THE EFFECT OF DIPHENYLHYDANTOIN IN VITRO ON THE METABOLISM OF TESTOSTERONE BY RAT LIVER SUBCELLULAR FRACTIONS

By
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ABSTRACT

The effect of diphenylhydantoin (DPH) in vitro on the "metabolite profile" of testosterone-4-14C has been studied utilizing incubates of high speed supernatant and microsomal fractions of rat liver. In contrast to earlier experiments with rat liver slices, in which a standard amount of DPH resulted in increased non-polar metabolite production, in the present experiments, when separate subcellular fractions are incubated, no such DPH effect can be detected under the conditions utilized. When certain mixtures of supernatant and microsomal fractions are incubated, however, there is evidence that the DPH augmentation of non-polar metabolite production can again be manifest. An explanation for these results can only be speculated upon at present.

In a previous report from this laboratory (Sholiton et al. 1967) we noted the marked difference in less polar metabolite formation produced from incubation of testosterone* with rat liver slices in the presence and absence of diphenylhydantoin (DPH). With female rat liver slices in the presence of added DPH, androsterone (A) and allo-tetrahydrotestosterone (allo-THT) formation was

* Glossary of trivial names and abbreviations:
Testosterone (T): androst-4-en-17β-ol-3-one.
Androsterone (A): 5α-androstan-3α-ol-17-one.
Allo-tetrahydrotestosterone (Allo-THT): 5α-androstan-3α,17β-diol.
Aetiocholanolone (E): 5β-androstan-3α-ol-17-one.
Tetrahydrotestosterone (THT): 5β-androstan-3α,17β-diol.
significantly enhanced whereas with the male, a definite increase in aetiocholanolone (E) and tetrahydrotestosterone (THT) production was noted.

The purpose of the present study was to attempt to localize the site of action of DPH on the hepatic metabolism of testosterone by utilizing subcellular fractions of rat liver.

METHODS AND MATERIALS

Preparation of Subcellular Fractions

Liver was obtained from adult virgin male and female Wistar rats (averaging 300 g body weight) which had been fed ad libitum. A paté of liver was prepared at 4°C using a tissue press. Homogenization and cell disruption were then accomplished using a teflon homogenizer with a clearance of 0.03 cm at 1000 rpm using 7 to 10 passes while cold in 0.25 M sucrose suspension (10 ml sucrose per g liver). A preliminary centrifugation to separate cellular debris, mitochondria and nuclear fragments was performed in a Spinco preparative ultracentrifuge (Model L-2) at 100 000 g for 5 min at -5°C. The resultant supernatant was then subjected to centrifugation at 100 000 g for 60 min, thereby providing a »soluble fraction« and a microsomal pellet. Microsomes were washed with 0.25 M sucrose and resuspended in 10 ml of 0.25 M sucrose.

Incubation constituents were identical to those described in our previous communication. Approximately 250 000 cpm of testosterone-4-14C (3.3 μg) were incubated with each aliquot of liver subcellular fraction to which a NADPH regenerating system buffered in Krebs-Ringer bicarbonate at pH 7.4 was added. A 95 per cent O2:5 per cent CO2 atmosphere was provided during the course of a 5-hour incubation. Paired samples, one containing DPH (2.5 mg) and the other DPH diluent (control), were incubated and analyzed simultaneously.

Extraction of metabolites was carried out with ethyl acetate and chloroform in a manner previously described (Sholiton et al. 1967). Chloroform extracts were partitioned on a Florisil® column with elution by 4 per cent methanol in chloroform to secure the less polar metabolites, followed by 10 per cent and 25 per cent methanol in chloroform fractions to secure the more polar metabolites.

Chromatographic separation of column eluates on paper was achieved using the systems previously described. Radio-metabolites were located by scanning with a Nuclear-Chicago Actigraph II (Figs. 1 and 2) and eluted with absolute methanol. Identification of radio-metabolites was accomplished by chromatography of acetylated derivatives on paper or by recrystallization to constant specific activity with added reference standards, as previously outlined (Sholiton et al. 1967).

