Metabolism *in Vitro* of [4-¹⁴C] Testosterone by Liver Homogenates of Normal and Carbon Tetrachloride Injured Rats*

Nobuko Sato, Minoru Ota and Kijuro Obara

*Department of Biochemistry (Prof. K. Obara),
Iwate Medical College, Morioka*

[4-¹⁴C] testosterone was incubated with liver homogenates of normal or carbon tetrachloride (CCl₄) injured male rats in the presence of NAD at 37°C for 90 min. The metabolites produced were identified by thin layer chromatography and chemical transformation.

In the normal liver homogenates, 5 α -androstane-3 α , 17 β -diol, epiandrosterone and 16 α -hydroxy testosterone were the predominant metabolites amounting to as much as 72% of the total metabolites.

In the injured liver homogenates, an unmetabolized substrate was detected in a large amount. Δ^4 -androstene-3,17-dione, 5 α -androstane-3,17-dione and androsterone were present in larger amounts, while the amount of their end product, 5 α -androstane-3 α , 17 β -diol, was less than that in the normal. 16 α -, 7 α - and 6 β -hydroxy testosterone were in smaller amounts compared with those in the normal.

These findings suggest that the hydroxylation of testosterone and the reduction of the 17-ketone to a 17-hydroxyl, *i.e.*, the conversion of androsterone to 5 α -androstane-3 α , 17 β -diol, might be impaired by the administration of CCl₄.

The metabolism of [¹⁴C] or [³H] testosterone in human subjects under normal and various pathological states has been studied¹⁻³ and the metabolism *in vivo* of testosterone in rats has also been studied.^{4,5} However, the overall metabolism *in vitro* of this substance by normal and CCl₄-injured liver tissues of rats is not yet clear. In the present study, [¹⁴C] testosterone was incubated with a normal or CCl₄-injured liver homogenate, and the metabolites were identified and their radioactivities were measured.

MATERIALS AND METHODS

Tissue preparation and incubation

Forty-eight hours after the intraperitoneal injection of corn oil (0.33 ml per 100 g body weight) to a male Wistar rat weighing about 200 g, the rat was decapitated. The liver was removed quickly and 0.5 g of the median lobe was homogenized with 1.5 ml of

Received for publication, April 9, 1969.

* Supported in part by a grant-in-aid for scientific research from the Ministry of Education.

0.1 M Krebs-Ringer phosphate buffer (pH 7.4) in a Potter-Elvehjem type glass homogenizer at 4°C. One μ c of [$4\text{-}^{14}\text{C}$] testosterone (specific activity: 50.6 mc/mM, purchased from the New England Nuclear Corp., Boston, Mass., U.S.A.) dissolved in 0.1 ml of ethyl alcohol and 500 μ g NAD in 1.0 ml of 0.1 M Krebs-Ringer phosphate buffer (pH 7.4) were added to a flask containing 1.5 ml of the homogenates. The mixture was incubated at 37°C for 90 minutes under an atmosphere of 95% oxygen and 5% carbon dioxide, with continuous shaking.

The liver of the CCl_4 injured rat was processed in the same manner as mentioned above at 48 hours after the intraperitoneal injection of CCl_4 (0.33 ml. of 10% (v/v) CCl_4 in corn oil per 100 g body weight).

Extraction and separation of products

The incubation mixture was shaken four times with 10 ml portions of dichloromethane. The combined dichloromethane extract was washed with 10 ml of 1 N NaOH and 10 ml portions of water three times. The extracts were then evaporated to dryness under a stream of nitrogen. After addition of 10 ml of water to the dried residue, an extraction was made three times with 20 ml portions of ether. The combined ether phases were evaporated and then the residue was redissolved in 2.0 ml of ethyl alcohol. An 0.1 ml aliquot of the ethanol solution and suitable reference steroids were applied to a thin layer of silica gel about 0.25 mm in thickness (Wakogel B-5 containing 5% calcium sulfate as a binder, Wako Pure Chemical Industries, Ltd., Japan) and were chromatographed at room temperature, using mostly a benzen-aceton (80:20 in vol.) system suitable for separation of C_{19} -steroid metabolites (B. Tamaoki, personal communication).

