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TESTOSTERONE INHIBITION OF 6β-, 7α- AND 16α-HYDROXYTESTOSTERONE METABOLISM BY RAT LIVER MICROSOMES*

M. JACOBSON,† W. LEVIN and R. KUNTZMAN

Wellcome Research Laboratories, Burroughs Wellcome & Co. (U.S.A.) Inc., Tuckahoe, N. Y. 10707, U.S.A.

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Abstract—Testosterone-4-^{14}C (900 mμmoles) is metabolized to 6β-, 7α- and 16α-hydroxytestosterone *in vitro* by liver microsomes from adult male rats, but not to compounds more polar than these three products. The formation of 6β-, 7α- and 16α-hydroxytestosterone increases linearly with tissue concentration and incubation time. However, in the absence of testosterone, 6β-, 7α- and 16α-hydroxytestosterone-4-^{14}C are metabolized by enzymes in liver microsomes to more polar compounds that absorb ultra-violet light. This metabolism of 6β-, 7α- and 16α-hydroxytestosterone to polar compounds is inhibited by the addition of testosterone to the incubation medium. The profile of testosterone metabolites formed by liver microsomes depends on the concentration of testosterone in the incubation mixture. When a high concentration of testosterone -4-^{14}C is used as the substrate, less of the 6β-, 7α- and 16α-hydroxytestosterone formed is further metabolized to highly polar substances than when a low concentration of testosterone-4-^{14}C is used as the substrate. This inhibition of the metabolism of 6β-, 7α- and 16α-hydroxytestosterone by testosterone is shown to be competitive.

THE HYDROXYLATION of testosterone in the 6β-, 7α- and 16α-positions by liver microsomal enzymes has been studied in recent years to acquire more knowledge about the enzyme systems responsible for microsomal oxidations. In the course of these studies, differences in the regulation of these three pathways of testosterone metabolism by liver microsomes have been found.[1–5] Since the rate of testosterone hydroxylation was measured in the above experiments by assay of 6β-, 7α- and 16α-hydroxytestosterone formation, it was necessary that these three products not be further metabolized under the incubation conditions which were used.

The studies presented here indicate that 6β-, 7α- and 16α-hydroxytestosterone are rapidly metabolized by liver microsomal enzymes in the absence of testosterone and that this metabolism is blocked in the presence of testosterone, so that in the usual incubation system, the three hydroxylated products formed from testosterone accumulate.

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MATERIALS AND METHODS

Enzyme assays. Liver from adult male rats was homogenized in 2 vol. of 0·25 M sucrose solution and microsomes were prepared as previously described.[6] The microsomal pellets were washed twice by resuspension in 0·25 M sucrose solution followed by recentrifugation. The microsomes were then resuspended in 0·1 M KH_2PO_4–K_2HPO_4 buffer, pH 7·4, so that each ml contained microsomes from 1 g of liver.

Microsomes obtained from adult male rat liver were incubated aerobically for 5, 10 or 15 min with either testosterone-4-^{14}C, 6β-hydroxytestosterone-4-^{14}C, 7α-hydroxytestosterone-4-^{14}C or 16α-hydroxytestosterone-4-^{14}C at 25° in the presence of an NADPH-generating system previously described.[7] A 2-ml portion of the 2·5-ml incubation mixture was taken at zero time, or after incubation, and transferred to tubes containing 30 ml of dichloromethane. The tubes were shaken for 30 min and centrifuged. Although testosterone and 6β-, 7α- and 16α-hydroxytestosterone were completely extracted into the dichloromethane, in experiments where more polar metabolites of the three hydroxylated compounds were formed, 90–100 per cent of the total radioactivity was recovered. A 20-ml portion of the organic phase was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 1·0 ml of methanol. An exact portion (100 μl) was applied to Whatman No. 1 filter paper for chromatography.

