# Identification of 7α-Hydroxylated Androgens as the Metabolites of Androstenedione by Testicular Microsomal Fraction of Rats*

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ABSTRACT: After incubation of androstenedione with testicular microsomal fraction (10,000–105,000*g* precipitate) of rats in the presence of reduced nicotinamide–adenine dinucleotide phosphate and in the atmosphere of $O_2$–$CO_2$ mixture (95:5, v/v), one of the major metabolites which was designated as I was identified as 7α-hydroxyandrostenedione by the following biochemical and physicochemical procedures. (1) By double-isotope dilution method, I was proved as being directly derived from androstenedione. (2) Under anaerobic condition or CO–atmosphere, the formation of I was severely reduced, suggesting that a mixed-function oxidase or a monooxygenase was involved in the transformation of androstenedione to I. (3) Through the chemical behaviors of I against the several reagents, it was considered as a hydroxylated androstenedione. Particularly, after treatment of I with methanolic KOH, formation of a conjugated dienone was proved by ultraviolet spectrometric analysis, suggesting that the hydroxylated position of I was C-7. (4) Configuration of the hydroxy group at C-7 of I was determined as α by thin-layer chromatography, in which 7α-hydroxyandrostenedione was separable from its 7β epimer. (5) Compound I and its derivatives were identical with the authentic 7α-hydroxyandrostenedione and its respective derivatives in thin-layer chromatography as well as by mass spectrometric analysis. (6) Finally, when the radioactive I which was obtained as a metabolite of [4-$^{14}$C]androstenedione was repeatedly crystallized with the authentic 7α-hydroxyandrostenedione, specific activities of crystals remained constant. Similarly, by some of the above stated procedures, 7α-hydroxytestosterone was tentatively identified as a minor metabolite of androstenedione. From the chemical structures of these metabolites, activity of the 7α-hydroxylase which introduced hydroxy group to androstenedione and testosterone was demonstrated in the microsomal fraction of rat testes.

It was reported that the testicular enzymes related to testosterone production from pregnenolone were exclusively concentrated into the microsomal fraction of the interstitial cells by Tamaoki *et al.* (1969). The above-mentioned androgen-producing enzymes and the cytochrome P-450 which was an essential component of the steroid hydroxylating system were recently found to be localized in the smooth-surfaced microsomal fraction, but not in the rough-surfaced one of rat testicular glands (Inano *et al.*, 1970).

When enzyme kinetic studies of the 17β-hydroxysteroid dehydrogenase in the state of the testicular microsomes were carried out in the presence of $NADPH_2$ and under the mixture of oxygen and carbon dioxide (95:5, v/v), a significant amount of the metabolite which was more polar than the expected product or testosterone in the thin-layer chromatogram was obtained. The metabolite was coincidental with one of the unidentified products of progesterone obtained in this laboratory by incubation of a testicular cell suspension (Tamaoki and Shikita, 1966) and other testicular preparations of rats.

In this paper, we report elucidation of the chemical structures of the metabolite and the other with biochemical, physicochemical, and radiochemical methods.

## Materials and Methods

*Tissue Preparations.* Male rats (3–4-months old) of the Wistar strain, bred in this Institute, were used for the experiments. After decapitation of the rats, the testes were isolated, and homogenized with a loose-fitting Teflon-glass homogenizer. The homogenates were centrifuged at 10,000*g* for 20 min. The supernatant fluid was then centrifuged at 105,000*g* for 1 hr to obtain the microsomal fraction as the precipitate.

*Steroids and Reagents* [4-$^{14}$C]Androstenedione (specific activity 34.8 mCi/mmole) and [7-$^{3}$H]androstenedione (specific activity 5880 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, England. [1,2-$^{3}$H]Testosterone (specific activity 43,000 mCi/mmole) was a gift of Endocrinology Study Section of National Institutes of Health, Bethesda, Md. All the radiochemical purities of these steroids were confirmed by thin-layer chromatography shortly before use. Radioactive steroids were diluted with the corresponding nonradioactive steroids to appropriate specific activities, and were used as the substrates. Authentic 7α-hydroxyandrostenedione was kindly supplied by Dr. D. H. Peterson, Upjohn Co., Kalamazoo, Mich., Dr. K. Singh, Ayerst Laboratories, Montreal, Canada, and Dr. S. Kraychy, Searle Co., Chicago, Ill. 7β-Hydroxyandrostenedione was kindly supplied by Dr. S. Kraychy. 2β-Hydroxyandrostenedione was kindly offered by Dr. R. M. Dadson, University of Minnesota, Minn. 7α-Hydroxytestosterone was kindly supplied from Dr. K. Irmscher, E. Merck, Darmstadt, Germany. Bismuth trioxide was kindly supplied by Mr. K. Kurotaki in this Institute. Other chemical reagents used in this investigation were commercially obtained.