After completing the development of the thin layer plate, the radioactive spots were detected by an autoradiographic method of exposing x-ray film to the coated phase of the plate for a week. The silica gel scraped from the areas of the radioactive steroids on the chromatogram detected by autoradiography was transferred into small test tubes and the steroids were extracted by ethyl alcohol from the silica gel. The ethyl alcohol extracts were evaporated and a steroid fraction was analyzed for radioactivity with a gas flow counter (Aloka TDC-2, J.R.C., Tokyo, Japan).

The following procedures were also used for the spots which were difficult to identify by Rf values. The spot on a thin layer chromatogram, which was scraped off with a spatula and transferred into a small tube, was extracted with ethyl alcohol, and a portion of the extract obtained was used for acetylation or oxidation.

Acetylation. The extract was evaporated under a stream of nitrogen and the dried residue was dissolved in acetic anhydride and pyridine (1:3). The acetylated steroids were extracted with chloroform. After the solvent was evaporated, the residue was redissolved in ethyl alcohol. The ethanol solution was applied to a thin layer plate and the shifting of the Rf value was determined.

Oxidation. The dried residue was dissolved in acetic acid and an aqueous solution of chromic acid according to Liberman *et al.*⁶

Detection of authentic steroids

The plate was sprayed with a 50% sulphuric acid in methanol, followed by heating at 110°C for 15 minutes.

The standard steroids were purchased from Sigma Chemical Co., Mann Research Laboratories Inc., and Ikapharm, Dr. Ickelheimer & Co., Ltd.

RESULTS

The Rf values of authentic standards on thin layer chromatogram were 0.04 for 16 α , 17 β -dihydroxy- Δ^4 -androst-3-one (16 α -hydroxy testosterone), 0.12 for 6 β , 17 β -dihydroxy- Δ^4 -androst-3-one (6 β -hydroxy testosterone), 0.16 for 6 β -

hydroxy- Δ^4 -androstene-3, 17-dione, 0.17 for 5β -androstane-3 α , 17 β -diol, 0.29 for 17 β -hydroxy- Δ^4 -androstene-3-one (testosterone), 3 α -hydroxy- 5β -androstane-17-one (etiocholanolone) and Δ^4 -androstene-3 β , 17 β -diol, 0.32 for 3 β -hydroxy- 5α -androstane-17-one (epiandrosterone), 0.33 for 17 β -hydroxy- 5β -androstane-3-one, 0.36 for 3 α -hydroxy- 5α -androstane-17-one (androsterone), 0.37 for 17 β -hydroxy- 5α -androstane-3-one, 0.42 for Δ^4 -androstene-3,17-dione, 0.51 for 5α -androstane-3,17-dione and 0.57 for $\Delta^{4,16}$ -androstadiene-3-one. The autoradiogram of the metabolites of [$4\text{-}^{14}\text{C}$] testosterone obtained by incubation of liver homogenates of male rats is illustrated in Fig. 1. The autoradiographic pattern of the metabolites obtained from the liver impaired by CCl_4 was essentially similar to that from the normal liver and no additional spot was detected.

Fig. 1. Autoradiogram of the thin-layer chromatogram of the radioactive metabolites of [$4\text{-}^{14}\text{C}$] testosterone obtained from liver homogenate of a male rat incubated at 37°C for 90 min.

The metabolites located in Zones 1-3

These highly polar steroids in and near the origin of the chromatogram were acetyltable and oxidizable by the procedures previously mentioned. From their chromatographic behavior, it seemed that they could be 16 α -, 7 α - and 6 β -hydroxy testosterone. The steroids in Zones 2 and 3 were estimated to be 16 α -hydroxy testosterone and 6 β -hydroxy testosterone, respectively, because of the same Rf value as that of the corresponding authentic steroid. The steroid in Zone 1 was presumed to be 7 α -hydroxy testosterone, based on the Rf value and the data reported by Conney and Klutch⁷.