When the amount of 6β-, 7α or 16α-hydroxytestosterone formed from testosterone was to be quantified individually, the extracts were chromatographed descending for 48 hr with the organic phase of a 2,2,4-trimethylpentane:toluene:methanol:water (3:5:4:1) system after overnight equilibration with the aqueous phase (Fig. 1). Under

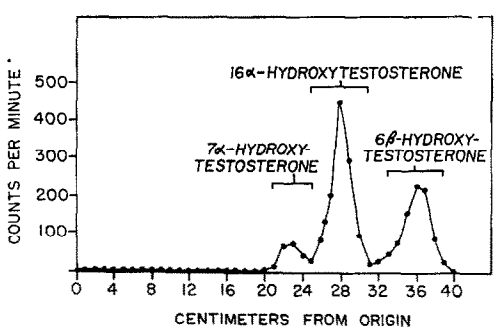

FIG. 1. Liver microsomal metabolism of testosterone to 6β-, 7α- and 16α-hydroxytestosterone. Microsomes from 100 mg of liver were incubated aerobically with 900 mμmoles (0·2 μc) of testosterone-4-^{14}C for 15 min at 25° in the presence of an NADPH-generating system as described under Materials and Methods. The separation of 6β-, 7α- and 16α-hydroxytestosterone from testosterone and its other metabolites was accomplished by 48-hr descending chromatography in the organic phase of a 2,2,4-trimethylpentane : toluene : methanol : water (3:5:4:1) system.

these conditions, testosterone and several metabolites of this steroid ran off the end of the filter paper. When the total amount of 6β-, 7α and 16α-hydroxytestosterone formed from testosterone and the amount of polar metabolites formed from the three hydroxylated compounds were to be quantified, the extracts were chromatographed descending with the organic phase of a system consisting of toluene:methanol:water

(2:1:1) after overnight equilibration with the aqueous phase (Fig. 2). The chromatography was stopped when the solvent front reached the end of the filter paper. The chromatograms were cut into 1- or 2-cm strips and the radioactivity measured in a liquid scintillation spectrometer utilizing the scintillation mixture of Bray.[8]

Isolation of 6β-, 7α- and 16α-hydroxytestosterone to be used for metabolic studies. Five flasks, each containing 14 μmoles (40 μc) of testosterone-4-^{14}C, were incubated for 15 min at 37° with microsomes from 6·66 g of adult male rat liver in the presence of an NADPH-generating system.[6] The volume of each incubation mixture was 114 ml. A 100-ml aliquot was taken from each incubation mixture and extracted by shaking for 45 min with 7 vol. of dichloromethane. After centrifugation, the dichloromethane extracts were pooled and evaporated to dryness in a 1 l. round bottom flask using a rotary vacuum evaporator. The residue was transferred with four washes of 10 ml of methanol each to a 60-ml round bottom flask. The 40 ml of methanol solution were evaporated to dryness and the residue was dissolved in 5 ml of methanol. Portions of 1·25 ml were applied at the origin over a 13-cm distance to each of four sheets of Whatman No. 3MM chromatography paper alongside reference standards of 6β-, 7α- and 16α-hydroxytestosterone. The 6β-, 7α- and 16α-hydroxytestosterone-4-^{14}C were separated by descending chromatography (48 hr) in a 2,2,4-trimethylpentane: toluene:methanol:water (3:5:4:1) system. The radioactivity corresponding to authentic 6β-, 7α- and 16α-hydroxytestosterone was eluted from the four chromatograms with methanol and the eluates of each metabolite were combined. Rechromatography of a small amount of each solution in the 2,2,4-trimethylpentane:toluene:methanol: water: (3:5:4:1) system and the toluene:methanol:water (2:1:1) system indicated that