RESULTS

Table 1 depicts the effect of adding a standard amount of DPH (2.5 mg/in incubate) on the metabolism of testosterone-4-14C by the »soluble fraction« and microsomes of female and male rat liver. Although there are some minor differences in mean values, no significant difference in less polar metabolite formation is produced in either male or female »high speed« supernatants of rat liver by the addition of this amount of DPH.
Table 1.
Per cent conversion of testosterone-4-\(^{14}\)C to metabolites by rat liver subcellular fractions

<table>
<thead>
<tr>
<th></th>
<th>Soluble fraction</th>
<th></th>
<th>Microsomes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diluent</td>
<td>DPH</td>
<td>Diluent</td>
<td>DPH</td>
</tr>
<tr>
<td></td>
<td>(\bar{x})</td>
<td>Range</td>
<td>(\bar{x})</td>
<td>Range</td>
</tr>
<tr>
<td>1. Females</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HO-T</td>
<td>3.1</td>
<td>(2.5–3.7)</td>
<td>3.7</td>
<td>(3.2–4.0)</td>
</tr>
<tr>
<td>THT</td>
<td>27.7</td>
<td>(22.8–32.4)</td>
<td>31.4</td>
<td>(24.2–34.6)</td>
</tr>
<tr>
<td>Allo-THT</td>
<td>16.7</td>
<td>(11.8–22.0)</td>
<td>14.8</td>
<td>(12.3–18.6)</td>
</tr>
<tr>
<td>E(^*)</td>
<td>4.8</td>
<td>(0.7–13.2)</td>
<td>5.2</td>
<td>(2.2–13.4)</td>
</tr>
<tr>
<td>A(^*)</td>
<td>21.2</td>
<td>(10.8–31.7)</td>
<td>27.3</td>
<td>(17.1–37.5)</td>
</tr>
<tr>
<td>2. Males</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HO-T</td>
<td>2.1</td>
<td>(0.9–3.2)</td>
<td>2.8</td>
<td>(1.6–4.0)</td>
</tr>
<tr>
<td>THT</td>
<td>8.9</td>
<td>(7.8–10.0)</td>
<td>7.9</td>
<td>(6.2–9.6)</td>
</tr>
<tr>
<td>E(^*)</td>
<td>8.5</td>
<td>(3.3–15.6)</td>
<td>10.2</td>
<td>(6.8–14.0)</td>
</tr>
</tbody>
</table>

\(\*\) Recovery factors utilized

There are striking differences, however, in the less polar metabolite »profile« between the male and female soluble fractions. In the female (as shown in Figs. 1 and 2 and in Table 1) there are considerable amounts of both 5\(\alpha\)- and 5\(\beta\)-ring-A reduced metabolites evident, while in the male only the 5\(\beta\)-reduced forms are noted in sizeable quantity, with only slight amounts of 5\(\alpha\)-reduced end-products detected.

An appraisal of more polar (HO-T) production reveal about 3 per cent conversion in the soluble fraction of both sexes with no DPH effect evident. The significance of this finding cannot be evaluated since only a portion of these polar derivatives is contained in the 4 per cent methanol in chloroform column eluent.

Incubation of microsomes from either sex under the conditions of these experiments result in primarily more polar derivative formation (Table 1, Figs. 1 and 2) with virtually no less polar metabolites evident.

When varying ratios of microsomes and soluble fractions are utilized a DPH effect somewhat comparable to that noted with whole liver slice can be produced (Fig. 3). Whereas mixtures of equivalent amounts of soluble fraction and microsome (gram for gram) result only in polar metabolite production, when the microsomes secured from 0.25 g of liver are added to the soluble fraction secured from 1.0 g of liver, less polar metabolites are formed. With
Radioscans of paper chromatograms of the extracted metabolites of testosterone-4-\(^{14}\)C following simultaneous incubation of female rat liver subcellular fractions. Chromatograms were run for 6 h in system: iso-octane:methanol:water (10:8:2). The peak at the non-polar end represents the radioactivity of \(^{14}\)C ink used as a marker.

this latter mixture, as depicted in Fig. 3, the formation of less polar derivatives is definitely increased by the addition of the standard amount of DPH to the incubation.

**DISCUSSION**

That marked differences exist in testosterone metabolite formation by male and female rat liver has been documented in the past. *Rubin & Dorfman*
Fig. 2.
Radioscans of paper chromatograms of the extracted metabolites of testosterone-4-$^{14}$C following simultaneous incubation of male rat liver subcellular fractions. Chromatograms were run for 6 h in system: iso-octane:methanol:water (10:8:2). The peak at the non-polar end represents the radioactivity of $^{14}$C ink used as a marker.

