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AROMATIZATION AND 19-HYDROXYLATION OF ANDROGENS BY RAT BRAIN CYTOCHROME P-450

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SUMMARY. The oxidative metabolism of androgens in the rat brain includes aromatization preceded by the requisite 19-hydroxylation. We have examined the transformation of [19-C^3H_3]androstenedione and [4-^{14}C]testosterone by the semipurified cytochrome P-450 fraction of the rat brain. [19-C^3H_3]Androstenedione generated tritiated water and formic acid in a ratio of 8 to 1 indicating that 19-hydroxylation in the brain far exceeds that necessary for aromatization. This was confirmed by the results of the ^{14}C-testosterone incubation in which the ^{14}C labeled 19-hydroxy and 19-oxo derivatives which were isolated exceeded the yield of ^{14}C-estrogens by several fold. Thus the rat brain has the capacity to form in situ 19-hydroxylated androgens which are not available to it from the circulation.

The aromatization of androgens to estrogens in the CNS has been implicated as playing an important role in the sexual differentiation of the brain (1, 2). The aromatization process in the rat brain was recently shown to exhibit features different from the classical model of this biotransformation in the human placenta (3). In the latter tissue the initial sequential 19-hydroxylations are followed by an essentially stoichiometric aromatization so that there is little or no accumulation of the 19 oxygenated androgens and the estrogens are virtually the sole products of the reaction (Fig. 1). In contrast, in rat brain homogenates and microsomes 19-hydroxylation greatly exceeds subsequent aromatization so that the formation of the 19 oxygenated androgens is several fold greater than that of the estrogens in this tissue. It is unknown whether the excess 19-hydroxylation is accomplished by a component of the aromatase complex or by a separate and unrelated enzyme. Because of the low enzyme activity in the brain (3, 4) there was some concern that these disproportionate reactions reflected low substrate concentration necessarily employed and were artifactual in nature. For this reason the transformation of testosterone with particular attention to 19 oxidation and

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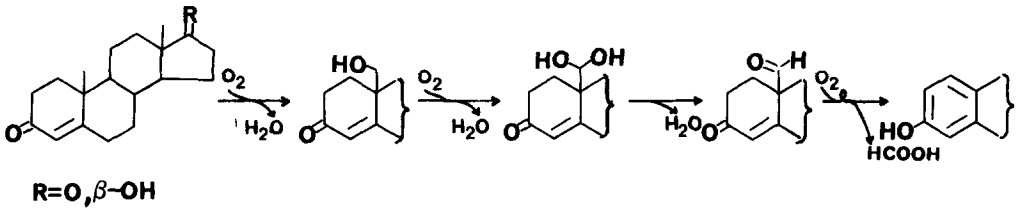

Fig. 1. Estrogen biosynthesis sequence.

aromatization was reexamined with the cytochrome P-450 fraction concentrated from rat brain microsomes which contains much higher enzyme activity.

MATERIALS AND METHODS

[19-C^3H_3]Androst-4-ene-3,17-dione (AD) (specific activity, 60 Ci/mmol) was prepared as described (5) and [4-^{14}C]17β-hydroxyandrost-4-en-3-one (T) (specific activity, 56.9 mCi/mmol) was purchased from Amersham. NADPH, dilauroyl-3-L-phosphatidyl choline and HEPES was obtained from Sigma. Estrone (E_1), estradiol (E2), AD, T, 2α-hydroxy(OH)-T, 6α-OHT, 7α-OHT and 16α-OHT were obtained from Steraloids, Inc. 19-Hydroxytestosterone (19-OHT) and 19-oxotestosterone (19-oxoT) were prepared by convenient methods from 19-OHAD which was a gift from Syntex Corp. The partially purified cytochrome P-450 fraction from rat brain microsomes was prepared as reported (6) and the P-450 activity quantified by the method of Omura and Sato (7). NADPH-cytochrome P-450 reductase was prepared from rat liver microsomes according to the method of Yasukochi and Masters (8).