*Incubation.* The radioactive steroids dissolved in volatile solvents were transferred into the incubation flasks, into

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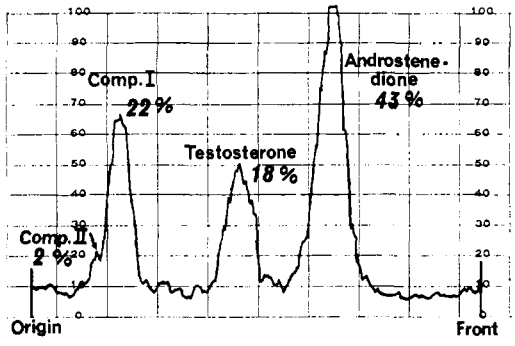


FIGURE 1: Radioscannogram of the metabolites of [4-$^{14}$C]androstenedione (175 mμmoles, 5.3 × $10^4$ cpm) obtained by incubation of it with testicular microsomal fraction of rats in the presence of NADPH (1 mg) and in the atmosphere of $O_2$–$CO_2$ (95:5, v/v) mixture for 1 hr at 37°.

which two drops of propylene glycol per flask were added. Shortly before the incubation, the volatile solvent was removed under reduced pressure. Then, to each flask, the mixture of 4 ml of the testicular microsomal suspension (corresponding to two animals) in 0.25 M sucrose solution and 1 ml of the isotonic sucrose solution which contained Tris-HCl (0.05 M, pH 7.4), $MgCl_2$ (0.005 M), and NADPH (1 mg) (Boehringer, Mannheim, Germany) was added. The incubation was carried out at 37° for 1 hr in the atmosphere of $O_2$–$CO_2$ (95:5, v/v) and other gas.

*Extraction and Isolation of the Metabolites.* Immediately after incubation, 15 ml of methylene dichloride was added into a flask and the mixture was vigorously shaken to arrest further enzymic reaction. The metabolites were isolated and separated by the method previously described (Inano and Tamaoki, 1966).

*Quantitation of the Metabolites.* Suitable aliquots of the extracts in counting glass vials (Wheaton Glass Co., Millville, N. J.) were evaporated, and the radioactivity of each steroid was measured with a liquid scintillation spectrometer (System 725, Nuclear-Chicago, Des Plaines, Ill.), in the toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene system (Shikita *et al.*, 1964).

*Mass Spectrometric Analysis.* Mass spectrometric analyses of the steroids obtained from the incubation mixture were performed with the mass spectrometers Model RMU-6 (Hitachi-Perkin-Elmer) and RMS-4 (Hitachi). Purified metabolites and reference steroids were dissolved in appropriate volumes of methylene dichloride and transferred into the glass sample tubes for mass spectrometry, respectively. After removal of the solvent under a stream of $N_2$ gas and then reduced pressure, the tubes were connected to the vaporization system of the mass spectrometer, and the samples were analyzed under the following conditions: oven temperature 200°, chamber temperature 200°, inlet pipe heater 200°, target current 60 μA, chamber voltage 75 eV, and main vacuum, 6 × $10^{-7}$ mm, for the mass spectrometer Model RMU-6; in case of Model RMS-4, oven temperature 230°, chamber temperature 230°, inlet pipe heater 250°, target current 80 μA, chamber voltage 70 eV, and main vacuum, 3 × $10^{-7}$ mm.

TABLE I: Metabolism of [4-$^{14}$C]Androstenedione and [1,2-$^3$H]Testosterone by Testicular Microsomal Fraction of Rats.[a]

| | Substrate Recovered and Products | | | |
|---|---|---|---|---|
| | Androstenedione | Testosterone | I | II |
| $^{14}$C | 8,700 | 18,000 | 4,600 | 1,400 |
| $^3$H | 46,300 | 94,200 | 14,500 | 12,200 |
| $^3$H:$^{14}$C | 5.32 | 5.23 | 3.15 | 8.71 |

[a] Figures in the table represent the counts per min of radioactivities of radiocarbon and tritium.

## Results

*Bioconversion of Androstenedione by the Testicular Microsomal Fraction of Rats.* After [4-$^{14}$C]androstenedione (175 mμmoles, 5.3 × $10^4$ cpm) was incubated with the testicular microsomal fraction, several radioactive metabolites were detected on thin-layer chromatogram, as shown in Figure 1. In this experiment, about 73.5 mμmoles of the substrate was consumed by the microsomal fraction, and testosterone was formed from it in a significant amount (31.5 mμmoles). A metabolite which was localized in the more polar position than the testosterone was obtained as one of the major products. This metabolite is called I in this paper, while a minor metabolite which was more polar than I is designated hereafter II.

*Pathways of Formation of I and II from the Androgens.* In order to examine the pathways of transforming androstenedione into I and II in the testicular microsomal fraction, the following double tracer technic was employed. [4-$^{14}$C]-Androstenedione (105 mμmoles, 39.8 × $10^3$ cpm) and [1,2-$^3$H]testosterone (105 mμmoles, 216.9 × $10^3$ cpm) were jointly incubated with the testicular microsomal fraction of rats. Thereafter, the radioactivities of $^{14}$C and $^3$H which were present in the isolated and purified fractions were separately measured as shown in Table I. The ratio of $^3$H to $^{14}$C in the testosterone fraction was almost same as the ratio in the androstenedione fraction, indicating that those two substrates had been mutually convertible by oxidoreduction of the 17β-hydroxysteroid dehydrogenase, and reached the equilibrium at the end of incubation. In the fraction of I, however, the ratio of $^3$H to $^{14}$C was found to be lower than the above ratios of the two substrates. This result indicates that $^{14}$C due to the added androstenedione had been more rapidly incorporated into I than the $^3$H of the testosterone added as the substrate, suggesting that I was directly derived from androstenedione, without reduction of 17-oxo group of the androstenedione.