The metabolites located in Zones 4 and 5

There were two zones which were very close or occasionally partially overlapped. As these zones were unseparable by thin layer chromatography, they were acetylated with acetic anhydride and pyridine after they were combined with carrier 5β -androstane-3 α , 17 β -diol and 6 β -hydroxy- Δ^4 -androstene-3,17-dione. Two

spots appeared on the autoradiogram—one was dark, less polar, indicating a large amount of a radioactive metabolite and the other was very faint. The former coincided with the acetylated 5β -androstane-3 α , 17 β -diol and the latter with the acetylated 6 β -hydroxy- Δ^4 -androstene-3,17-dione. Using a chloroform-ethyl alcohol (90:10 in vol.) solvent system, where 6 β -hydroxy- Δ^4 -androstene-3,17-dione showed a lower polarity than 5β -androstane-3 α , 17 β -diol, the radioactive spot was concentrated mainly at the location of the carrier 5β -androstane-3 α , 17 β -diol and partially overlapped with 6 β -hydroxy- Δ^4 -androstene-3,17-dione. Using a chloroform-ethyl alcohol (95:5 in vol.) solvent system, Rf was 0.43 for testosterone, 0.43 for 16 α -hydroxy- Δ^4 -androstene-3,17-dione and 0.30 for 6 β -hydroxy- Δ^4 -androstene-3,17-dione, respectively. Because of its higher polarity than these metabolites located in Zones 3 and 4, the possibility of 16 α -hydroxy- Δ^4 -androstene-3,17-dione was ruled out.

The metabolite located in Zone 6

The steroid located in Zone 6 was oxidizable and acetylable, and the mobility of the oxidized or acetylated product on thinlayer chromatography showed the same value as for the corresponding product of 5α -androstane-3 α , 17 β -diol or 5α -androstane-3 β , 17 β -diol. However, this steroid in Zone 6 was considered to be 5α -androstane-3 α , 17 β -diol, because it is a more likely metabolite than 5α -androstane-3 β , 17 β -diol in testosterone metabolism.

The metabolites located in Zone 7

Zone 7 covered a wide dark area on the autoradiogram. There was a possibility that 5α -androstane-3 β , 17 β -diol (Rf 0.27), testosterone (Rf 0.29), epiandrosterone (Rf 0.32) and 17 β -hydroxy- 5β -androstane-3-one (Rf 0.33) were involved in Zone 7 in the solvent system previously mentioned. After acetylation of Zone 7, two radioactive spots appeared on thin layer chromatography. One of them, which was in a large amount, had the same Rf as for the acetylated substances of etiocholanolone and epiandrosterone. The acetylated product of the other spot which was present in trace amounts in the normal liver and in large amounts in the injured liver showed similar polarity to acetylated testosterone. The Rf of acetylated Δ^4 -androstene-3 β , 17 β -diol was 0.60, whereas that of the acetylated product of the main steroid in Zone 7 was 0.57.

After oxidation, two spots appeared on chromatography. The one showed the same Rf as for 5α -androstane-3,17-dione, whereas the other showed the same Rf as for Δ^4 -androstene-3,17-dione.

Using an ether solvent system where Rf was 0.41 for epiandrosterone, 0.34 for testosterone and 0.29 for etiocholanolone, the mobility of the substance which existed in large amounts showed the same Rf as for epiandrosterone, and a trace of the substance in the normal liver. The latter was, however, detected in a large amount in the injured liver.

By using the Girard reagent in the usual manner, testosterone, etiocholanolone

and epiandrosterone were present in the ketonic fraction, whereas Δ^4 -androstene-3 β , 17 β -diol, 5 α -androstane-3 β , 17 β -diol and 17 β -hydroxy-5 β -androstan-3-one were present in the nonketonic fraction. After the reaction of the substances in Zone 7 with the Girard T reagent, the ratio of radioactivities in the ketonic and the non-ketonic fraction was approximately 5:1 in the normal and injured livers.

In the normal liver, the substance in the ketonic fraction was presumed to be epiandrosterone and the substance in the non-ketonic fraction was thought to be 17 β -hydroxy-5 β -androstan-3-one. A trace of testosterone was also found. In the injured liver, a large amount of testosterone was detected in the ketonic fraction.

