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STEREOCHEMISTRY OF 1,2-HYDROGEN LOSS DURING AROMATIZATION IN THE BRAIN

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Summary—The stereochemistry of hydrogen loss from C-1 and C-2 during aromatization in rat brain was studied using androstenedione containing a known distribution of isotopic label. Comparison of the tritium content of the estrone obtained from the aromatization of androstenedione labeled predominantly in the 1α,2α positions with that in estrone obtained from a parallel incubation using substrate with label in the 1β,2β orientation gave an estrone α/β ratio of 3.6. This ratio compares with a calculated value of 4.3 for an aromatization mechanism involving loss of the 1β,2β-hydrogens. The distortion from the predicted value is due to the loss of tritium from the α-substrate which is unrelated to aromatization. The ratio determined experimentally is compatible with 2β-tritium loss since random or α-elimination from C-2 would yield α/β ratios of 2.2 and 1.3 respectively. In an analogous manner the stereochemistry of tritium loss at C-1 was determined using [1α-^{3}H] and [1β-^{3}H]androstenedione. The α/β ratio of the isolated estrone was 3.6 which is in good agreement with the calculated value of 3.3 for 1β-tritium elimination. Our results therefore show that estrogen formation in the brain occurs with the same stereospecificity of hydrogen loss at C-1 and C-2 as in placental microsomes.

INTRODUCTION

The aromatization sequence for the conversion of androgens to estrogens has been studied and characterized in human placental microsomal preparations [1–5]. The presence of a similar aromatization of androgens to estrogens in discrete regions within the central nervous system has been described and its physiological importance evaluated [6–9]. The question whether the biochemical mechanism deduced for the placental aromatization applies also to the transformation of androgens in the brain is relevant since it bears on the identity of the enzyme complexes involved in the multi-sequence transformation in the two tissues. It is therefore important to characterize the brain aromatase sequence and identify any differences from the placental process since these may be critical to an understanding of the regulation of estrogen formation in the central sites. One criterion that can be employed to assess congruence of the enzymes in the placenta and the brain is the stereochemistry of hydrogen loss from C-1 and C-2 from the androgen precursors. In the placental aromatase system, the reaction has been shown to involve the *cis* elimination of the β-hydrogens from these sites [10, 11]. Most investigators have assumed that the stereochemistry of the transformation in central sites parallels that described in the placenta but the issue has not been experimentally resolved. We have studied the conversion of androstenedione to estrone in the rat hypothalamus and amygdala employing substrates with a known stereospecific distribution of the isotope label in an effort to identify the stereochemistry of the conversion in these tissues. In this communication we report that this biotransformation in the central sites is stereospecific, involves loss of the 1β- and 2β-hydrogen and is therefore analogous at least in this respect to the process in the placental microsomes.

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EXPERIMENTAL

[1β,2β-^{3}H]Testosterone (sp. act. = 50.4 Ci/mmol) with a distribution of tritium of 1β = 49%; 2β = 33%; 1α = 10%; 2α = 8% and [1α,2α-^{3}H]testosterone (sp. act. = 49.0 Ci/mmol) with a tritium distribution of 1α = 37.4%; 2α = 31.8%; 1β = 11.5%; 2β = 11.2%; other = 8.1% were obtained from New England Nuclear Corp. The distribution of the isotope as determined by tritium NMR analysis was a personal communication from the supplier.

The corresponding [1β-^{3}H]androstene-3,17-dione and [1α-^{3}H]androst-3,17-dione were prepared from the respective [1,2-^{3}H]testosterone substrates by the following method. The [1,2-^{3}H]testosterone (100 μCi) was dissolved in absolute ethanol (5.0 ml) and 5.0 μl were removed for determination of radioactive content. Sodium hydroxide (1 N solution, 25 μl) was then added, and the reaction was heated under reflux with a positive pressure of nitrogen for 30 min. A 5.0 μl aliquot was pipetted and evaporated *in vacuo* to remove any ^3H_2O which was formed by elimination of tritium from C-2. The decrease in radioactivity corresponded to that calculated from the distribution of tritium at C-2 in the respective testosterone substrates.

After dilution by water and extraction three times with chloroform, the organic phase was dried, filtered

and evaporated *in vacuo*. The residue was purified on analytical silica gel thin layer chromatography (TLC) plates (Analabs, North Haven, CT) using chloroform–ethanol (95:5, v/v) as the solvent system. The eluted product corresponding to authentic testosterone was dissolved in methylene chloride (5 ml) and pyridinium dichromate (10 mg) was added [12]. The reaction was stirred at room temperature for 2 h, evaporated *in vacuo* and purified on TLC plates as above to yield [1α-^{3}H]- or [1β-^{3}H]androstenedione. The homogeneity of the isolated radiolabeled products was determined by reverse isotope dilution and found to be at least 98%. In a similar oxidation, [1α,2α-^{3}H]androstenedione and [1β,2β-^{3}H]androstenedione were prepared from their respective testosterone analogs.