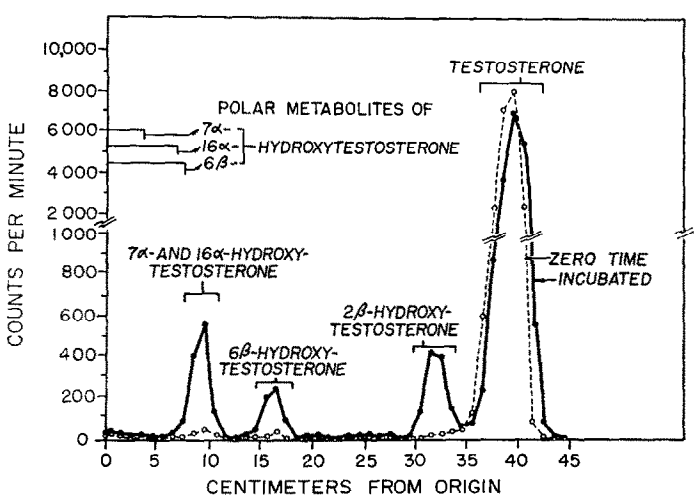

FIG. 2. Liver microsomal metabolism of testosterone to polar metabolites. Microsomes from 100mg of liver were incubated aerobically with 900 mμmoles (0·2 μc) of testosterone-4-^{14}C for 15 min at 25° in the presence of an NAPDH-generating system as described under Materials and Methods. The extracts were chromatographed descending in the organic phase of a toluene : methanol : water (2:1:1) system until the solvent reached the end of the paper. The brackets labeled "Polar Metabolites of 7α-, 16α- and 6β-Hydroxytestosterone" indicate the areas where polar metabolites of these three hydroxylated compounds would have been found had they been formed.

the radioactivity in each eluate had the same mobility as authentic 6β-, 7α- or 16α-hydroxytestosterone and absorbed ultraviolet light.

RESULTS

The metabolism of testosterone to 6β-, 7α- and 16α-hydroxytestosterone by rat liver microsomes. Testosterone is metabolized by male rat liver microsomes in the presence of an NADPH-generating system to 6β-, 7α- and 16α-hydroxytestosterone,[6, 9, 10] but not to metabolites more polar than these three hydroxylation products (Figs. 1 and 2). The formation of the three hydroxylated metabolites from 900 mμmoles of testosterone in the presence of microsomes from various amounts of liver can be seen in Fig. 3. The formation of 6β-, 7α- and 16α-hydroxytestosterone was linear with the concentration of microsomes, and was also linear with respect to incubation time (Fig. 4). Although these results suggest that 6β-, 7α- and 16α-hydroxytestosterone are not further metabolized by liver microsomal enzymes in the presence of testosterone under the above incubation conditions, further experiments were done to determine whether this is so.

Hepatic metabolism of 6β-, 7α- and 16α-hydroxytestosterone to polar metabolites. Large quantities of ^{14}C-labeled 6β-, 7α- and 16α-hydroxytestosterone were prepared by incubating testosterone-4-^{14}C with liver microsomes, and these products were subsequently isolated by extraction, chromatography and elution as described in the Materials and Methods section. When 22 mμmoles of 6β-, 7α- and 16α-hydroxytestosterone were incubated with liver microsomes, the three compounds were metabolized to ultraviolet absorbing products chromatographically more polar than the starting substrates (Fig. 5). Thus, a disparity exists between these data and the data

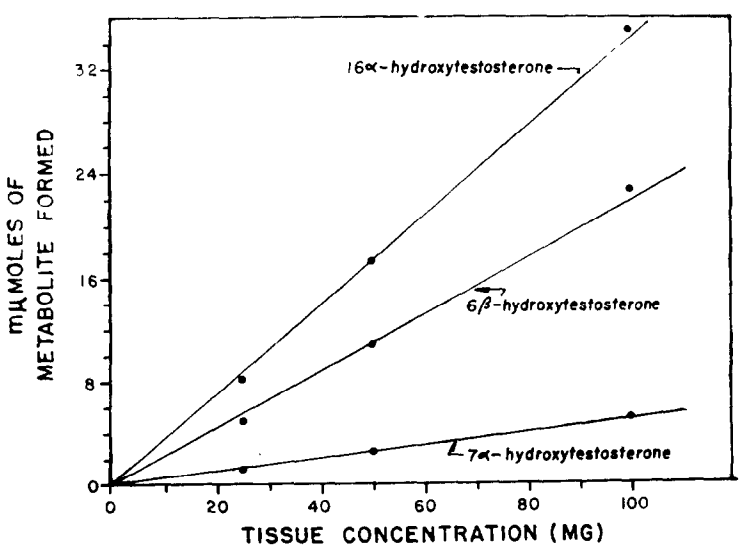

FIG. 3. The effect of tissue concentration on the metabolism of testosterone to 6β-, 7α- and 16α-hydroxytestosterone. Testosterone-4-^{14}C (900 mμmoles/0·2 μc) was incubated aerobically with microsomes obtained from various amounts of liver for 15 min at 25° in the presence of an NADPH-generating system. The formation of 6β-, 7α- and 16α-hydroxytestosterone was quantified as described under Materials and Methods.