On the contrary, the ratio of $^3$H to $^{14}$C in the fraction of II was higher than the ratios of the two substrates, indicating that II was derived from androstenedione after reduction of its 17-oxo group, or transformed directly from testosterone.

*Effect of Aerobic, Anaerobic, and CO Atmospheres upon Testicular Microsomal Metabolism of Androstenedione.* [4-$^{14}$C]Androstenedione (175 mμmoles, 6.6 × $10^4$ cpm) was incubated with the testicular microsomal fraction of rats, under three kinds of gas, namely, $O_2$, Ar, and CO. Under the oxygen-enriched atmosphere, I was produced from androstenedione in the highest yield among those obtained

under the three gas phases, as shown in Table II. When the gas phase was replaced with Ar, production of I was severely inhibited, suggesting that the enzyme reaction by which androstenedione was transformed to I required molecular oxygen besides NADPH. Furthermore, if CO was employed as the incubation atmosphere, formation of I was almost completely diminished, indicating that I was produced from androstenedione by an $O_2$-requiring enzyme or most likely hydroxylase which was inhibited by CO like other microsomal hydroxylases such as testicular 17α- and adrenal 21-hydroxylases (Inano *et al.*, 1969, 1970). On the other hand, the 17β-hydroxysteroid dehydrogenase activity which was expressed as the yield of testosterone produced from androstenedione was apparently elevated in the atmosphere of Ar and also of CO.

*Ultraviolet Light Absorption.* After I (40 μg) was dissolved in 4.0 ml of absolute methanol, ultraviolet absorption spectrum was obtained by a recording spectrophotometer (Cary Instrument, Model 14). As the results, the following optical characteristics of I were obtained: $\lambda_{max}^{CH_3OH}$ 240 mμ, $\epsilon_{1cm}^{1\%}$ 532, and $\epsilon$ 16,100, when calculated as monohydroxyandrostenedione.

*Chemical Reactions.* ACETYLATION. Compound I was found to be acetylatable with acetic anhydride and pyridine at room temperature overnight. When the acetylated product was chromatographed on thin-layer plate coated with silica gel in the benzene–acetone (4:1, v/v) solvent system, the $R_F$ value of I acetate was 0.47, while the $R_F$ of initial I was 0.21. The acetylated I was resistent to the oxidation with 0.5% $CrO_3$ in 90% aqueous acetic acid solution.

$CrO_3$ OXIDATION. After oxidation of radioactive I with $CrO_3$ in aqueous acetic acid solution for 10 min at room temperature, no radioactive product was extracted with methylene dichloride from the reaction mixture, suggesting that degradation of the steroid nucleus occurred, in agreement with the previously reported result on 7α-hydroxyandrostenedione (Gold and Garren, 1964).

TREATMENT WITH $Bi_2O_3$ IN ACETIC ACID. To the solution of 80 μg of I in 2.0 ml of glacial acetic acid was added 50 mg of bismuth trioxide. Then the mixture was heated according to the previously reported procedure (Baran, 1958). When the product was subjected to spectrometric analysis, the following characteristics were obtained: $\lambda_{max}^{CH_3OH}$ 282 mμ and $\epsilon_{1cm}^{1\%}$ 902.

TREATMENT OF I WITH METHANOLIC KOH. Treatment of I (50 μg) with 0.1 N KOH in methanol (1.5 ml) at 60° for 3 hr resulted a product which showed the absorption maximum at 283.5 mμ in the ultraviolet spectrum. This was the significant shift from the absorption maximum at 240 mμ of the initial product. The $R_F$ value of the product was 0.88 in the thinlayer chromatogram developed in the benzene–acetone (3:1, v/v) system, which coincided with the dehydrated product of authentic 7α-hydroxyandrostenedione. Provided that the reaction product be androsta-4,6-diene-3,17-dione as suggested from the above observation, molecular extinction coefficient of the product was calculated as 27,000.

*Removal of the Tritium Labeled at C-7 Position of Androstenedione by the Testicular Microsomal Hydroxylase and Chloranil.* 7-Tritiated androstenedione (63.53 × $10^4$ cpm) was mixed with [4-$^{14}$C]androstenedione (8.94 × $10^4$ cpm) and nonradioactive one (350 mμmoles), and then the mixture was incubated with the testicular microsomal fraction. After incubation, the recovered substrate and the isolated products were analyzed for two kind of radioactivities due to the different nuclides. While the testosterone obtained as a metabolite showed the same ratio of tritium to radiocarbon as the ratio of the recovered substrate, the ratios of $^3H:^{14}C$ in the fractions of I and II were remarkably reduced, indicating that the 7-tritiated androstenedione was partly converted into 7α-hydroxyandrostenedione, after removal of a part of the tritium labeled at C-7 by replacement of it with hydroxy group (Table III).