The metabolite located in zone 8

The acetylated product of the radioactive steroid located in Zone 8 had similar polarity to acetylated androsterone, 17 β -hydroxy-5 α -androstan-3-one and 17 β -hydroxy-5 β -androstan-3-one. By oxidation, androsterone and 17 β -hydroxy-5 α -androstan-3-one to 5 α -androstane-3,17-dione, and 17 β -hydroxy-5 β -androstan-3-one converted to 5 β -androstane-3,17-dione. The oxidized product of this spot showed a mobility similar to 5 α -androstane-3,17-dione and its epimer. As 17 β -hydroxy-5 α -androstan-3-one and the 5 β -epimer do not react in the usual way with the Girard reagent, this spot was reacted with a Girard T reagent and the radioactivities in the ketonic and non-ketonic fractions were determined. The radiocount in the ketonic fraction was about 540 times higher than in the non-ketonic fraction. Therefore, this substance in Zone 8 was presumed to be androsterone.

The metabolites located in zones 9 and 10

Zones 9 and 10 were identified as Δ^4 -androstene-3,17-dione and 5 α -androstane-3,17-dione, respectively, from their characteristic Rf values.

The metabolites located in zones 11 and 12

Traces of less polar radioactive substances were found in Zones 11 and 12. Zone 11 was presumed as $\Delta^{4,16}$ -androstadien-3-one from its characteristic Rf value. No attempt was, however, made to characterize a substance in Zone 12.

Percentages of the specific radioactivities of the individual metabolites to the total radioactivity

The free metabolites of [4- 14 C] testosterone identified in the present study are summarized in Table 1. The average percentages of the radioactivities of the individual metabolites to the total radioactivity obtained in three normal animals or in three CCl₄ injected animals are also shown in this table.

Examination of hepatic function

The glutamic pyruvic transaminase in the serum obtained at the decapitation was 66 and 1,600 King-Armstrong units in the normal and the injured rats,

TABLE 1. *Identified metabolites of [4-¹⁴C] testosterone by normal and CCl₄-injured rat liver homogenates**

Zone	Metabolites	Percentage of radioactivity	
		normal	CCl ₄ -injured
		%	%
1	7 α -hydroxy testosterone (?)	8.4	1.2
2	16 α -hydroxy testosterone	16.0	2.2
3	6 β -hydroxy testosterone	3.4	1.7
4	6 β -hydroxy- Δ^4 -androstene-3,17-dione	0.1	0.1
5	5 β -androstane-3 α , 17 β -diol	4.4	2.6
6	5 α -androstane-3 α , 17 β -diol	38.6	20.1
7	testosterone	0.1	9.6
	epiandrosterone	17.5	20.8
	17 β -hydroxy-5 β -androstan-3-one	3.4	6.3
8	androsterone	5.6	15.4
9	Δ^4 -androstene-3,17-dione	1.2	13.5
10	5 α -androstane-3, 17-dione	0.8	5.8
11	$\Delta^4,16$ -androstadien-3-one	0.3	0.1
12	unidentified	0.3	0.8

* [4-¹⁴C] testosterone was incubated with liver homogenates of male rats administered with and without CCl₄. See the details in the text.

respectively. There were, however, no differences in the thymol turbidity and the cephalin-cholesterol flocculation tests.

DISCUSSION

The metabolism of androgens by slices and homogenates of human or other mammalian livers has not been studied as thoroughly as C₂₁-steroids and even the identification of the metabolites has not been established completely. In earlier studies, Kochakian *et al.*⁸ reported that a homogenate of male guinea pig liver was able to metabolize testosterone to Δ^4 -androstene-3, 17-dione, and no other metabolites were detected. They also found an increase of the metabolic rate by the addition of nicotinamide and NAD to the incubation medium. Stylianou *et al.*⁹ identified five metabolites, Δ^4 -androstene-3,17-dione, 5 β -androstane-3 α , 17 β -diol, 5 α -androstane-3 α , 17 β -diol, 5 α -androstane-3 β , 17 β -diol and $\Delta^4,16$ -androstadien-3-one, by incubation of [4-¹⁴C] testosterone with a homogenate of a human liver biopsy sample. Using thin layer chromatography and various color reactions, Lisboa *et al.*¹⁰ identified the following ten metabolites— Δ^4 -androstene-3, 17-dione, 5 α -androstane-3, 17-dione, 5 β -androstane-3, 17-dione, 17 β -hydroxy-5 α -androstan-3-one, 17 β -hydroxy-5 β -androstan-3-one, 5 α -androstane-3 α , 17 β -diol, 5 α -androstane-3 β , 17 β -diol, 5 β -androstane-3 α , 17 β -diol, 6 β -hydroxy- Δ^4 -androstene-3, 17-dione and 16 α -hydroxy- Δ^4 -androstene-3,17-dione—as the metabolites of testosterone by human liver slices without NAD or a NADPH-generating system.