in Figs. 1–4, which had suggested that the 6β-, 7α- and 16α-hydroxytestosterone formed during incubations with testosterone were not further metabolized. These observations prompted studies to determine whether testosterone could inhibit the metabolism of 6β-, 7α- and 16α-hydroxytestosterone.

Hepatic metabolism of 6β-, 7α- *and* 16α-*hydroxytestosterone in the presence of testosterone.* When 6β-, 7α- and 16α-hydroxytestosterone-4-^{14}C were incubated in the presence of various amounts of nonlabeled testosterone, an inhibition of the metabolism of hydroxylated testosterone was observed (Table 1). It is seen that 9·6 mμmoles, 7·3 mμmoles and 11·3 mμmoles of polar metabolites were formed from 22 mμmoles of 6β-, 7α- and 16α-hydroxytestosterone, respectively, in the absence of testosterone, while only 0·2–0·4 mμmoles of polar metabolites were formed in the

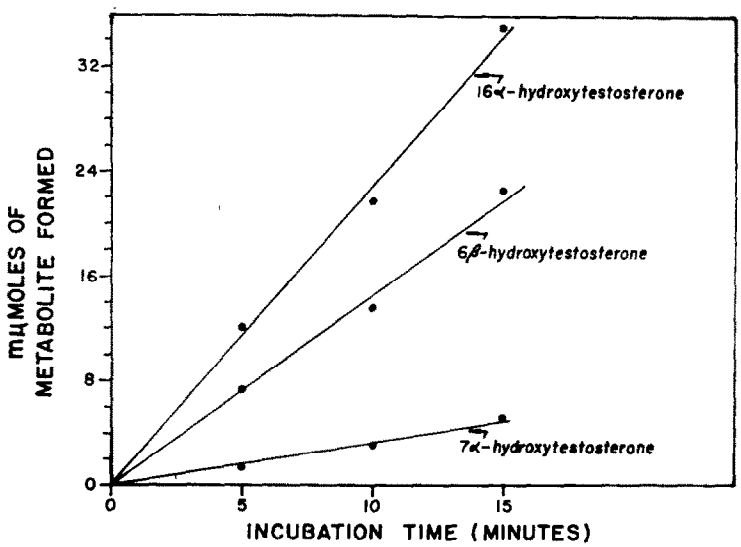

FIG. 4. The effect of incubation time on the metabolism of testosterone to 6β-, 7α- and 16α-hydroxytestosterone. Microsomes from 100 mg of liver were incubated aerobically with 900 mμmoles (0·2 μc) of testosterone-4-^{14}C for varying periods of time at 25° in the presence of an NADPH-generating system. The formation of 6β-, 7α- and 16α-hydroxytestosterone was quantified as described under Materials and Methods.

presence of 700 mμmoles of testosterone. Several concentrations of 6β-, 7α and 16α-hydroxytestosterone were incubated alone or with 50 mμmoles of nonlabeled testosterone. The amount of polar metabolites formed from 6β-, 7α- and 16α-hydroxytestosterone was quantified and plotted against initial substrate concentration according to the method of Lineweaver and Burk.[11] The results shown in Fig. 6 indicate that the testosterone inhibition of the metabolism of 6β-, 7α- and 16α-hydroxytestosterone to polar metabolites is competitive.

