THE LESS-POLAR METABOLITES PRODUCED
BY INCUBATION OF TESTOSTERONE-4-14C WITH RAT
AND BOVINE BRAIN

By
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ABSTRACT

Rat and bovine brain have been incubated with testosterone-4-14C under standard conditions. With use of paper chromatography, the extracted metabolites were noted to fall into less-polar, iso-polar, and more polar fractions. The components of the less-polar fraction were separated by acetylation and thin-layer chromatography and the major end-products identified by recrystallization to constant specific activity or constant 3H/14C ratios.

Androst-4-enedione and 5a-dihydrotestosterone were formed consistently under the conditions utilized. Trace amounts of other less-polar metabolites were noted occasionally.

That mammalian brain is capable of metabolizing steroid substrates under in vitro conditions has been well-established (Grosser & Bliss 1963, 1966; Peterson et al. 1965; Sholiton et al. 1965, 1966). When cortisol, cortisone, 11β-hydroxy-androstenedione or adrenosterone are used as substrate, the major enzymatic activity of rat brain appears to be the equilibration of the 11-keto:11-hydroxy position. More recently, we have incubated rat brain with testosterone-4-14C, a steroid in which no oxygen function is present at the C-11 position (Sholiton et al. 1966). Under the conditions utilized, about 10 per cent of the original radioactive substrate was metabolized to end-products more polar and less-polar than the substrate (Fig. 1).

The purpose of the present study was to attempt to define some of the less-polar metabolites of testosterone formed by rat brain in vitro and to ascertain
if brain from another animal source (in this instance, bovine) would produce the same or different metabolites under similar conditions of incubation.

METHODS AND MATERIALS

Incubation of 1 gram aliquots of rat and bovine brain with a standard amount of testosterone-4,14C was performed as previously described (Sholiton et al. 1966). Rat brain was freshly resected from male and female adult virgin Wistar rats immediately upon decapitation. Bovine brain was secured at a local abattoir immediately upon removal and chilled with ice. With the latter, 1 gram aliquots for incubation were obtained from cerebral cortex, cerebral peduncles and hypothalamus. Homogenization was performed with a 10 ml glass homogenizer and incubation was carried out in a Dubnoff shaker (atmosphere: 95% oxygen, 5% CO₂) for a 5-hour period at 37°C. The incubation was fortified with a NADPH-generating system provided by the addition of the following: nicotinamide-adenine-dinucleotide (NADP), 10⁻³ m, and glucose-6-phosphate (G-6-P), 10⁻² m. To each incubation 10 ml of freshly prepared Krebs-Ringer bicarbonate buffer (pH 7.4) was added. The incubation was terminated by the addition of glacial acetic acid followed by immediate freezing.

Extraction was accomplished with the use of ethyl acetate following addition of sodium sulfate, 20% by weight. The organic phase was evaporated to dryness under reduced pressure and the extract was then taken up with re-distilled chloroform. The chloroform extract was applied to a Florosil® column (15 mm diameter, packed to 88 mm in height) and eluted with 4% methanol in chloroform. The latter was taken to dryness and re-diluted with 1 ml of absolute methanol. Recovery of radioactivity in the above manipulations was determined in several incubates. About 95% of original substrate radioactivity was noted in the ethyl acetate extract with 75% of initial radioactivity obtained in the 4% methanol in chloroform fraction. Further column elution with 10% methanol in chloroform and 25% methanol in chloroform yielded less than 2% of total radioactivity. Hence, only the 4% methanol in chloroform eluate was utilized for further chromatographic separation.

Paper chromatographic separation of the methanolic extract was accomplished on

* Glossary of Trivial Names:
  Adrenosterone: androst-4-ene-3,11,17-trione.
  Aetiocholanolone: 3α-hydroxy-5β-androstan-17-one.
  5α-Androstanedione: 5α-androstan-3,17-dione.
  Androst-4-enediol: 3α,17β-dihydroxy-androst-4-ene.
  Androst-4-enedione: androst-4-ene-3,17-dione.
  Androsterone: 3α-hydroxy-5α-androstan-17-one.
  Cortisol: 11β,17,21-trihydroxy-pregn-4-ene-3,20-dione.
  Cortisone: 17,21-trihydroxy-pregn-4-ene-3,11,20-trione.
  Dehydroepiandrosterone: 3β-hydroxy-androst-5-en-17-one.
  5α-Dihydrotestosterone: 5α-androstan-17β-ol-3-one.
  Epitestosterone: 17α-hydroxy-androst-4-ene-3-one.
  20β-Hydroxycortisol: 11β,17,20β,21-tetrahydroxy-pregn-4-ene-3-one.
  Testosterone: 17β-hydroxy-androst-4-ene-3-one.
  Tetrahydrocortisol: 3α,11β,17,21-tetrahydroxy-5β-pregn-20-one.
Whatman No. 3 MM paper strips (3 cm wide) in system iso-octane:methanol:water (10:8:2) for 6 h at 22°C. A radioscan of the chromatogram revealed three primary peaks of metabolic radioactivity (Fig. 1) for both the bovine and rat incubates and in the rat a small secondary peak which migrated further than the less-polar peak (peak No. 2).