TABLE II: Effect of Aerobic, Anaerobic, and CO Atmospheres on Testicular Microsomal Metabolism of Androstenedione.[a]

| Steroids | Gas Phase | | |
|---|---|---|---|
| | $O_2$ | Ar | CO |
| Substrate Unchanged | 24.2 | 25.7 | 22.8 |
| Metabolites | | | |
| Testosterone | 40.6 | 69.8 | 102.6 |
| Compound I | 51.1 | 24.9 | 1.6 |
| Compound II | 21.2 | 11.7 | 0.9 |

[a] [4-$^{14}$C]Androstenedione (175 mμmoles, 6.6 × $10^4$ cpm) was incubated with testicular microsomal fraction of rats (corresponding to four animals) in the presence of NADPH (1 mg) under the three different atmospheres for 60 min. Yields of steroid were expressed in millimicromoles, which were estimated from the radioactivities.

On the other hand, the mixture of nonradioactive androstenedione (7 μmoles), 4-$^{14}$C-labeled one (8.94 × $10^4$ cpm), and 7-tritiated one (63.53 × $10^4$ cpm) was dissolved in 2.0 ml of *t*-butyl alcohol, and then to the solution was added 500 mg of chloranil (tetrachloro-*p*-benzoquinone). The reaction was performed according to the method of Campbell and Babcock (1959). The reaction product, or androsta-4,6-diene-3,17-dione which was purified with the thin-layer chromatography showed $\lambda_{max}^{CH_3OH}$ 284 mμ in the ultraviolet absorption spectrum, and the ratio of $^3H:^{14}C$ of the product was remarkably reduced, being in agreement with the ratios of I and II obtained from [4-$^{14}$C,7-$^3$H]androstenedione by the testicular microsomal fraction. Simultaneously, the mixture of [4-$^{14}$C]androstenedione (10.4 × $10^4$ cpm), [1,2-$^3$H]-androstenedione (74.1 × $10^4$ cpm), and nonradioactive androstenedione (350 mμmoles) was incubated under the same condition, no significant decrease of the tritium relative to the radiocarbon was observed in the fractions of 7α-hydroxylated androstenedione and testosterone.

*Configuration of the Hydroxy Group Introduced to C-7 of Androstenedione.* 7α-Hydroxyandrostenedione and its 7β epimer were mutually separable on thin-layer chromatogram developed in the benzene–acetone (8:2, v/v) solvent system. When the radioactive I which was derived from [4-$^{14}$C]-androstenedione was mixed with nonradioactive 7α- and 7β-hydroxyandrostenedione, and the mixture was subjected to the above-stated thin-layer chromatographic analysis, the radioactivity due to I was found exclusively on the spot of the carrier 7α-hydroxyandrostenedione. This configuration was further supported by the result obtained by crystalliza-

TABLE III: Removal of the Tritium Labeled at C-7 of Androstenedione by Testicular Microsomal Hydroxylase and Chloranil.

| Mixture | Ratio of the Two Nuclides, $^3H:^{14}C$ | | | | | |
|---|---|---|---|---|---|---|
| | Incubation | | | | Chloranil Treatment | |
| | Substrate (Androstenedione) | Testosterone | I | II | Before (Androstenedione) | After (Androsta-4,6-diene-3,17-dione) |
| [7-$^3$H]Androstenedione and [4-$^{14}$C]androstenedione | 7.11 | 7.11 | 3.94 | 3.94 | 7.11 | 3.92 |
| [1,2-$^3$H]Androstenedione and [4-$^{14}$C]androstenedione | 7.12 | 7.10 | 7.00 | 7.43 | | |

TABLE IV: Chromatographic Mobilities of I and Authentic 7$\alpha$-Hydroxyandrostenedione and Their Derivatives.

| | Solvent System A[a] | | | | | Solvent System B[b] | |
|---|---|---|---|---|---|---|---|
| | Free | *O*-Methyloxime | | $(CH_3)_3Si$ | *O*-Methyloxime-$(CH_3)_3Si$ | *O*-Methyloxime-$(CH_3)_3Si$ | |
| Authentic 7$\alpha$-hydroxyandrostenedione | 0.23 | 0.41[c] | 0.50[c] | 0.73 | 0.93 | 0.44[c] | 0.54[c] |
| Compound I | 0.21 | 0.43 | 0.53 | 0.74 | 0.93 | 0.41 | 0.52 |

[a] Solvent system A, benzene–acetone (4:1, v/v). [b] Solvent system B, *n*-heptane–ethyl acetate (5:2, v/v). [c] *syn–anti* isomer.

tion of I with 7$\alpha$-hydroxyandrostenedione which will be discussed later.

*O-Methyloxime and Trimethylsilyl Derivatives of I.* *O*-Methyloxime of I was prepared as follows: To I was added 1 ml of pyridine solution contained 10 mg of *O*-methylhydroxylamine hydrochloride (Tokyo Chemical Industry Co., Tokyo). The reaction mixture was heated at 60° with stirring for 4 hr or overnight at room temperature (Fales and Luukkainen, 1965). At the end of reaction, pyridine was evaporated to dryness under a stream of nitrogen and then to the residue was added 30 ml of ethyl acetate. Ethyl acetate extract was washed with 5 ml of 10% NaCl–5% $Na_2CO_3$ aqueous solution, three times. As the *O*-methyloxime of I, the two stereoisomers or *syn–anti* isomers with the different $R_F$ values on thin-layer chromatogram were obtained.

