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EVIDENCE THAT ESTRADIOL-2/4-HYDROXYLASE ACTIVITIES IN RAT HYPOTHALAMUS AND HIPPOCAMPUS DIFFER QUALITATIVELY AND INVOLVE MULTIPLE FORMS OF P-450: ONTOGENETIC AND INHIBITION STUDIES

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Summary—Estradiol-2/4-hydroxylase activity was studied in hypothalamic and hippocampal microsomes of male and female Wistar rats between 22 and 75 days after birth. The activity exhibited substrate saturation (50–100 μM estradiol) required NADPH and was inhibited by carbon monoxide, α-naphthoflavone and metyrapone. With 30 μM estradiol, 50% inhibition required 50–70 μM α-naphthoflavone compared to 200 μM metyrapone. Metyrapone exhibited biphasic inhibition curves which did not differ significantly between hypothalamus and hippocampus, whereas α-napthoflavone was a more potent inhibitor of the hippocampal enzyme than of the hypothalamic enzyme. The K_m and V_{max} of the hippocampal estradiol-2/4-hydroxylase were significantly greater than that of the hypothalamus in both sexes at all ages studied. In female rats the K_m of the hypothalamic enzyme changed from 14 μM at 23 days of age, to 47 μM at 71 days, but remained constant at about 29 μM in males. The K_m of the hippocampal enzyme showed no significant change with age in either sex. The present findings indicate that catecholestrogen formation in the brain is catalyzed by multiple forms of microsomal P-450. They also suggest that these enzyme activities in the rat hypothalamus and hippocampus differ qualitatively. Ontogenetic changes in the K_m of estradiol-2/4-hydroxylases appeared to be limited to the female hypothalamus. This might reflect a specific biological requirement of the female hypothalamus during critical stages of sexual differentiation of the brain. The relatively high hippocampal activity might reflect the catalytic versatility of different P-450 isozymes and does not necessarily imply a physiologically meaningful role with respect to catecholestrogen biosynthesis in this particular brain area.

INTRODUCTION

There is substantial evidence that the formation of catecholestrogens (2- and 4-hydroxyestrogens) is catalyzed by microsomal P-450 [1–3]. There are also indications that more than one form of P-450 may be involved in the reaction [3–4]. These findings are important because the expression or catalytic activities of different forms of P-450 are affected independently by variables such as age, sex, tissue or species [5–7]. The question therefore arises as to whether, and to what extent, these variables affect catecholestrogen-forming activity.

Ontogenetic studies have indicated that estradiol-2/4-hydroxylase activities in rat brain and liver exhibit sex differences, but that these differences appear at a later development stage in the brain than in the liver [3]. We have found that hepatic estradiol-2/4-hydroxylase activity in female, but not in male rats, undergoes qualitative changes during sexual maturation. During puberty the enzyme activity in liver microsomes of female rats shows a transition from linear to non-linear kinetic behaviour and a progressive decrease in apparent K_m [8].

The above observations prompted us to investigate the estradiol-2/4-hydroxylase activities in discrete brain areas of male and female rats at different ages. Preliminary studies [9] indicated that the hippocampus has the highest, and the hypothalamus the lowest activity of 6 discrete brain areas examined. For more detailed studies we therefore chose the hypothalamus and hippocampus. The main objective of these studies was to compare possible ontogenetic changes and sex differences in the kinetic parameters of estradiol-2/4-hydroxylase in two functionally different brain regions with ontogenetic changes previously observed in rat liver [8]. A second objective was to confirm that microsomal P-450 catalyzes catecholestrogen formation in rat brain and to determine whether more than one form of P-450 is involved. The results of these studies form the substance of the present article.

EXPERIMENTAL

Chemicals

All chemicals were of analytical grade and were obtained from E. Merck, Darmstadt (unless otherwise specified). Estradiol, α-naphthoflavone (7,8-benzoflavone) and metyrapone (2-methyl-1,2-di-3-pyridyl-propanone) were purchased from Sigma, St

Louis, MO and NADPH from Boehringer, Mannheim. Catechol-*O*-methyltransferase (COMT) was prepared by us from rat liver essentially as described by Nikodejevick *et al.* [10]. *S*-[^{3}H]methyl adenosylmethionine (sp. act. 8–15 Ci/mmol) was obtained from New England Nuclear, Cambridge, MA.

Animals and tissue preparation

Locally bred Wistar rats (22–75 days old) were housed in plastic cages with wire mesh flooring under diurnal lighting and had free access to food and water.