Only free metabolites were studied in the present experiment, because the authors have demonstrated that the majority of the metabolites of testosterone were present in a free form after 90 minutes incubation.¹¹

5 α -androstane-3 α , 17 β -diol, 16 α -hydroxy testosterone and epiandrosterone were predominant testosterone metabolites by normal liver homogenates in the present study. Conney and Klutch⁷ isolated polar products of testosterone metabolism including 7 α , 6 β -, 16 α - and 2 β -hydroxy testosterone and demonstrated that androgen hydroxylase in liver microsomes of rats required reduced NADP and oxygen for activity. In the present study, hydroxy testosterone, 16 α -, 6 β - and 7 α -hydroxy testosterone occupied one-fifth of the total metabolites in the normal liver.

In the metabolites produced by the CCl₄-injured liver, these hydroxy testosterone were remarkably lower than those by the normal liver and occupied only 5% of the total metabolites. These findings indicated that hydroxylation of testosterone is impaired by the administration of CCl₄.

In the normal rat, 5 α -androstane-3 α , 17 β -diol and epiandrosterone accounted for as much as 56% of the total testosterone metabolites by liver homogenates, whereas Δ^4 -androstene-3,17-dione and 5 α -androstane-3,17-dione were present in far less amounts. This indicates that the reduction of the 4-5 double bond and the subsequent reduction of the carbonyl group at C-3 occurred rapidly. Scarcely any of the unmetabolized substrate, testosterone, remained in the normal liver. This might be accounted for by the long period of incubation of this substrate with liver homogenates. The results also indicate that, under the conditions used *in vitro*, the liver tissue had both 3 α - and 3 β -hydroxysteroid dehydrogenases.

Androsterone was easily converted into 5 α -androstane-3 α , 17 β -diol by reduction of the carbonyl groups at C-17, whereas its 3 β -epimer seemed to be the end-product in the pathway of 3 β -hydroxy steroid.

Because of the fact that C₁₉-steroids with a 5 α -H configuration predominated over their isomers, it could be assumed that the Δ^4 -5 α -hydrogenase activity was significantly higher than the Δ^4 -5 β -hydrogenase in the liver of male rats.

In the CCl₄-injured liver, a large amount of testosterone was unmetabolized, and Δ^4 -androstene-3,17-dione, 5 α -androstane-3,17-dione and androsterone were detected in a larger amount compared to those in the normal liver. On the other hand, their further metabolite, 5 α -androstane-3 α , 17 β -diol, was in a smaller amount. These observation might suggest that the conversion of androsterone into 5 α -androstane 3 α , 17 β -diol, namely the reduction of the 17-ketone to a 17-hydroxyl, is impaired by CCl₄.

In the present study, a less polar radioactive metabolite was found in a trace amount (Zone 12). This might well be a monooxygenated C₁₉-steroid, because of its lower polarity in the chromatographic behavior.

Etiocolanalone which is a common metabolite in human urine could not be detected in the present study. It has been shown that only male rat livers are capable of converting Δ^4 -androsterone-3,17-dione into 5 β -androstane-3,17-dione.

Mosebach *et al.*¹² demonstrated large amounts of etiocholanolone and androsterone in the livers of male rats as the initial metabolites of intraperitoneally injected testosterone. In the present study, no evidence was obtained for the presence of etiocholanolone, at least not in detectable amounts.