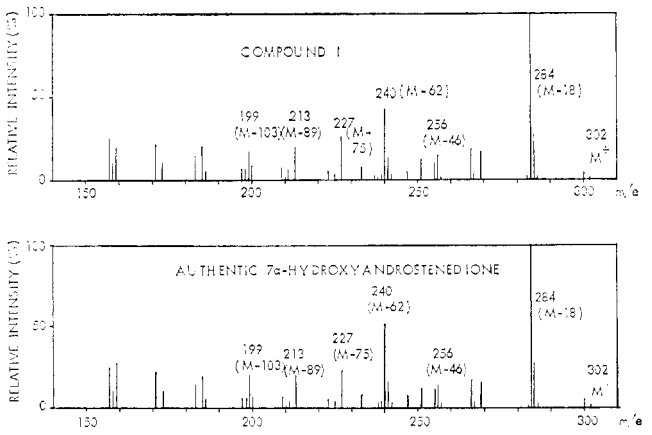


FIGURE 2: Mass spectra of I (190 $\mu$g) and authentic 7$\alpha$-hydroxyandrostenedione (150 $\mu$g) obtained with a mass spectrometer (Model RMS-4). For detail, see Materials and Methods.

A part of the isomer mixture of I *O*-methyloxime was further subjected to formation of trimethylsilyl ether by the following procedure; to the sample which was dried under reduced pressure in a desiccator, 0.5 ml of *N,O*-bis(trimethylsilyl)acetamide (Tokyo Chemical Industry Co., Tokyo) was added and left at room temperature for 20 min. Then, the *O*-methyloxime trimethylsilyl ether of I was purified by thin-layer chromatography. As shown in Table IV, the $R_F$ values of the free and several derivatives of I on the thin-layer chromatography agreed, respectively, with the mobilities of authentic 7$\alpha$-hydroxyandrostenedione and its corresponding derivatives.

*Mass Spectrometric Analysis of I.* The mass spectrometric analysis revealed that I was identical with authentic 7$\alpha$-hydroxyandrostenedione, as shown in Figure 2. The authentic 7$\alpha$-hydroxyandrostenedione gave no significant intensity as a molecular ion at *m/e* 302 either, but one of main peaks was observed at *m/e* 284, which corresponds to the dehydrated fragment (M − 18). The hydroxy group at the 7$\alpha$ position was easily and characteristically dehydrated by pyrolysis and/or electron shower. However, the *O*-methyloxime of I

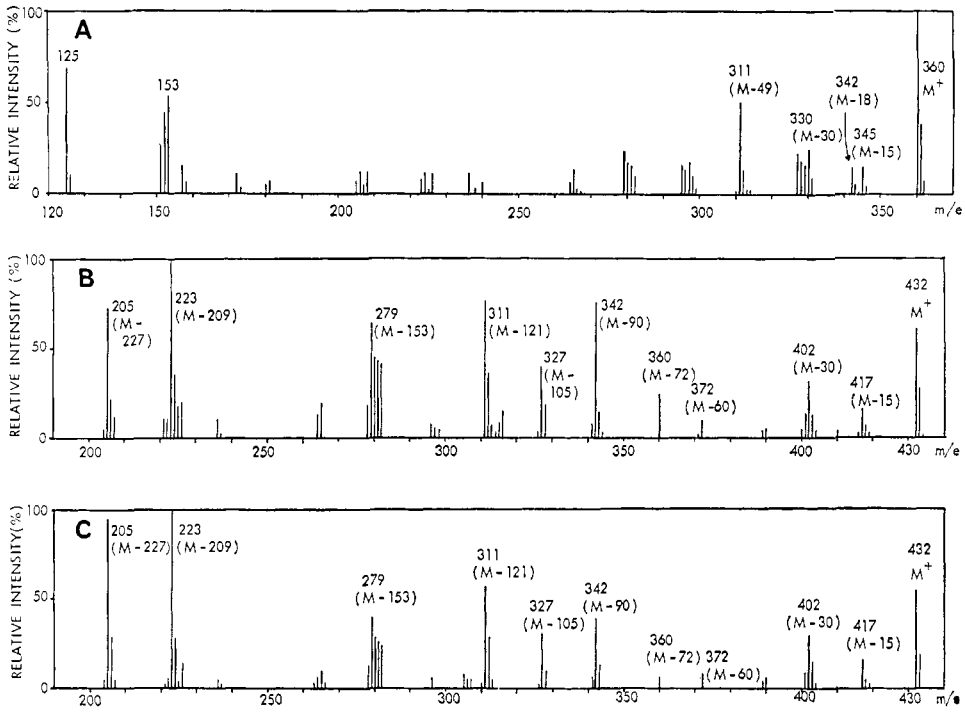


FIGURE 3: Mass spectra of (A) I-*O*-methyloxime (40 μg) and (B) I-*O*-methyloxime trimethylsilyl ether (10 μg) using mass spectrometer (Model RMU-6), and (C) authentic 7α-hydroxyandrostenedione-*O*-methyloxime trimethylsilyl ether (200 μg) measured with mass spectrometer (Model RMS-4). For detail, see Materials and Methods.

showed relatively strong intensity at *m/e* 360, which was regarded as the molecular ion peak, as shown in Figure 3. Fragment peaks were also observed at *m/e* 311 (M − 49) or [M − (30 + $H_2O$ + H)], 153 (M − 207) and 125 (M − 235). The fragment at *m/e* 311 is produced due to the loss of water and hydrogen from the fragment peak at *m/e* 330 or (M − 30). The loss of a fragment whose mass number was 30 is characteristic to *O*-methyloxime and is caused due to cleavage of the N–O bond.