The rats were killed by decapitation and livers and whole brains were rapidly removed and placed in ice-cold 0.154 M KCl–50 mM Tris–HCl buffer (pH 8.0 at 37°C). Discrete brain areas were dissected out on ice-cooled glass plates and placed in the above buffer (pH 8.0 buffer). For each age group and sex studied, brain areas from 10–15 animals per experiment were pooled. The pooled brain areas (200–1000 mg wet wt) were homogenized in 10 ml of the "pH 8.0 buffer" and microsomes separated by differential centrifugation as described previously [8]. The microsomes were suspended in the above buffer to give microsomal protein concentrations of about 1 mg/ml suspension and kept on ice until assayed (30–60 min). Standard differential centrifugation methods were used to prepare mitochondria (12,000 *g* pellet) and soluble fractions (105,000 *g* supernatant) from brain and liver homogenates. Protein concentrations were measured by the method of Lowry *et al.*[11] using crystalline bovine serum albumin as reference standard.

Estradiol-2/4-hydroxylase assay and determination of kinetic parameters

Estradiol-2/4-hydroxylase activity in brain and liver microsomes was assayed by the radio-enzymatic method of Paul *et al.*[1] as previously described [8, 12]. The method was slightly modified by performing the assay at pH 8.0 instead of pH 7.4 [9]. To determine kinetic parameters (K_m and V_{max}) the enzyme activity was assayed at different concentrations of estradiol (1–50 μM) and Lineweaver–Burk plots constructed by means of linear regression analysis. For this purpose an Apple II computer and a program, supplied by Dr R. Horton, St Georges Hospital, London, were used.

TLC analysis of products assayed

The radioactive products measured in the radio-enzymatic assay were analyzed by means of TLC (Merck Silicagel 60F255 on aluminium foil) using chloroform–methanol–acetic acid (96:3:1, by vol.) as solvent system [1]. Authentic stable 2-methoxyestradiol and 2-hydroxyestradiol-3-methylether (Sigma) were used as markers. Chromatographed stable components were visualized by staining with I_2 vapour and lightly marked with a pencil. The positions of radioactive products were determined by cutting chromatograms into 1 cm strips and counting the radioactivity contained in each strip.

RESULTS

Characteristics of the estradiol-2/4-hydroxylase assay

In the present study the radio-enzymatic assay yielded blank values which were less than 10% of test values obtained in brain microsomes. The activity in blank samples containing no tissue and in blank samples containing heat-inactivated microsomes did not differ significantly. The relatively low blank values can be attributed mainly to the use of a COMT preparation with very little contaminating estradiol-2/4-hydroxylase activity.

The effect of pH on the assay was studied by performing the assay at different pH values between 6.0 and 8.4 at 37°C, using 0.15 M KCl–50 mM

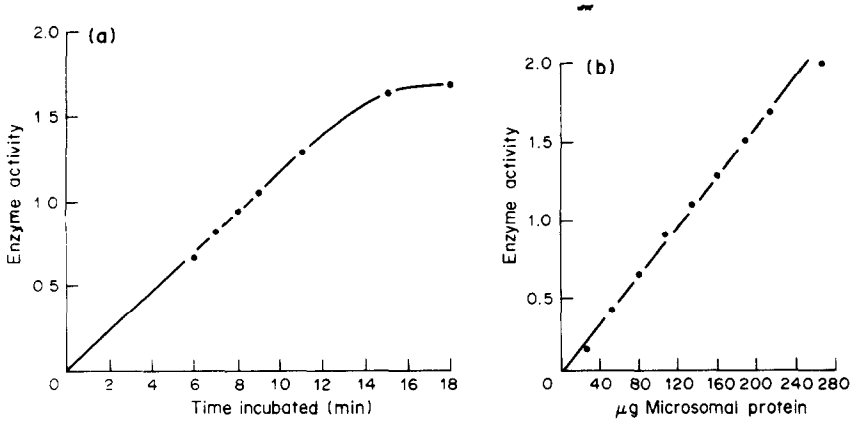

Fig. 1. Effects of time (a) and increasing microsomal protein concentration (b) on product formation in the radio-enzymatic assay of estradiol-2/4-hydroxylase activity in brain microsomes of 60-day old male rats. In the time studies (a) 100 μg microsomal protein were assayed. Assays were performed at pH 8.0 at 37°C in the presence of 50 μM estradiol. Enzyme activity shown is pmol 2-methoxyestradiol per mg microsomal protein (a), or per 10 min incubation (b).