Radiometric analysis: 0.5 nmole of AD containing 0.53 x 10^6 dpm of [19-C^3H_3]AD was mixed with 0.5 nmole of cytochrome P-450, 0.25 unit of NADPH-cytochrome P-450 reductase, 30 μg of dilauroyl-L-3-phosphatidylcholine and 100 μmole of HEPES (pH 7.4) in a total volume of 0.45 ml. The mixture was incubated at 37°C for 2 min, and then the reaction was initiated by the addition of 50 μl of NADPH (8 mM in water). After incubation for 10 min, the resultant mixture was added 1.5 ml of 1 M phosphoric acid and lyophilized after flash-frozen. Quantities of tritiated water and formic acid in the sublimed fraction were determined as described previously (5).

Product isolation analysis: 2.22 nmole of [4-^{14}C]T was incubated in the double volume of the same system above. The reaction was terminated by the addition of 0.5 ml of 2% ascorbic acid solution in chilled water and products were extracted into 3 ml of ethyl acetate 3 times. The organic extracts were combined, dried over anhydrous sodium sulfate and evaporated under a nitrogen stream. The residue was subjected to two dimensional TLC separation together with non-radioactive authentic standards. The plate was developed with a mixture of hexane-ethyl acetate (2:3) in the first dimension and chloroform-ethyl acetate-ethanol (100:20:7) in the second dimension. After chromatography, the TLC plate was exposed to a sheet of LKB Ultrafilm for 48 hr and the film was then developed. The non-radioactive standards were visualized by sulfuric acid. The appropriate radioactive regions were scraped into each counting vial for determination of radioactivity by liquid scintillation counting.

RESULTS AND DISCUSSION

Incubation of the partially purified brain cytochrome P-450 fraction in a reconstituted system with [19-C^3H_3]androstenedione yielded tritiated water and

formic acid in a ratio of 7.9 which is similar to the results obtained with the crude brain microsomal preparation (3, 4) and which indicates that 19-hydroxylation is proceeding at several times the level of aromatization in this system. The formation of greater quantities of 19 oxygenated androgens than of the estrogens was confirmed in a study in which the brain P-450 fraction was incubated with [4-^{14}C]testosterone, a substrate which could not be employed in the previous microsomal studies because of its relatively low specific activity. With the more active P-450 preparation the substrate was transformed effectively and the products were separated by two dimensional thin layer chromatogaphy and were visualized by autoradiography (Fig. 2). The structural assignments follow from correlation of the radioactive spots with authentic standards. The amounts of the individual products formed were determined semiquantitatively by elution and counting of the appropriate regions. The results are listed in Table 1 and show that the amount of 19 oxygenated androgens identified exceeds almost 4 fold that of estradiol, which was the only estrogen formed. Calculation employing the formula ([19-OHT] + 2[19-oxoT] + 2[E_2])/[E_2] showed that the expected H_2O/HCOOH ratio should be 7.5 which is in good agreement with the observed ratio of 7.9 in the radiometric experiment using [19-C^3H_3]AD. It is of interest that with the ex-

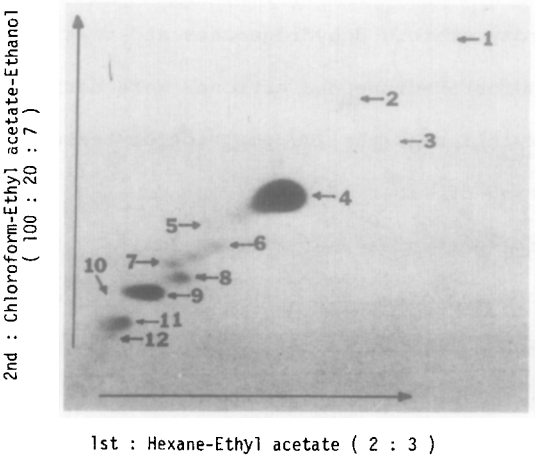

Fig. 2. Metabolic profile of testosterone by partially purified rat brain cytochrome P-450. A reconstitution of the cytochrome P-450 fraction and TLC analysis followed by autoradiography were described in Materials and Methods. The spots are identified by standards and shown in Table 1.