*Hepatic metabolism of high and low concentrations of testosterone-*4-^{14}*C.* Various concentrations of radioactive testosterone were incubated with liver microsomes. Total 6β-, 7α- and 16α-hydroxytestosterone and total polar metabolites of 6β-, 7α- and 16α-hydroxytestosterone were separated and quantified as described in

the Materials and Methods section. The results of the studies are shown in Table 2 and indicate that the pattern of metabolite formation depends on the substrate concentration. When 16 mμmoles of testosterone were incubated with microsomes from 100 mg of liver, 19·5 per cent of the 6β-, 7α- and 16α-hydroxytestosterone formed was metabolized to compounds more polar than the monohydroxylated testosterones, whereas none of the 6β-, 7α- and 16α-hydroxytestosterone was further metabolized to more polar compounds when 164 mμ moles of testosterone was incubated with liver

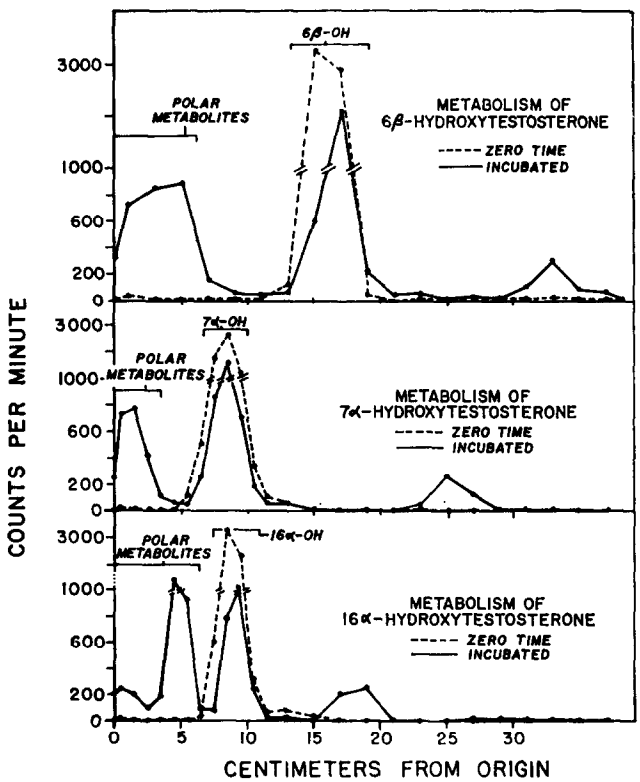

FIG. 5. Liver microsomal metabolism of 6β-, 7α- and 16α-hydroxytestosterone to polar metabolites. Microsomes from 100 mg of liver were incubated aerobically with 22 mμmoles (0·07 μc) of 6β-, 7α- and 16α-hydroxytestosterone-4-^{14}C for 15 min at 25° in the presence of an NADPH-generating system. The polar metabolites formed were separated from 6β-, 7α- and 16α-hydroxytestosterone by descending chromatography in the organic phase of a toluene : methanol : water (2:1:1) system as described under Materials and Methods.

microsomes Similar results were obtained when testosteroesne metabolismw as determined in the presence of microsomes obtained from 167 mg of liver: 75·8 per cent of the 6β-, 7α- and 16α-hydroxytestosterone formed from 16 mμmoles of testosterone was metabolized to more polar compounds, while only 11·6 per cent of the 6β-, 7α- and 16α-hydroxytestosterone formed from 164 mμmoles of testosterone was metabolized to more polar compounds. Thus, the ratio of the amount of 6β-, 7α- and 16α-hydroxytestosterone to the amount of more polar metabolites increased with increasing

TABLE 1. TESTOSTERONE INHIBITION OF THE METABOLISM OF 6β-, 7α- AND 16α-HYDROXYTESTOSTERONE BY RAT LIVER MICROSOMES*

Amount of testosterone (mμmoles)	mμmoles Polar metabolites formed from		
	6β-hydroxy-testosterone	7α-hydroxy-testosterone	16α-hydroxy-testosterone
0	9·6	7·3	11·3
50	2·9	2·6	4·3
200	1·0	1·3	0·9
700	0·4	0·4	0·2

*Twenty-two mμmoles (0·07 μc) of each substrate were incubated aerobically with liver microsomes obtained from 100 mg of liver for 15 min at 25° in the presence of an NADPH-generating system. The amount of polar metabolites formed was quantified as described under Materials and Methods.