The *O*-methyloxime trimethylsilyl derivatives of I gave molecular ion at *m/e* 432. The base peak is at *m/e* 223 (M − 209) and significant peaks are also observed at *m/e* 402 (M − 30) and *m/e* 372 or [M − (30 × 2)]. The loss of a fragment with mass 30 is due to cleavage of the N–O bond. The peak at *m/e* 342 or (M − 90) represents the loss of trimethylsilanol or $(CH_3)_3SiOH$. Pronounced peaks are found at *m/e* 360 or (M − 72), 327 or [M − (90 + 15)], 311 or [M − (90 + 30 + hydrogen)], 279 or (M − 153) and 205 or (M − 227).

*Sulfuric Acid Chromogen of I.* Compound I was dissolved in the concentrated sulfuric acid so as to be 10 μg/ml as the final concentration. After a certain time interval, the spectra were recorded over the range between 200 and 600 mμ using a recording spectrophotometer (Cary Instrument, Model 14), with concentrated sulfuric acid alone as the reference. The wavelengths of the characteristic maxima and minima in the spectra of the sulfuric acid chromogen of I and authentic 7α-hydroxyandrostenedione were found identical with each other, as shown in Table V.

*Recrystallization of I.* From the above-stated results, I was most likely identical with 7α-hydroxyandrostenedione. Therefore, the radioactive I was repeatedly crystallized with authentic 7α-hydroxyandrostenedione, and the specific activities of the crystals obtained through the repeated crystallization were found to be constant within the experimental error, as shown in Table VI.

Though the available amount of II (as shown in Figure 1) was limited for the identification, II was tentatively identified as 7α-hydroxytestosterone from the following results: (1) By the double-isotope experiment as shown in Table I, compound II was suggested as derived directly from testosterone. (2) As II formation of II was remarkably reduced under anaerobic condition and CO atmosphere, II was postulated as a hydroxylated testosterone (Table II). (3) The $R_F$ values of II and its derivatives on thin-layer chromatograms were identical with those of 7α-hydroxytestosterone and its corresponding derivatives. (4) The radioactive II was repeatedly crystallized with authentic 7α-hydroxytestosterone, and the specific activities of the crystals obtained through repeated crystallization were found to be constant within the experimental error, as shown in Table VI.

From the above-stated experimental results, I and II were identified, respectively, as 7α-hydroxylated androstenedione and testosterone.

## Discussion

Compound I had an ultraviolet absorption maximum at 240 mμ, indicating that the metabolite still retained the $\Delta^4$-3-oxo group in the A ring. Therefore, the possibility that I be of 4-hydroxy-$\Delta^4$-3-oxo structure could be excluded, as this structure characteristically absorbs ultraviolet light at 280 mμ in the enolized form (Dorfman, 1953). The results of acetylation indicated that I retained acetylatable hydroxy

TABLE V: Maximum and Minimum Wavelengths of Sulfuric Acid Chromogen Spectra of I and Authentic 7α-Hydroxyandrostenedione.

| Steroid | Time (min) | Absorption Max. (mμ) ($\epsilon^{1\%}_{1\,cm}$) | Absorption Min (mμ) |
|---|---|---|---|
| Authentic 7α-hydroxyandrostenedione | 13 | 284.0 (116) | 234.6 |
| | | 355.2[a] | 325.0 |
| | | 372.9 (75) | |
| | 120 | 285.9 (320) | 235.9 |
| | | 357.9[a] | 322.8 |
| | | 374.7 (319) | 410.0 |
| | | 452.4 (75) | |
| Compound I | | | |
| | 13 | 286.7 (194) | 238.4 |
| | | 358.2[a] | 334.9 |
| | | 374.5 (76) | |
| | 120 | 286.7 (534) | 239.4 |
| | | 358.8[a] | 331.7 |
| | | 374.4 (350) | 410.0 |
| | | 450.0 (37) | |

[a] Shoulder.