Further separation of the metabolites of the less-polar metabolic pool was obtained in the following manner. The less-polar peaks were eluted from the paper chromatogram with methanol. The eluates were then acetylated using a standard technique involving overnight incubation with anhydrous pyridine, acetic anhydride (1:1 v/v) (Kliman & Peterson 1960). The acetylated derivatives were separated by the application to an alumina-gel G thin-layer chromatographic system (methylene-chloride: chloroform 97:3) run at room temperature for 45 min. Unexposed X-ray film (Ansco high-speed Supreme Emulsion) was applied to the chromatographic plate for 72 h (Fig. 2). The X-ray film was then developed and exposed areas were correlated with identical areas on the alumina-gel plate. These areas were removed from the plate and eluted with ethyl-acetate, sodium-sulphate and water. The organic phase was then pipetted into an evaporating dish and taken to dryness. The radiometabolites were reconstituted with methanol and an aliquot counted in toluene phosphor with a Packard Tri-Carb liquid scintillation spectrophotometer.

Identification of radiometabolites was accomplished by using established techniques for comparison of chromatographic mobilities of unknowns and acetylated derivatives against standard reference compounds, by derivative formation, and finally by recrystallization to constant specific activity (Tables 1 and 2). Recrystallization to constant 3H/14C ratios was accomplished with both androst-4-enedione and aetiocholanolone by the use of tritiated standards to which the 14C radiometabolite had been added.

RESULTS

Although the paper chromatographic system utilized for separation of metabolite pools in this study failed to adequately separate many less-polar derivatives of testosterone, it was apparent that alumina-gel thin-layer chromatography could readily accomplish this separation, particularly after acetylation of some of these compounds.

Two metabolites were consistently found in the less-polar metabolite pool produced by rat and bovine brain incubation with testosterone-4-14C. These were identified as androst-4-enedione and 5α-dihydrotestosterone (Figs. 1 & 2, Table 1). Moreover, in the rat trace amounts of aetiocholanolone and androstone were occasionally noted. In both rat and bovine incubates at least one unidentified trace metabolite was found.

In the rat, no sex differentiation was noted. In bovine brain incubates, no qualitative distinction could be ascertained between brain samples obtained from various areas.

Marked variation in conversion was obtained from incubate to incubate, probably reflecting the difficulty in maintaining absolutely identical conditions for the incubation of these biological materials. Moreover, it should be
Radioscan of representative paper chromatograms of the extracted homogenates with a testosterone-4-14C substrate and chromatographed on system iso-octane:methanol:water (10:8:2) for 6 h (standard setting for Actigraph II, 300 counts (full scale) at 10 s time constant and 1/8" window aperture).

emphasized that metabolite production represented by the iso-polar and polar radioactive peaks noted on paper chromatography has yet to be evaluated.

**DISCUSSION**

Results of this study indicates that both rat and bovine brain contain dehydrogenases which under *in vitro* conditions convert testosterone to several less-polar metabolites. The conditions of these incubates provide a long-term generation of NADPH, but at least early in the incubation there is an excessive amount of NADP. This could possibly act as a means of shifting the 17-keto:17-hydroxy equilibration in favour of 17-keto formation. The presence of androst-4-enedione indicates that a 17-carbon dehydrogenase is present in rat and bovine brain homogenates. Also, it appears that 4-reductases are present to form the di-hydro compound in bovine and rat brain incubates, as well as aetiocholanolone and androstolone in the latter.

There appear to be some differences in the end-product formation between the two species. Rat brain occasionally formed at least one 5β-reduced end-product, aetiocholanolone, where none was noted in the bovine less-polar metabolite pool. Moreover, as represented by less-polar peak 2 (Fig. 1), a small amount of androsterone was noted in the rat, whereas none was discernible with bovine
Representative thin-layer chromatogram run for 45 m at room temperature on alumina gel G plates in system methylene chloride:chloroform (97:3). The standards have been stained with a modified Zimmermann reagent. The radio-metabolites have been demarcated on X-ray film applied unexposed to the plate for 72 h and then developed.

Key to symbols:
I = androst-4-enedione
II = 5α-dihydrotestosterone acetate
III = aetiocholanolone acetate
IV = unknown
V = unknown

Rat 1. Chromatogram of a pool of less-polar radiometabolites of testosterone-4-14C from a group of rat brain incubates.
Rat 2. Chromatogram of a pool of less-polar radiometabolites of testosterone-4-14C from a different group of rat brain incubates.
Bovine. Chromatogram of a pool of less-polar radiometabolites of testosterone-4-14C from a group of bovine brain incubates.