Aromatization in rat brain homogenates

Male CD rats (Charles River Laboratories) 4 days of age or adult rats weighing 200–250 g were sacrificed by decapitation. The brain was removed and chilled on ice to facilitate removal of the hypothalamus and amygdala. The CNS dissection was carried out as described by Glowinski and Iverson[13]. The tissues were pooled, homogenized in 0.05 M Tris–HCl (pH 7.4) and used in a final concentration of 50 mg/ml in each incubation. In addition to tissue, the incubation mixture contained NADPH (3 mg), glucose-6-phosphate dehydrogenase (10 units) and glucose-6-phosphate (15 μmol) in a final volume of 10 ml. Incubations were carried out in duplicate and were started by the addition of an aliquot of 24×10^6 dpm of [1,2-^{3}H]androstenedione and 5×10^6 dpm of 1-^{3}H-substrate. Control incubations were conducted as above except that tissues were replaced by an equal volume of Tris–HCl buffer. Incubations were carried out at 37°C under air for 60 min. Aliquots (1.0 ml) were removed for analysis of ^3H_2O formation. These were added to 1% charcoal in Tris–HCl buffer (1.0 ml) and allowed to sit on ice for 10 min prior to sedimentation by centrifugation. An aliquot of the supernatant was flash frozen, lyophilized and the ^{3}H content in 0.5 ml samples of the lyophilized water was determined by counting in a scintillation counter.

The remainder of the incubation was mixed with estrone (10.1 mg) containing a known amount of [^{14}C]estrone and then extracted three times with chloroform (90 ml). The organic extracts were dried over sodium sulfate, filtered and evaporated *in vacuo*. The residues were dissolved in pyridine (1.0 ml) and treated with acetic anhydride (1.0 ml). The solution was allowed to stand at room temperature for 15 h and then evaporated *in vacuo*. The residues were submitted to preparative TLC on silica gel using ethyl acetate–cyclohexane (30:70, v/v) as the solvent. The purified steroid was then crystallized to constant isotope ratio using ether/petroleum ether as the solvent.

Aromatization in placental microsomes

Incubations were carried out as above using microsomes prepared from human term placentas [14] in a final concentration of 5 mg/ml. Incubations were begun by the addition of 3.5×10^6 dpm radiolabeled steroid. In this tissue estradiol was isolated and measured since it is the major aromatized product with the amount of estrone being less than 5% of that of the estradiol. The estradiol was purified by preparative TLC on silica gel using chloroform–ethanol (95:5, v/v) as the solvent. The isolated steroid was further purified by preparative TLC using ethyl acetate–cyclohexane (1:1, v/v) as the solvent. The repurified estradiol was then crystallized to constant specific activity with benzene as the solvent.

RESULTS AND DISCUSSION

The most suitable method for determining the stereochemistry of aromatization is to measure the decrease in $^3H/^{14}C$ isotope ratio which accompanies the conversion of a mixture of [1,2-^{3}H]androstenedione and [^{14}C]androstenedione to estrogen. This technique is limited however, by the 1000-fold lower specific activity of the [^{14}C]androgen relative to the tritiated compound. Thus in the brain where the biotransformation occurs in very low yield, and the enzyme is saturated at low concentrations of substrate, it is not possible to use a sufficient amount of the [^{14}C]androgen which would permit the detection of label in the aromatized product.

We therefore sought to study the stereochemistry of the brain biotransformation by comparing the aromatization of [^{3}H]androstenedione labeled predominantly in the 1α,2α position to a substrate with label in the 1β,2β orientation. If the brain reaction proceeds by the same β stereochemistry established for the placental aromatization, the tritium content of the estrogen formed and the amount of ^3H_2O that is generated can be compared between the two parallel reactions on the basis of a knowledge of the isotope distribution in the androgen substrates. Comparison of the yields from the two incubations with the expected values would reveal whether the stereochemistry of aromatization in brain and placenta is similar or not.