TABLE VI: Identification of Radioactive I and II by Recrystallization.

| No. of Recrystallization | Specific Activities of Crystals (cpm/mg) | |
|---|---|---|
| | I | II |
| First | 707 | 302 |
| Second | 683 | 300 |
| Third | 684 | 295 |
| Fourth | 683 | 300 |
| Fifth | 678 | |
| Calculated | 686[a] | 315[b] |

[a] Calculated specific activity was estimated from the radioactivity 4600 cpm divided by 6.7 mg of authentic 7α-hydroxyandrostenedione. [b] Calculated specific activity was estimated from the radioactivity 3560 cpm divided by 11.3 mg of authentic 7α-hydroxytestosterone.

group(s) with acetic anhydride in pyridine, but no other hydroxy group such as 11β-hydroxy group which is not acetylated with the above reagent, as the acetate of I was resistant to the $CrO_3$ oxidation. The result due to the treatment of I with bismuth trioxide suggested that I is not 2α-hydroxy-$\Delta^4$-3-oxosteroid, whose oxidation product by the above procedure, or 2-hydroxy-$\Delta^{1,4}$-3-oxo compound showed the absorption maximum at 252–254 mμ (Baran, 1958). Therefore, the shift of absorption maximum from 240 to 282 mμ by the treatment of I with this reagent in glacial acetic acid occurred, not because of oxidation, but due to dehydration by the acetic acid used as solvent at 95°. If the product was assumed as androsta-4,6-diene-3,17-dione, the molecular extinction coefficient was calculated as ε 25,600 from the experimental value $\epsilon^{1\%}_{1cm}$ 902, in agreement with the value calculated from the spectrum of the dehydrated I by treatment with methanolic alkali. On the other hand, the androstenediones, hydroxylated at 6β, 7β, 11β, and 16α positions run on the thin-layer chromatogram with the different mobilities to the $R_F$ value of I, when developed in the benzene–acetone (4:1, v/v) system. By the mass spectrometric analysis, monohydroxylated compounds of androstenedione at other positions such as C-15 (Zaretskii *et al.*, 1966) and C-19 (Eggers, 1965) showed significant intensity as the molecular ion peak at *m/e* 302 and the relatively low intensity at *m/e* 284 as the dehydrated fragment peak. The *O*-methyloxime and *O*-methyloxime trimethylsilyl derivative of I showed relatively strong intensity at *m/e* 360 and 432 which were regarded as the molecular ion peaks, respectively. These results suggested that I itself was monohydroxy diketone of $\Delta^4$-$C_{19}$ steroid. By the treatment of I with methanolic KOH, the absorption maximum in the ultraviolet spectrum is significantly shifted from 240 to 283.5 mμ, and molecular extinction coefficient increased remarkably. These are characteristic of the conjugated dienone, or 4,6-dien-3-one structure, which was produced by dehydration of 7α-hydroxy-$\Delta^4$-3-oxosteroid, introducing another double bond between C-6 and C-7 (Bernstein *et al.*, 1959). In this connection, the conjugated dienone structure showed its maximum around 281 to 284 mμ while molecular extinction coefficient become 25,000–30,000 (Dorfman, 1953).

$\Delta^4$-3-Oxosteroid which has been subjected to dehydrogenation by chloranil is generally converted into the corresponding $\Delta^6$ derivative after removal of two protons at 6β and 7α position (Campbell and Babcock, 1959; Agnello and Laubach, 1960; Brodie *et al.*, 1965). By dehydrogenation of [4-$^{14}$C, 7-$^3$H]androstenedione with chloranil, the reduced ratio of $^3H$:$^{14}C$ in the fraction of androsta-4,6-diene-3,17-dione is in agreement with the ratios of I and II converted from the mixture of [4-$^{14}$C,7-$^3$H]androstenedione by the microsomal fraction of rat testes. Those results would indicate that the hydroxylated position in I would be C-7, being consistent with the previous result of the dienone formation of I with methanolic KOH.

As the reason why I was produced even in the atmosphere of Ar, it was considered that, as the molecular oxygen which was dissolved in the liquid phase was not completely removed by evacuation, the remaining oxygen was utilized by the enzyme for the hydroxylation. On the other hand, the cytochrome P-450 was converted into the reduced cytochrome P-450–CO complex in the presence of $NADPH_2$ by introduction of carbon monoxide, and accordingly the hydroxylating system was completely inactivated even in the presence of the dissolved oxygen. Therefore, the cytochrome P-450 detected spectrometrically in the testicular smooth-surfaced microsomal fraction was related not only to the 17α-hydroxylase and $C_{17}$-$C_{20}$-lyase (Inano *et al.*, 1970), but also to the 7α-hydroxylase. The 7α-hydroxylase in the homogenates of rat liver which converted dehydroepiandrosterone into 7α-hydroxydehydroepiandrosterone was completely inhibited by addition of Amphenon B or 3,3-bis(*p*-aminophenyl)-2-butanone dihydrochloride in a concentration of 5 ×

$10^{-4}$ M (Stárka and Kůtová, 1962). Conney *et al.* (1968) reported that the metabolism of testosterone to 7α-hydroxytestosterone by rat liver microsomal fraction was inhibited by CO.

The result that the 17β-hydroxysteroid dehydrogenase activity was apparently enhanced along with decrease of the 7α-hydroxylase activity due to the anaerobic condition indicated that atmospheric concentration of oxygen in the course of incubation has a significant role in controlling the testosterone production by regulating the two activities of the 17β-hydroxysteroid dehydrogenase and the 7α-hydroxylase, both of which utilized androstenedione as the common substrate.

