THE EFFECT OF DIPHENYLHYDANTOIN IN VITRO ON THE FORMATION OF THE POLAR METABOLITES OF TESTOSTERONE BY RAT LIVER

By

Leon J. Sholiton, Emile E. Werk and Joseph MacGee

ABSTRACT

Significant inhibition of the conversion of testosterone to polar hydroxylated derivatives can be produced in rat liver slice incubations if large enough amounts of diphenylhydantoin are added. The amount needed to produce this effect is greater in male than in female incubates. The latter under control conditions convert a greater percentage of testosterone to polar metabolites but this difference is eliminated by the addition of diphenylhydantoin at each of the three concentrations utilized in this study.

When the hydroxylation of cortisol or testosterone is induced in the human by the administration of diphenylhydantoin (DPH) or certain barbiturates, ring-A reduction of these steroids is significantly decreased (Werk et al. 1964; Southren et al. 1969). Paradoxically, this relationship in cortisol metabolism can be reversed by incubating DPH with rat liver slices and a cortisol substrate. In this circumstance, \( \Delta^4 \)-reductase activity is enhanced and the formation of 6-hydroxycortisol is substantially diminished (Sholiton et al. 1964).

That a similar phenomenon may exist with testosterone as substrate is suggested by the increase in ring-A reduced metabolites of testosterone produced by incubating 2.5 mg of DPH per gram of liver under the conditions utilized in the cortisol experiments (Sholiton et al. 1967). However, this amount of DPH in vitro failed to result in a reciprocal decrease in polar hydroxylated testosterone derivatives (HO-T). Indeed, in male liver incubates total polar metabolite formation was enhanced by this level of DPH.

The purpose of the present study was to determine if increasing the amount
of DPH added to a standard liver incubate could produce an inhibition of overall hydroxylation of a testosterone substrate.

METHODS AND MATERIALS

Incubation of adult male and female rat liver slices with radioactive testosterone was performed in a manner previously described (Sholiton et al. 1967). In each incubation flask, approximately 250 000 cpm of [4-14C]testosterone (3.3 µg) was incubated with a 1 g aliquot of liver to which a NADPH regenerating system, buffered in 10 ml of 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-h incubation. From each liver paired aliquots were incubated simultaneously with the following amounts of DPH in diluent or with diluent alone (control):

<table>
<thead>
<tr>
<th>Diluent</th>
<th>DPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 100 µl</td>
<td>2.5 mg</td>
</tr>
<tr>
<td>2. 500 µl</td>
<td>12.5 mg</td>
</tr>
<tr>
<td>3. 1000 µl</td>
<td>25.0 mg</td>
</tr>
</tbody>
</table>

Extraction of the hydroxylated derivatives (HO-T) was carried out with ethyl acetate and chloroform as previously described (Sholiton et al. 1967). Chloroform extracts were partitioned on a Florisil® column and eluted in succession with 4 per cent, 10 per cent and 25 per cent methanol in chloroform.

Paper chromatographic separation of column eluates was achieved using the system: iso-octane:methanol:water (10:8:2) at 22°C for 6 h. In this system, the hydroxylated derivatives of testosterone are localized at the origin, whereas the non-hydroxylated ring-A reduced metabolites migrate dependent on their polarities.

The hydroxylated derivatives were eluted with absolute methanol after localization by radio-scanning. The combined polar fraction from the 4 per cent, 10 per cent and 25 per cent methanol in chloroform eluates were quantitated by counting aliquots in toluene phosphor with a liquid scintillation spectrometer. Conversion to HO-T was calculated by the following formula:

\[
\text{% conversion} = \frac{\text{CPM in combined polar fractions} \times 100}{\text{CPM in substrate per incubate}}
\]

RESULTS

The data presented in Table 1 and graphically depicted in Fig. 1 were subjected to an analysis of variance with the following findings:

A. When diluent alone is added in amounts comparable to that in which DPH was diluted:

1. The females obtain values of HO-T which are significantly higher than the males (F = 24.99, P < 0.001). The average conversion to hydroxylated derivatives of testosterone is 58.8 per cent; that for males 35.5 per cent.