Table 1 shows the yield of ^3H_2O from placental incubations of the two radiolabeled substrates. The transfer of ^{3}H into water from the α-substrate is expected to be 23% of the total label while the corresponding value for the β-compound is 82%. The expected α/β ratio of ^3H_2O is therefore 0.26. The ratio calculated from the data in Table 1 is 0.25 in good agreement with the expected value. These values confirm the original assignment of ^{3}H location and stereochemistry on the basis of tritium NMR. Table 1 also shows the amount of [^{3}H]estradiol produced from placental incubations containing either [1α,2α-^{3}H]androstenedione or [1β,2β-^{3}H]androstenedione. The expected tritium content of the

Table 1. Aromatase activity in placental and adult brain preparations using [1,2-^{3}H]androstenedione as substrate

Sample		Total yield: [^{3}H]Estradiol (dpm) crystallization no. 2	3	^3H_2O (dpm)
Placenta	α-1	2.0×10^6	1.9×10^6	4.5×10^5
	α-2	2.1×10^6	2.0×10^6	4.5×10^5
	β-1	4.7×10^5	4.7×10^5	1.8×10^6
	β-2	4.9×10^5	4.8×10^5	1.8×10^6
		[^{3}H]Estrone (dpm) ^{3}H	^{14}C	^{3}H/^{14}C	
Brain	α-1	10,600	5390	1.97	12,900
	α-2	11,400	5720	1.99	12,300
	β-1	6270	5410	1.16	21,400
	β-2	5810	5050	1.15	20,000

Tissues were incubated in duplicate with either [1,2α-^{3}H] or [1,2β-^{3}H]androstenedione as described in the Experimental section. Placental estradiol was crystallized to constant specific activity. Brain estrone was crystallized to constant isotope ratio. ^3H_2O values are the average of two aliquots removed from the individual incubations.

[^{3}H]estradiol generated from the [1α,2α]androgen is 77% of the original amount present, while the estrogen obtained from the 1β,2β-substrate should retain only 18% of the initial label. Therefore, the ratio of [^{3}H]estradiol produced from the α-substituted androgen relative to that from the β-substrate should be 4.3 (77/18). The ratio obtained experimentally (Table 1) is 4.2 which is again consistent with the above value.

The corresponding yields of [^{3}H]estrone and ^3H_2O from the isomeric labeled substrates in brain incubations are also given in Table 1. The α/β ratio obtained from the respective [^{3}H]estrone values is 1.8 which is significantly lower than the expected ratio of 4.3. This difference may be attributed to a loss of tritium from the α-substrate which is unrelated to aromatization. Although such a release of label probably occurs also in the placental system, the much lower aromatase activity in the brain makes the impact of this tritium loss on the α/β ratio much greater in the central tissue. Analysis of the ^3H_2O produced from brain incubations shows an α/β ratio of 0.61 in contrast to an expected value of 0.26 which again may be justified by a non-aromatization accompanied loss from the α-substrate.

An alternative possibility that was considered which could account for the difference in α/β ratios from the expected values was that an isotope effect might accompany the loss of tritium from C-1 and C-2. Even if the isotope effect were small, the impact could be large in the brain since the yield of estrogen is very low in this tissue. To examine whether an isotope effect exists, we compared the ^{3}H/^{14}C ratios of estradiol isolated from incubations with placental microsomes using high yield (60 min) and low yield (0.5 min) conditions. An increase in the ^{3}H/^{14}C ratio of the estradiol at the shorter time period relative to the longer incubation time would be indicative of an isotope effect. The substrates used were [1α,2α-^{3}H] and [1β,2β-^{3}H]androstenedione and [1α-^{3}H] and [1β-^{3}H]androstenedione. The results (data not shown) indicate that for each substrate no isotope effect is operative, and therefore the difference in α/β ratios of brain estrone in Table 1 from the expected values is not explained by a differential loss of tritium versus hydrogen from the androgen substrate.

Table 2. Aromatase activity in neonatal brain preparations using [1,2-^{3}H]androstenedione as substrate

Sample	Total yield [^{3}H]estrone (dpm) ^{3}H	^{14}C	^{3}H/^{14}C
α	93,840	22,140	4.24
	88,470	21,940	4.03
β	25,640	22,810	1.12
	25,160	22,120	1.14

Neonatal brain homogenates were incubated as described in the Experimental section. Estrone was crystallized to constant isotope ratio.

Aromatase activity is known to be higher in neonatal rats compared to mature animals [8]. We therefore repeated the studies described in Table 1 using brain homogenates obtained from 4-day old male rats to see if the α/β ratio of the [^{3}H]estrone obtained from the isomeric labeled substrates would better correspond to the calculated value. Table 2 shows that the α/β ratio determined from the yields of [^{3}H]estrone isolated from neonatal brain preparations is 3.6. This value is much closer to the expected ratio of 4.3 than the α/β ratio of 1.8 calculated from the [^{3}H]estrone isolated from adult brain incubations. We speculate that even though the yield of estrone is higher in the neonate study, there is still a sufficient loss of label from the α-substrate which is unrelated to aromatization so that the α/β ratio is still distorted from the expected value. The ratio of 3.6 however, does lead to the conclusion that the stereochemistry of tritium elimination must be β since random or α loss at C-2 would result in α/β values of 2.2 (67/30) and 1.3 (56/43) respectively.