Table 1. Rat brain cytochrome P-450 metabolites of testosterone

Spot No.*	Standard	Amount of metabolite formed (pmole/10 min/nmole P-450)
1	E_1	0
2	AD	14
3	E_2	2
4	T	-
5	19-OxoT	4
6	2α-OHT	13
7	6β-OHT	7
8	Unknown-1	25
9	Unknown-2	130
10	19-OHT	3
11	7α-OHT	34
12	16α-OHT	8

*Spot No. corresponds to the number on Fig. 2.

ception of androstenedione, comprising 0.6% of the incubated substrate, all of the other identified transformation products contained the 17β-hydroxyl functional group and that the unchanged testosterone represented 89% of the recovered radioactivity. Similarly in the previous study of the transformation of estradiol by this brain P-450 preparation (6) all of the significant products contained the 17β-hydroxyl functions with the exception of a 0.4% yield of estrone. These results imply that the P-450 fraction was not contaminated with 17β-hydroxy steroid dehydrogenases and that the respective 17-keto products formed, androstenedione and estrone, were derived by a cytochrome P-450 dependent hydroxylation at the 17α-position of testosterone or estradiol followed by elimination of water (9).

The results of these studies confirm that the rat brain is an active organ of 19-hydroxylation of androgens and that the products of this reaction are to a large extent not aromatized. Thus the previous results obtained with much less active microsomal preparations were not due to artifactual causes such as substrate concentration, lipid peroxidation or absence of sufficient reducing equivalents but represented the actual biotransformations of brain tissue at least under in vitro conditions (3, 4). The results however do not shed any

further light as to whether the 19-hydroxylation is achieved by an enzyme related to the aromatization process or by an unrelated 19-hydroxylase which has been described so far only in the adrenal (10, 11). In either event however the evidence obtained offers a powerful argument that the 19-hydroxy androgens constitute a significant product of brain androgen metabolism and that they may have specific neuronal or neuroendocrine functions (12, 13).

The results of the study using [4-^{14}C]testosterone demonstrate that androgens can be hydroxylated by the brain P-450 fraction in multiple other sites besides C-19. Thus 2α-, 6β-, 7α- and 16α-hydroxylations are all significant transformations exceeding the 19-hydroxylations in quantity. Two major products of unknown structure were also observed. It should, however, be noted that whereas all of the other identified hyroxylations achieved by the rat brain P-450 fraction are also duplicated by liver cytochrome P-450's 19-hydroxylation has not been found in the latter tissue (5, 15) and thus these metabolites are available to the brain only via in situ formation and could therefore have paracrine or autocrine CNS functions.

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REFERENCES

1. McEwen, B.S., Lieberburg, I., Chaptal, C., and Krey, L.C. (1977) Horm. Behav. 9, 249-263.
2. MacLuskey, N.J., and Naftolin, F. (1981) Science 211, 1294-1303.
3. Hahn, E.F., Miyairi, S., and Fishman, J. (1985) Proc. Natl. Acad. Sci. USA 82, 2728-2730.
4. Michnovicz, J.J., Hahn, E.F., and Fishman, J. (1987) Endocrinology 121, 1209-1214.
5. Miyairi, S., and Fishman, J. (1985) J. Biol. Chem. 260, 320-325.
6. Sugita, O., Miyairi, S., Sassa, S., and Kappas, A. (1987) Biochem. Biophys. Res. Commun. 147, 1245-1250.
7. Omura, T., and Sato, R. (1964) J. Biol. Chem. 239, 2370-2378.
8. Yasukochi, Y., and Masters, B.S.S. (1976) J. Biol. Chem. 251, 5337-5344.
9. Suhara, K., Fujimura, Y., Shiroo, M., and Katagiri, M. (1984) J. Biol. Chem. 259, 8729-8736.
10. Sato, H., Ashida, N., Suhara, K., Itagaki, E., Takemori, S., and Katagiri, M. (1978) Arch. Biochem. Biophys. 190, 307-314.
11. Kosaki, T., Yarborough, C., and Osawa, Y. (1986) 68th Annual Meeting of the Endocrine Society (Anaheim) Abstract no. 331.
12. Parrott, R.F. (1974) J. Endocrinol. 61, 105-115.
13. Roselli, C.E., Horton, L.E., and Resko, J.A. (1985) Endocrinology 117, 2471-2477.
14. Jansson, I., Mole, J., and Schenkman, J.B. (1985) J. Biol. Chem. 260, 7084-7093.