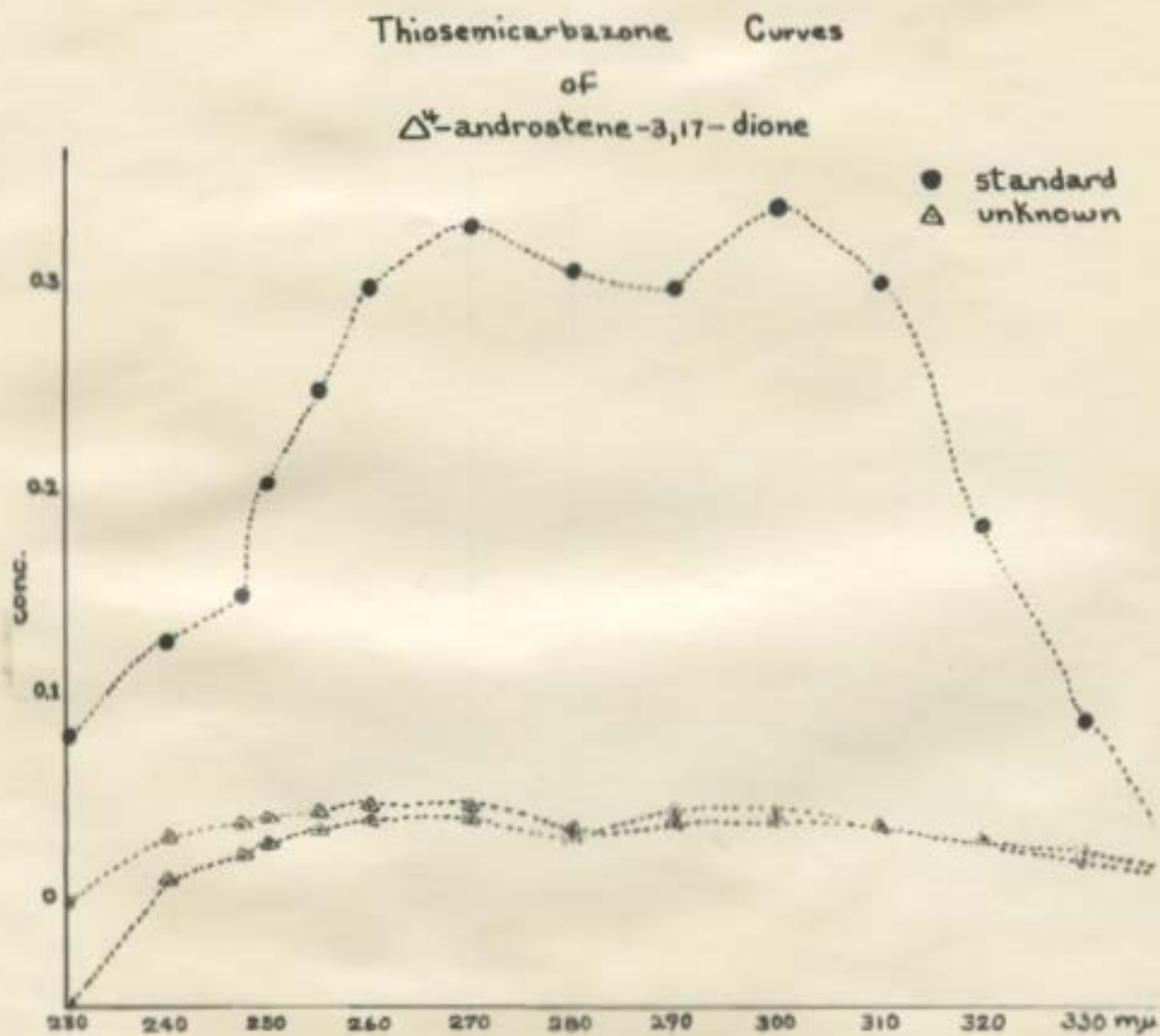