To study the stereochemistry of tritium loss from C-1 we simplified the system and eliminated contributions from unrelated reactions at C-2 by employing [1-^{3}H]androstenedione as the substrate. This study was carried out only with brain tissue since the distribution of label in the 1,2-^{3}H-substrates had previously been validated in the placental system. Although the amount of tritium at C-1 and its absolute distribution does not change in the [1-^{3}H]androgens, the specific activity of these compounds is different than that of the 1,2-^{3}H-analogs. These values are 28.0 Ci/mM for 1α-androstenedione and 29.7 Ci/mM for 1β-androstenedione, because 43 and 41% of the total radioactivity respectively is lost from C-2 on treatment of the [1,2-^{3}H]androgen with base. The specific activity of the [^{3}H]estrone isolated from brain incubations can then be expected to be 18.2 Ci/mM for the 1α-compound since 45% of the label contained in the 1α-androstenedione is retained if β elimination takes place. The corresponding specific activity of the [^{3}H]estrone produced from the

Table 3. Aromatase activity in adult brain homogenates using [1-^{3}H]androstenedione as substrate

	Total yield [^{3}H]estrone (dpm)			
	^{3}H	^{14}C	^{3}H/^{14}C	^3H_2O (dpm)
α-1	1470	3290	0.45	3210
α-2	1640	3320	0.49	3080
β-1	480	3200	0.15	1690
β-2	470	3330	0.14	1720

Brain homogenates were incubated in duplicate with either [1α-^{3}H]- or [1β-^{3}H]androstenedione as described in the Experimental section. Estrone was crystallized to constant isotope ratio. ^3H_2O values are the average of two aliquots removed from the individual incubations.

1β-androstenedione is computed as 5.0 Ci/mM since only 10% of the tritium is expected to remain.

Table 3 lists the yields of [^{3}H]estrone isolated from the respective incubations. The expected α/β ratio obtained from the specific activities of the α and β estrogens is 3.6. The calculated ratio from Table 3 is 3.3 which is in good agreement with the predicted value. Therefore, the amount of tritium label present in the [^{3}H]estrogen is consistent with an aromatization mechanism involving loss of the 1β-hydrogen from the androgen. These results also indicate that [1-^{3}H] and not [1,2-^{3}H]androstenedione should be used to study brain aromatization, since use of the former substrate avoids overestimation of the extent of the reaction.

Table 3 also shows the measured values for ^3H_2O release from the two substrates. The expected α/β ratio is 0.24 since 12% of the tritium in the 1α-androstenedione should be transferred into water compared to 49% from the 1β-substrate. The calculated α/β ratio of ^3H_2O from the data in Table 3 is 1.8, indicating that an excessive amount of tritium is released from the [1α-^{3}H]androstenedione. The fact that the [^{3}H]estrone α/β ratio is in accord with the expected value argues that the discrepancy in the ^3H_2O ratio must originate prior to aromatization and therefore occurs on the androgen substrate. Since the radiolabeled androstenedione is present in a much greater concentration than the [^{3}H]estrogen, even a small amount of non-aromatization related release of tritium from the former would be expected to distort the α/β ratio of ^3H_2O. To further characterize the nature of this tritium transfer into water we incubated brain homogenates with [1α-^{3}H] or [1β-^{3}H]androstenedione in the presence or absence of cofactors. The results (data not shown) indicated a significant production of ^3H_2O from [1α-^{3}H]androstenedione in the absence of cofactors, a situation not matched by the [1β-^{3}H]androstenedione substrate. This suggests that the tritium release from the 1α-^{3}H-compound is non-enzymatic and contributes to the disproportionate ratios observed. It is quite possible that this loss occurs from the 8% of label located at positions other than C-1 and C-2.

In conclusion we demonstrated using [1,2-^{3}H]- and [1-^{3}H]androstenedione containing a known distribution of isotopic label that estrogen formation in the brain occurs with the same stereospecificity of hydrogen loss at C-1 as in the placental microsomes. Although this feature of the brain aromatization mechanism corresponds to the placental stereochemistry, it does not ensure that the entire sequences are identical. Studies are currently being carried out to further characterize the aromatase enzyme in central tissues.

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