2. The apparent slight changes in hydroxylation with increasing diluent are
Table 1.
Effect of DPH on % conversion of testosterone to polar metabolites (HO-T) by rat liver incubates.

<table>
<thead>
<tr>
<th></th>
<th>Diluent control</th>
<th>DPH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \bar{x} )</td>
<td>SD</td>
</tr>
<tr>
<td>Males</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ( \mu l )*</td>
<td>32.4 ( \pm 6.9 )</td>
<td>2.5</td>
</tr>
<tr>
<td>500 ( \mu l )</td>
<td>35.9 ( \pm 8.2 )</td>
<td>12.5</td>
</tr>
<tr>
<td>1000 ( \mu l )</td>
<td>38.4 ( \pm 3.8 )</td>
<td>25.0</td>
</tr>
<tr>
<td>Females</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N = 5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 ( \mu l )</td>
<td>58.7 ( \pm 7.7 )</td>
<td>2.5</td>
</tr>
<tr>
<td>500 ( \mu l )</td>
<td>59.9 ( \pm 12.0 )</td>
<td>12.5</td>
</tr>
<tr>
<td>1000 ( \mu l )</td>
<td>58.1 ( \pm 10.0 )</td>
<td>25.0</td>
</tr>
</tbody>
</table>

\( \bar{x} \) = Mean
SD = Standard deviation
* = Volume of DPH in diluent utilized. Concentration of DPH = 25 mg/ml

Table 1
not significant statistically, either for the data as a whole or for the sexes taken separately. The difference in trend between the sexes is not significant.

B. When DPH is added in amounts of 2.5 mg, 12.5 mg and 25 mg per gram of liver:

1. There is a significant decrease in hydroxylation with increasing dose (\( F = 66.17 \)). Treating the present doses as equally spaced there is both a significant linear and a significant quadratic effect. That is to say, the drop in hydroxylation from 2.5 mg of DPH to 12.5 mg is greater than from 12.5 to 25.0 mg.

2. The difference in sex noted above disappears, so that the conversions are not significantly different between the sexes, either on the average or at specific dose levels (\( F = 0.64 \)). The average value for females is 40.3 per cent; that for males is 36.1 per cent.

C. Using the difference between the value obtained with DPH and the appropriate diluent control as a measure of drug-dosage response, the following can be concluded:

1. The response to DPH is significantly different in the two sexes; the decrement for females is significantly greater than the males at each dose level (\( F = 21.81, P < 0.001 \)).
The percentage conversion of [4-14C]testosterone to polar derivatives (HO-T) with graduated amounts of DPH added to each liver incubation. Each point represents the mean of 5 incubations. The diluent control represents that amount of DPH diluent which would contain the equivalent amount of DPH as follows: 100 λ = 2.5 mg; 500 λ = 12.5 mg; 1000 λ = 25 mg.

2. With 2.5 mg of DPH, there is significant increase in HO-T in the male but no change in the female.

In order to eliminate the possibility that DPH in itself was inhibiting the extractability of the polar metabolites of testosterone, in several experiments DPH in 2.5, 12.5 and 25 mg dosage was added after incubation of radio-testosterone and male or female rat liver slices. In every instance the addition of DPH failed to alter the amount of recovered HO-T pool using combined 4 per cent, 10 per cent and 25 per cent methanol in chloroform eluates as compared to the control incubates.

DISCUSSION

The paradoxical in vivo versus in vitro effect of a pharmacologic agent on steroid hydroxylation has been observed previously. Conney et al. (1965) have commented upon the fact that the addition of hexobarbital in vitro inhibited the hydroxylation of testosterone by rat liver microsomes, whereas its admini-
stration to the rat enhanced the hydroxylation of testosterone. The same effect on testosterone hydroxylation was produced with chlordane and DDT by Welch et al. (1967). More recently, Rubin & Lieber (1968) have found a similar effect on pentobarbital hydroxylation produced by ethanol, and utilize this relationship to help explain the tolerance of chronic alcoholics to barbiturates when sober, and their increased sensitivity to sedatives when inebriated.