(1956) reported that testosterone incubated with a liver homogenate from male rats yielded androsterone and epiandrosterone, while similar incubations with female rat liver homogenates yielded androsterone and 17$\beta$-hydroxyandrostane-3-one. Yates et al. (1958) concluded that the 5$\alpha$-$\Delta^{4}$-reductase activity in female rat liver for testosterone as well as other steroid substrates was much greater than in the male. More recently, Kuntzman et al. (1966) have shown that the hydroxylation of testosterone to polar metabolites by male rat liver microsomes was several-fold greater than with the female. In contrast, these latter
Fig. 3.
Radioscans of paper chromatograms of the extracted metabolites of testosterone-4-\textsuperscript{14}C following simultaneous incubation of representative mixtures of female rat liver subcellular fractions. A pellet of microsomes represents the microsomes secured from the centrifugation of 1 g of liver resuspended in 10 ml of 0.25 m sucrose.

Investigators found that female rat liver microsomes metabolized testosterone to non-polar ring-A reduced compounds.

The findings of the current study suggest that, under the conditions specified, rat liver microsomes and soluble fraction each yield strikingly different testosterone metabolite «profiles». The incubation of microsomes from either sex results in primarily polar metabolite formation whereas the soluble fractions from either sex produce sizeable percentages of less polar ring-A reduced end-products. These latter have been identified as aetiocholanolone (E) and tetrahydrotestosterone (THT) in the male (Fig. 1), while the female
soluble fractions yields E and THT as well as androsterone (A) and allo-THT (Fig. 2).

It has been effectively demonstrated by studies in the past that steroid $\Delta^4$-reductase activity resides in the subcellular fractions of rat liver (Tomkins & Isselbacher 1954; Forchielli et al. 1958, 1963; McGuire & Tomkins 1960). In a recent review of this subject, Dorfman & Ungar (1965) conclude that for each steroid there are two reducing enzymes in liver, one associated with the microsomal fraction of the cell which produces the $5\alpha$ isomer, and a soluble enzyme which produces the $5\beta$ form. In keeping with this conclusion, it is interesting to note that Chamberlain et al. (1965) have recently prepared aetiocholanolone-4-14C from testosterone-4-14C using a male rat liver soluble fraction and $5\alpha$-$\Delta^4$-reduced radio-metabolites of testosterone-4-14C by using female rat liver microsomes.

Our results demonstrate a $5\beta$-$\Delta^4$-reductase for testosterone to be evident in both male and female »high speed« supernatants. There is $5\alpha$-$\Delta^4$ reducing potential in both the male and female soluble fractions, however. This possibly can be attributed to incomplete separation of microsomes from the supernatant, or to solubilization of microsomal enzymes during fraction preparation. Moreover, the specific conditions under which our studies are carried out, namely, a 5-hour incubation period in the presence of a NADPH generating system at a pH of 7.4, are somewhat different from those of other workers.

When equivalent amounts of soluble fraction and microsomes are reconstituted and incubated, only more polar, presumably hydroxylated, derivatives are formed (Fig. 3). This observation is in keeping with the findings of Chamberlain et al. (1965) who noted the rapid hydroxylation of testosterone metabolites produced by rat liver microsomes. The degree of polar metabolite formation could be altered in our study by recombining varying ratios of microsomal and soluble fractions (Fig. 3).

The DPH effect noted with the liver slice (Sholiton et al. 1967) is not manifest in the present study, wherein a single subcellular fraction is the source of metabolizing enzyme. However, only the standard amount of DPH (2.5 mg per incubate) has been evaluated and it may be that increasing amounts of DPH in vitro could alter testosterone metabolism by these fractions. When the standard amount of DPH is added to mixtures of subcellular fractions (viz., 0.25 gram equivalent of microsomes with 1.0 gram equivalent of soluble fraction) augmentation of metabolite formation is again evident (Fig. 3). The enhancement of non-polar ring-A reduced metabolite production resulting from the addition of DPH under these circumstances suggests that this effect is due to inhibition by DPH of microsomal hydroxylation of testosterone or its reduced metabolites. The nature of this inhibition is speculative at present but the effect of DPH on intracellular electrolyte concentrations, on membrane
permeabilities, or its competition for cofactors or coenzymes while being metabolized, are all aetiological considerations.

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REFERENCES


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