5 β -androstane-3 α , 17 β -diol is produced by two pathways, one is through Δ^4 -androstene-3,17-dione — 5 β -androstane-3,17-dione — etiocholanolone, the other through 17 β -hydroxy-5 β -androstane-3-one. However, this fact does not explain the absence of one of its precursors, etiocholanolone, in our study, because of the low amount of 5 β -androstane-3 α , 17 β -diol. It is difficult to draw any conclusion about the 5 β -pathway due to the limited data of the experiment at present. However, attention should be paid to the reports by Stylianou *et al.*⁹ and Lisboa *et al.*¹⁰ in which etiocholanolone could not be detected in their studies of human liver tissues.

Acknowledgment

We are grateful to Dr. B. Tamaoki, National Institute of Radiological Sciences, and Prof. K. Shimizu, Tottori University School of Medicine, Japan for their advice. We are also indebted to Dr. D.V.K. Murthy, Synthex Research Center, Palo Alto, Calif., U.S.A., for the supply of 6 β -hydroxy testosterone and Δ^4 ,16-androstadien-3-one.

References

- 1) Dorfman, R.I. & Ungar, F. *Metabolism of the Steroid Hormones*. Burgess Publishing Co., Minneapolis., 1953.
- 2) Baulieu, E.E. & Mauvais-Jarvis, P. Studies on testosterone metabolism I. Conversion of testosterone 17 α -³H to 5 α - and 5 β -androstane 3 α , 17 β diol-17 α -³H: a new "17 β hydroxyl pathway". *J. biol. Chem.*, 1964, **239**, 1569–1577.
- 3) Baulieu, E.E. & Mauvais-Jarvis, P. Studies on testosterone metabolism II. Metabolism of testosterone-4-¹⁴C and androst-4-ene-3,17-dione-1,2-³H. *J. biol. Chem.*, 1964, **239**, 1578–1584.
- 4) ElAttar, T., Mosebach, K.-O. & Dirscherl, W. The initial metabolism of 4-¹⁴C-testosterone in rat. II. *Acta endocr.*, 1964, **45**, 437–446.
- 5) ElAttar, T., Dirscherl, W. & Mosebach, K.-O. The initial metabolism of 4-¹⁴C-testosterone in rat. III. *Acta endocr.*, 1964, **45**, 527–534.
- 6) Lieberman, S., Katzenellenbogen, E.R., Schneider, R., Studer, P.E. & Dobriner, K. Isolation of urinary steroids after cortisone and adrenocorticotrophic hormone. *J. biol. Chem.*, 1953, **205**, 87–91.
- 7) Conney, A.H. & Klutch, A. Increased activity of androgen hydroxylases in liver microsomes of rats pretreated with phenobarbital and other drugs. *J. biol. Chem.*, 1963, **238**, 1611–1617.
- 8) Kochakian, C.D., Raut, V. & Nall, D.M. Metabolism of testosterone by guinea pig liver and kidney homogenates. *Amer. J. Physiol.*, 1957, **189**, 78–82.
- 9) Stylianou, M., Forchielli, E., Tummillo, M. & Dorfman, R.I. Metabolism *in vitro* of 4-¹⁴C-testosterone by a human liver homogenate. *J. biol. Chem.*, 1961, **236**, 692–694.
- 10) Lisboa, B.P., Drosse, I. & Breuer, H. Stoffwechsel von Testosterone in Leberschnitten des Menschen. *Hoppe-Seyler's Z. physiol. Chem.*, 1965, **342**, 123–131.
- 11) Obara, K., Sato, N. & Ota, M. *In vitro* metabolism of testosterone in rat liver. I. Conjugation of testosterone in the injured liver induced by carbon tetrachloride. *Igaku to Seibutsugaku (Jap.)*, 1968, **76**, 159–162.
- 12) Mosebach, K.-O., Dirscherl, W., Rath, K. & Rieck, G. Die initialen Metabolite von Testosterone in den Zellfraktionen der Rattenleber und -niere *in vivo*. *Hoppe-Seyler's Z. physiol. Chem.*, 1966, **344**, 197–208.