That a species difference in the metabolism of steroids by brain may exist has previously been suggested by the work of Grosser & Bliss (1966). These investigators failed to note oxidation at C-11 when guineapig brain was incubated with cortisol or corticosterone, whereas activity was very much in evidence with rat and dog cerebral cortex when incubated under identical conditions. Moreover, in terms of testosterone, Kuntzman et al. (1966) have
Table 1.
Data on recrystallization.

<table>
<thead>
<tr>
<th>Metabolite:</th>
<th>Rat</th>
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<tr>
<td>Androst-4-enedione</td>
<td>Recryst. to CSA</td>
<td>M. L.</td>
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<tr>
<td></td>
<td>cpm/mg</td>
<td></td>
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<td>1st</td>
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<td>M. L.</td>
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<tr>
<td>2nd</td>
<td>615</td>
<td></td>
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<tr>
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<th>Recryst. to CSA</th>
<th>M. L.</th>
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<tbody>
<tr>
<td>5α-dihydrotestosterone</td>
<td>Calc. 775</td>
<td></td>
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<td>Calc. 452</td>
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<tr>
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<tr>
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<td></td>
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<td>2nd</td>
<td>312</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd</td>
<td>327</td>
<td>328</td>
<td></td>
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Key to abbreviations:
M. L. = Mother liquor
CSA = Constant specific activity
convincingly demonstrated the species differences in the metabolism of this steroid in vitro by liver microsomes of the guineapig, dog, rabbit, mouse and rat.

In the present study there was no attempt to differentiate metabolite production by the various parts of rat brain, although no significant qualitative differences were detected when 1 gram aliquots of bovine brain from three separate regions were utilized. It should be noted, however, that Grosser & Bliss (1966) in their studies on the metabolism of the 11β-hydroxy steroids by cerebral tissues, observed that rat sub-cortex and hypothalamus had distinctly less 11β-hydroxy steroid dehydrogenase activity than rat cortex or cerebellum. More recently Grosser & Axelrod (1968) reported that in the baboon 11β-hydroxysteroid dehydrogenase activity appears to be greatest in the cerebellum of the foetal and neonate brain incubates.

The enzymatic degradation of testosterone in several tissues other than liver and brain has been studied by other investigators. King et al. (1964b) reported on the metabolic breakdown of testosterone by normal and neoplastic rat breast tissue. They noted A4-5α-hydrogenase, 3-keto-steroid reductase, and 17β-hydroxy-steroid dehydrogenase activity. The same investigators (King et al. 1964a) had previously found testosterone acetate formation in rat and mouse mammary tissues. Blaquier et al. (1967a) observed a small amount of epi-testosterone formation from testosterone when incubated with human adrenal tissue and subsequently (Blaquier et al. 1967b) demonstrated androst-4-enedione and androst-4-enediol production under similar circumstances from whole blood. Rongone (1966) has reported a metabolic profile from the incubation of human male mammary skin with testosterone which is similar to that which we find with rat brain – viz., androst-4-enedione, 5α-androstane-dione, aetiocholanolone, and androsterone. It is interesting to consider this similarity of metabolic activity in view of the common embryonic origin of the two tissues.

An inherent weakness of any in vitro study such as that presented here is that it is very difficult to project in vitro findings into a physiologic setting. Although only small amounts of certain metabolites are detected in these experiments, as previously noted (Sholiton et al. 1966), under in vivo conditions such a degree of conversion should be of significant functional importance. The minute difference in the concentration of testosterone of peripheral blood between normal and hirsute women, for example, attests to the possibility that small changes in testosterone metabolism could produce marked physiologic alteration (Dignam et al. 1964; Lloyd et al. 1966).

Moreover, there have been three studies which suggest that brain may actively metabolize corticosteroids in vivo. Touchstone et al. (1966) reported the presence not only of large quantities of cortisol in human brain secured at autopsy, but also large amounts of such cortisol metabolites as tetrahydro-cortisol and 20β-hydroxy-cortisol. R. I. Henkin (1968, pers. communication) in
his studies of the dynamics of the uptake of radio-cortisol by the brain of the
eviscerated cat, observed the presence of cortisol radio-metabolites in the
nervous tissue of this animal. Knapstein et al. (1968) reported conversion of
15.9% of \textsuperscript{3}H-dehydroepiandrosterone to \textit{\textdelta}\textsubscript{5}-ring D hydroxylated metabolites
when perfused into the brains of rhesus monkeys. To our knowledge, no such
studies with testosterone have thus far been undertaken.

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