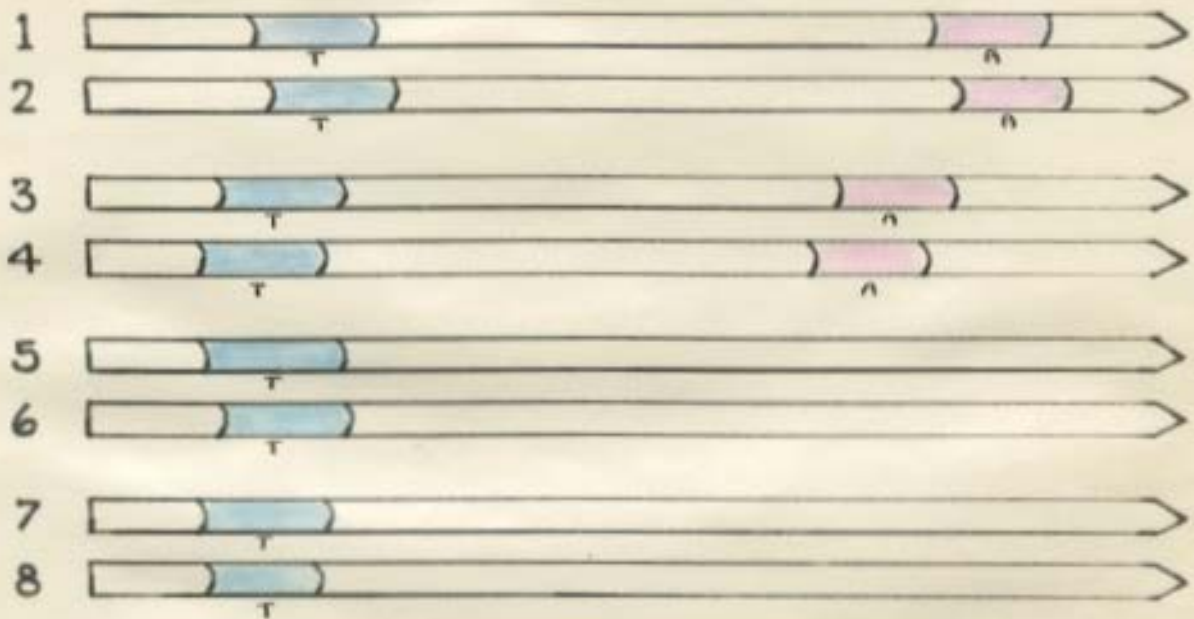