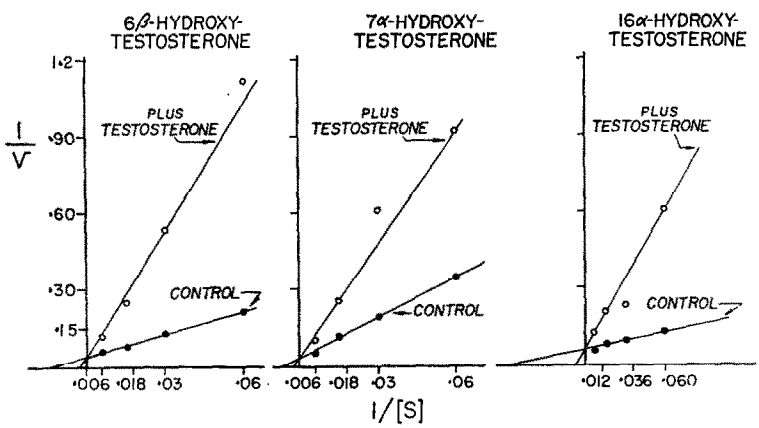

FIG. 6. 1/v versus 1/S diagram of the liver microsomal metabolism of 6β-, 7α- and 16α-hydroxytestosterone. Microsomes from 100 mg of liver were incubated aerobically for 15 min at 25° with 16, 33, 67 or 164 mμmoles of 6β-, 7α- and 16α-hydroxytestosterone-4-^{14}C alone or with 50 mμmoles of non-labeled testosterone in the presence of an NADPH-generating system. Each incubation flask contained the same amount of radioactivity (0·05 μc). The amount of polar metabolites formed was quantified as described under Materials and Methods. v = mμmoles of polar metabolites formed from 6β-, 7α- and 16α-hydroxytestosterone. S = mμmoles of substrate/2·5 ml incubation mixture.

testosterone concentration in the incubation mixture. These results demonstrate that in the presence of a high concentration of testosterone, 6β-, 7α- and 16α-hydroxytestosterone are not further metabolized by liver microsomes (Fig. 2), but that in the absence of testosterone, the monohydroxy metabolites are rapidly metabolized.

DISCUSSION

When testosterone-4-^{14}C is incubated with liver microsomes from male rats in the presence of an NADPH-generating system, 6β-, 7α- and 16α-hydroxytestosterone are formed.[6, 9, 10] The data presented in this paper show that metabolites more polar than

6β-, 7α- and 16α-hydroxytestosterone are not formed when 900 mμmoles of testosterone are incubated (Figs. 1 and 2) and that the formation of these three hydroxylated metabolites of testosterone is linear with time and with the concentration of liver microsomes (Figs. 3 and 4). However, when 6β-, 7α- and 16α-hydroxytestosterone were isolated from an incubation mixture and reincubated with male rat liver microsomes in the absence of testosterone, all three compounds were metabolized.

The disparity between these two sets of data was explained by experiments in which nonradioactive testosterone was added to an incubation mixture containing radioactive 6β-, 7α- and 16α-hydroxytestosterone. When the concentration of testosterone in the incubation medium was increased, the metabolism of 6β-, 7α- and 16α-hydroxytestosterone to polar metabolites decreased. These results suggest that testosterone

TABLE 2. PROFILE OF METABOLITES FORMED DURING THE INCUBATION OF HIGH AND LOW CONCENTRATIONS OF TESTOSTERONE*

	Experiment 1		Experiments 2	
Testosterone per incubation (mμmoles)	Total 6β-, 7α- and 16α-hydroxytestosterone formed (mμmoles)	Total polar metabolites of 6β-, 7α- and 16α-hydroxytestosterone formed (mμmoles)	Total 6β-, 7α- and 16α-hydroxytestosterone formed (mμmoles)	Total polar metabolites of 6β-, 7α- and 16α-hydroxytestosterone formed (mμmoles)
16	6·06	1·47	2.88	7·47
33	11·06	1·58	7·06	12·22
67	16·21	0·94	22·68	11·66
164	23·78	0·00	50·02	6·56