On the other hand, the 6β-hydroxylase (Tamaoki and Shikita, 1966), 15α-hydroxylase (Neher and Wettstein, 1960), 16α-hydroxylase (Oshima *et al.*, 1967), 17α-hydroxylase (Shikita *et al.*, 1964), 19-hydroxylase (Oh and Tamaoki, 1970), and 21-hydroxylase (Dominguez *et al.*, 1960) were also reported to exist in the testicular tissues. 7α-Hydroxylated products of testosterone (Conney and Klutch, 1963; Gustafsson *et al.*, 1968; Jagarinec *et al.*, 1967), dehydroepiandrosterone (Šulcová and Stárka, 1968; Stárka, 1965), androstenedione (Gold and Garren, 1964), and pregnenolone (Stárka *et al.*, 1966) were obtained by incubation with microsomal fraction of the liver. It was observed by Heinrichs *et al.* (1967) that the hepatic 7α-hydroxylase activities of rat and rabbit were uniformly distributed between the rough- and smooth-surfaced microsomal subfractions. In the microbiological study, progesterone and 11-deoxycorticosterone were converted into their 7α-hydroxylated analogs with a *Helminthosporium* culture and to their 7β-hydroxylated analogs with a *Cladiosporium* culture (McAleer *et al.*, 1958). Šulcová and Stárka (1968) isolated 7β-hydroxydehydroepiandrosterone besides its 7α epimer, when dehydroepiandrosterone was incubated with the hepatic microsomal fraction of rats. In the present experiment, however, exclusive 7α hydroxylation of androstenedione was demonstrated by incubation with testicular microsomal fraction of rats, while testosterone was also solely 7α hydroxylated by the hepatic microsomal fraction (Lisboa *et al.*, 1968); Conney *et al.*, 1969; Jacobson and Kuntzman, 1969).

As 7α-hydroxyandrostenedione was often obtained in higher yield than testosterone itself by the mature rat testes from androstenedione, physiological role of the metabolite in relation to testicular endocrine function and/or to regulation of androgen production and its secretion by the hydroxylated steroid would be postulated.

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# Sequence Analysis of Complex Protein Mixtures by Isotope Dilution and Mass Spectrometry*

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ABSTRACT: A volatile Edman reagent, methyl isothiocyanate, is used to degrade sequentially polypeptide mixtures from the N-terminal end. Standard mixtures of $^{15}N$-enriched amino acids or their methylthiohydantoin derivatives are added to the reaction. Increase in the $^{14}N:^{15}N$ isotope ratio is observed when $^{14}N$ amino acid methylthiohydantoins derived from the N-termini of the polypeptide chains dilute the standard mixture. The extracted methylthiohydantoins are transferred to the sample probe of a single-focusing mass spectrometer; the temperature of the probe is raised gradually and the methylthiohydantoin amino acids are volatilized sequentially into the ion beam, identified, and their $^{14}N:^{15}N$ isotope ratio established. By these means an artificial mixture of polypeptide chains has been shown to have four components; the quantity of each component present has been measured, the amino acid sequence of the N-terminal ten residues has been established for both of the two major sequences present, and the amino acids present at each locus have been determined for the first ten residues of the two minor sequences which were present in equimolecular proportions. This information is obtained simultaneously from a single operation which is less time consuming and laborious than the analysis of the first ten amino acid residues from a single polypeptide chain by conventional means. The rates of loss of reactive N-terminal groups have been studied when A and B chains of insulin were sequenced simultaneously. The loss rates were 2.5 and 0.9% per residue, respectively. Recovery rates of $^{15}N$-labeled methylthiohydantoin amino acids using $^{15}N$ amino acids or their methylthiohydantoin derivatives established that either may be used for isotope dilution and that losses of the thiazolinone intermediate to compounds other than the methylthiohydantoin derivatives are not quantitatively important. Enolase was shown to have identical sequences for the first eight amino acids from the amino-terminal end in each of the two subunits by this method.

Many proteins occur in a microheterogeneous state. Such proteins form families in which the individual members are distinguished from each other by single or relatively limited amino acid residue sequence changes. These changes may reflect genetic polymorphism in protein structure (Dixon, 1966), the action of enzymes on the protein, or may be concerned directly with the function of the protein. Sometimes a single amino acid replacement may cause a major change in the physical characteristics of the protein, facilitating isolation of the variant (Zuckerkandl, 1968). It has been established that antibodies are collections of heterogeneous proteins, differing in amino acid residue sequence in limited regions of the molecule (Edelman and Gall, 1969). It has not so far proved possible to separate these closely related proteins into single species by conventional means although less heterogeneous populations have been produced (Miller *et al.*, 1967; Richards *et al.*, 1969; Brenneman and Singer, 1969) or isolated from more heterogeneous populations (Eisen and Siskind, 1964).

A method for the quantitative sequential degradation of a mixture of proteins (or peptides) from the N-terminal residue is described in this paper. It is an adaption of the Edman procedure using volatile reagents, coupled with isotope dilution and the use of the single-focusing mass spectrometer employed in its triple capacity as a differential vacuum distillation apparatus, an isotope ratio assay instrument (Biemann, 1962) and employed for the determination of residue structure by means of the molecular ion and fragmentation peak pattern of the mass spectrum. This quantitative sequential degradation method is designed to study microheterogeneity both in antibodies (Richards *et al.*,

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