In a previous study with testosterone, DPH at the dose level of 2.5 mg per incubate failed to show an inhibition of hydroxylated derivative formation, although enhanced ring-A reduction of testosterone was readily produced (Sholiton et al. 1967). However, results of the present study clearly indicate that significant suppression of overall testosterone hydroxylation can be achieved when DPH concentration in five- and ten-fold increments are utilized. That milligram amounts of inhibitor in vivo are required to inhibit hydroxylation of microgram quantities of substrate has been noted by Rubin & Lieber (1968) regarding the in vitro inhibition of pentobarbital, aniline and benzpyrene hydroxylases by ethanol. Also, such a relationship in the inhibition of oestradiol hydroxylation by norethindrone has been described recently by Watanabe (1969).

It should be emphasized, however, that the polar fraction that we are measuring represents total testosterone hydroxylation and undoubtedly consists of many different hydroxylated end-products, both ring-A reduced and ring-A unsaturated. For example, Jacobson & Kuntzman (1969) have shown recently that both the rate and amount of 6β-, 7α- and 16α-hydroxylation of testosterone vary independently of one another as influenced by age, sex, castration and testosterone propionate treatment. It may very well be that with even small amounts of DPH, formation of individual components of the pool of hydroxylated derivatives are inhibited. Unfortunately, standards of hydroxylated ring-A reduced metabolites of testosterone are not available for use in isolating and quantitating such compounds.

Using rat liver, Kuntzman et al. (1965) have shown that the Michaelis constant (Km) for the hydroxylation of testosterone is 2.6 × 10⁻⁵ M. Also using rat liver, Kutt & Verbely (1969) have recently published a Km for DPH of 3.2 × 10⁻⁵ M, a value very similar to that of testosterone. Therefore, a difference in Km cannot be used to explain the need for large amounts of DPH to inhibit the hydroxylation of testosterone under the conditions of our incubations.

It is quite possible that the competition between substrate and inhibiting agent is not for the same hydroxylation enzyme but for some rate-limiting factor or factors such as NADPH, wherein utilization by the inhibiting agent on a mass action basis might at the proper concentration deprive the substrate of sufficient cofactor (s) for its metabolism.

Another interesting observation in the present study is the difference in the
hydroxylation noted between the sexes. Female liver produces a greater amount of hydroxylated derivatives than male liver in the control, but this difference is obviated by the addition of DPH. At all dose levels, DPH results in almost identical percentages of hydroxylation between the male and female, but the latter would appear to be more sensitive to the inhibiting effect of DPH. In the study by Jacobson & Kuntzman (1969) cited above, no unsaturated hydroxylated derivatives of testosterone were formed by female liver microsomes, although the male produced considerable quantities of the unsaturated derivatives. But the studies of Chamberlain et al. (1965) have demonstrated effectively that female rat liver microsomes rapidly convert testosterone and ring-A reduced metabolites of testosterone to polar, presumably hydroxylated, derivatives. We can only project from these and our own studies that female rat liver, as well as male, is able to hydroxylate testosterone or its derivatives. The difference probably lies in the ability of female liver to reduce testosterone in greater quantity at a more rapid rate. As a consequence, female rat liver may produce ring-A reduced hydroxylated derivatives primarily while the male forms ring-A unsaturated hydroxylated metabolites as its major pathway.

It is obvious that further study of the mechanisms involved, particularly after some of these compounds are available as standards, must be forthcoming before any definitive explanation of these interesting relationships can be established.

ACKNOWLEDGMENTS

We are indebted to Miss Ruth Noll for her excellent technical assistance, and to Mr. Richard L. Clark for preparation of the illustration. We particularly wish to thank Dr. Goldine Gleser of the Department of Psychology, University of Cincinnati, for her assistance in the statistical analysis of our data.

REFERENCES


Received on April 25th, 1969.