Figure 12 A

Testosterone Incubation

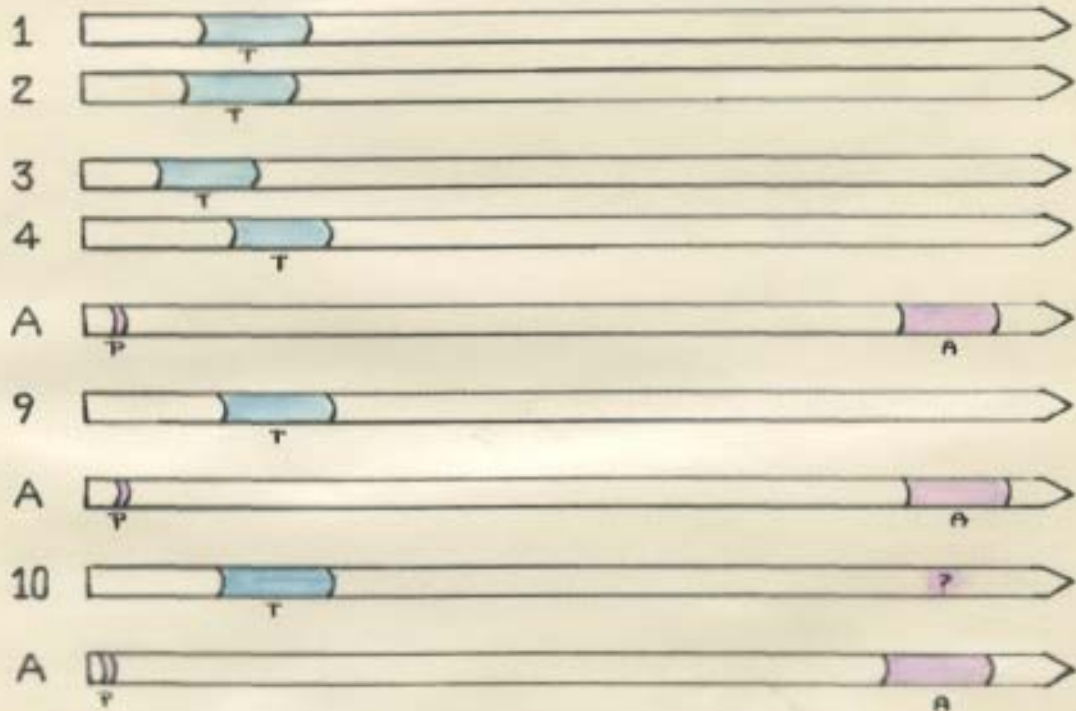


Incubation of testosterone without tissue

T = testosterone

A = compound believed to be Δ^4 -androstene-3,17-dione

Figure 12 B



Testosterone metabolism by microorganisms

T = testosterone

A = compound believed to be Δ^4 -androstene-3,17-dione

P = polar compound

Figure 13



Metabolism of testosterone by Pseudomonas pyocyaneus

T = testosterone

A = compound believed to be Δ^4 -androstene-3,17-dione

P = unknown ketonic polar compounds

ABSTRACT

From a series of in vitro incubations with human skin the existence of an enzyme system which metabolizes testosterone to Δ^4 -androstene-3,17-dione and "polar compounds" has been established.

Several enzyme systems are felt to be active in testosterone breakdown by human skin; some of these from "polar compounds", one of them forms Δ^4 -androstene-3,17-dione, while others form additional unknown ketonic compounds. These skin enzymes were found to vary quantitatively among different subjects.

Catabolism in a typical in vitro incubation of testosterone with skin ranged between twenty and twenty five per cent. The major metabolite of this reaction is believed to be Δ^4 -androstene-3,17-dione, although it accounts for less than half of the substrate loss.

The enzyme system responsible for the conversion of testosterone to Δ^4 -androstene-3,17-dione in human skin is remarkably heat resistant. Extensive boiling of the tissue showed little destruction of the enzyme, possible due to the formation of a protective enzyme protein or lipid complex.

At least twelve compounds appeared on the radioautogram (Figure 11 C) of skin incubated with testosterone.

Incubation solutions and skin were tested for bacterial

contamination and metabolism of testosterone. Where microorganisms were found a loss of steroid was observed. However, no production of Δ^4 -androstene-3,17-dione was seen.

A gram negative microorganism isolated from one specimen of skin was identified as Pseudomonas pyocyaneus. When this isolated bacteria was incubated with testosterone overnight a 22 per cent loss of steroid occurred, but again without the formation of Δ^4 -androstene-3,17-dione.

Microorganisms may be responsible for the slight degradation occurring in incubated testosterone without skin. However they are not felt to have significant effect on testosterone metabolism with tissue since 1.) no Δ^4 -androstene-3,17-dione was produced, 2.) the microorganisms were only isolated once, and 3.) they are normally present in very small concentrations (Pseudomonas being only a transient organism on the human skin).

An in vitro system where testosterone and human skin were incubated in a Krebs-ringer phosphate buffer at pH 7.4 was utilized to determine the enzymatic activity.

An adaptation of the "Warburg technique of the estimation of dehydrogenase activity" was proved to be useless for the determination of enzymatic activity. Regardless of the steroid preparation utilized, variation in compon-

ents and their concentrations, or change in incubation
time no valid enzyme curve could be established.

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