*Various amounts of testosterone-4-^{14}C were incubated with liver microsomes obtained from 100 mg (Experiment 1) and 167 mg (Experiment 2) of liver for 15 min at 25° in the presence of an NADPH-generating system. Each incubation flask contained the same amount of radioactivity (0·05 μc). The amount of 6β-, 7α- and 16α-hydroxytestosterone formed and the total amount of polar metabolites of 6β-, 7α- and 16α-hydroxytestosterone formed were calculated as described under Materials and Methods.

inhibits the further metabolism of the three hydroxylated compounds. Some of the inhibition caused by testosterone could have been due to isotope dilution by nonlabeled hydroxytestosterone formed during the incubation. However, calculations based on the amounts of 6β-, 7α- and 16α-hydroxytestosterone formed from 900 mμmoles of testosterone in 15 min (Fig. 4) indicated that isotope dilution could not have accounted for the 96, 93 and 98 per cent decreases in the metabolism of labeled 6β-, 7α- and 16α-hydroxytestosterone, respectively, shown in Table 1. The awareness that isotope dilution might be a source of error, however, prompted studies on the metabolism of high and low concentrations of radioactive testosterone. When a low concentration of testosterone-4-^{14}C was used as the substrate, the degree of further metabolism of the 6β-, 7α- and 16α-hydroxytestosterone to more polar compounds was high relative to the formation of 6β-, 7α- and 16α-hydroxytestosterone; however, when a high concentration of testosterone-4-^{14}C was used as the substrate, the degree of further metabolism of the three hydroxylated compounds was low relative to 6β-, 7α- and 16α-hydroxytestosterone formation. This inhibition of the metabolism of 6β-, 7α- and 16α-hydroxytestosterone to polar metabolites by testosterone

was further shown to be competitive. It is thus evident from the results presented in this paper that 6β-, 7α- and 16α-hydroxytestosterone are rapidly metabolized by enzymes in liver microsomes in the absence of testosterone, but that these compounds are not metabolized when formed during an incubation of rat liver microsomes with a high concentration of testosterone. This inhibition by testosterone could be due to the fact that a common microsomal drug-metabolizing system functions not only in the hydroxylation of testosterone, but also in the further oxidation of the hydroxylated testosterone metabolites. Drugs and steroids have been known to competitively act as alternative substrates for microsomal enzyme systems.[12–14] For instance, the liver microsomal *N*-demethylation of ethylmorphine has been shown to be competitively inhibited by drugs such as hexobarbital, zoxazolamine, phenylbutazone and 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A), while ethylmorphine and chlorpromazine have been shown to inhibit the metabolism of each other.[12, 13] Similarly, estradiol-17β, testosterone and hydrocortisone have been found to competitively inhibit the oxidative metabolism of ethylmorphine and hexobarbital by liver microsomes.[14] Although the most likely possibility is that testosterone inhibits the further metabolism of its products similarly by acting as an alternate substrate, the data presented in this paper do not rule out the possibility that another metabolite of testosterone may be the inhibitor. The observation that the polar metabolites of 6β-, 7α- and 16α-hydroxytestosterone absorb ultraviolet light suggests that the Δ^4-3-keto group of the steroids is intact and that the metabolites of the monohydroxy testosterones possess additional hydroxyl groups. Evidence for the metabolism of testosterone to dihydroxylated products by rat liver microsomes was recently presented by Lisboa *et al.*[10]

The ability of a substrate to inhibit the further metabolism of its metabolites is a general concept and several examples of this are known. Aminopyrine, which is metabolized to monomethyl-4-aminoantipyrine by rat liver microsomes, inhibits the conversion of the latter compound to 4-aminoantipyrine; i.e. the tertiary amine inhibits the conversion of the secondary amine to the primary amine.[15] Similarly, Levin and Conney,[16] in studying the hepatic metabolism of 7,12-dimethylbenz(α)-anthracene to monohydroxymethyl compounds, demonstrated a similar type of inhibition of the further metabolism of these hydroxylated compounds. Thus, it is apparent from these studies and those presented in this paper, that when a low concentration of substrate is used in an incubation medium, a different pattern of microsomal metabolism may be obtained than when a high concentration of substrate is used.

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