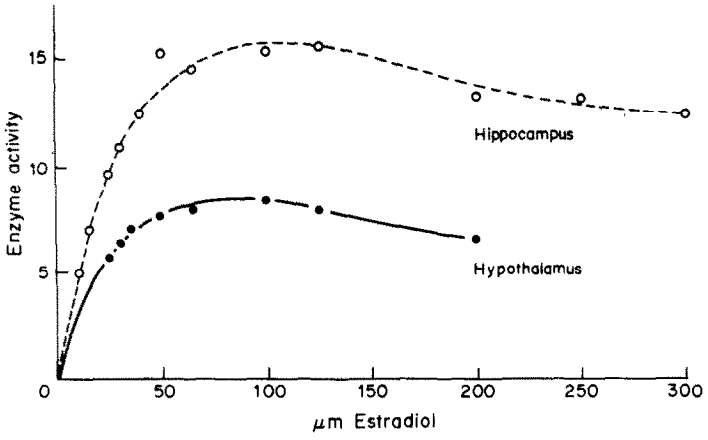

Fig. 2. Effects of increasing substrate concentrations on the rate of product formation in estradiol-2/4-hydroxylase assay. Hypothalamic and hippocampal microsomes (100 μg microsomal protein) of 50-day old male rats were assayed at pH 8.0. Enzyme activity shown is pmol 2-methoxyestradiol/mg microsomal protein/10 min.

Tris–HCl buffer. Maximum activity was observed at pH 8.0 and the assay was accordingly performed at this pH throughout the present study. Analysis by TLC indicated that the nature of the radioactive products measured in the assay was not affected to any significant extent by pH. More than 80% of the radioactive assay products, derived from assays of whole brain, hypothalamic and hippocampal microsomes, co-chromatographed with an authentic 2-methoxyestradiol marker, irrespective of whether the assay was performed at pH 7.0 or pH 8.0.

The NADPH requirements of 2/4-hydroxyestradiol formation by brain microsomes were also studied. In the absence of NADPH product formation was only 20% of that observed in the presence of saturating amounts of NADPH (400–800 μM). The latter concentration was therefore routinely used in the radio-enzymatic assay.

The effects of time and microsomal protein concentration on estradiol-2/4-hydroxlase activity were studied in hypothalamic microsomes of adult male rats. The results are shown in Fig. 1. Product formation was linear with time up to 10 min incubation at 37°C, and with increasing amounts of microsomes up to 200 μg microsomal protein. Incubation times of 10 min and 100 μg microsomal protein per assay tube were therefore routinely used for assaying estradiol-2/4-hydroxylase activity in brain microsomes. These conditions yielded amounts of product which were sufficient to permit accurate counting of radioactivity and also allowed reliable estimates of specific enzyme activity.

Substrate saturation is an important criterium for establishing that a reaction is enzymatically mediated. The effects of increasing estradiol concentrations on the rate of product formation by hypothalamic and hippocampal microsomes were therefore examined. The results of these experiments are shown in Fig. 2. Substrate saturation (beginning at about 50 μM estradiol) was exhibited by the estradiol-2/4-hydroxylase in microsomes of both hypothalamus and hippocampus. At estradiol concentrations greater than 100 μM, product formation was inhibited. To avoid the possibility of substrate inhibition 50 μM estradiol was routinely used when assaying activity at a single substrate concentration. In determining kinetic parameters (K_m and V_{max}) of estradiol-2/4-hydroxylase activity, estradiol concentrations between 1 and 50 μM were used. The curves presented in Fig. 2 also show that the estradiol-2/4-hydroxylase activity in microsomes of rat hippocampus was significantly greater than in hypothalamic microsomes as previously reported [9].

Subcellular distribution of enzyme activity

The subcellular distribution of estradiol-2/4-hydroxylase activity was studied in whole brain (minus cerebellum) and liver of adult male rats. Specific enzyme activities in microsomes, mitochondria and soluble fractions (105,000 *g* supernatant) were assayed under the conditions routinely used for brain microsomes. The results are summarized in Table 1. In both brain and liver 70–80% of the total activity in the three subcellular fractions was present in the microsomes. The microsomal fractions also exhibited the highest specific activity. Only 1–2% of the total activity was present in the soluble fractions of both brain and liver and the specific activity in these fractions was between 200- and 400-fold less than in microsomes.

Effects of P-450 inhibitors

To obtain evidence that microsomal P-450 catalyzes catecholestrogen formation in rat brain, we studied the effects of carbon monoxide, α-naphthoflavone and metyrapone on estradiol-2/4-hydroxylase activity in hypothalamic and hippocampal microsomes. For comparative purposes studies with carbon monoxide were also done on microsomes obtained from whole brain and liver. The effects of

Table 1. Subcellular distribution of estradiol-2/4-hydroxylase activity in rat brain and liver

Subcellular fractions	Estradiol-2/4-hydroxylase activity (pmol 2-methoxyestradiol/mg protein) and protein yields (mg/g tissue) of different subcellular fractions					
	Brain			Liver		
	Protein yield (mg)	Activity	% Of total	Protein yield (mg)	Activity	% Of total
Microsomes	5.6	43	73	25	1200	81
Mitochondria	24	3.5	26	35	187	17
Soluble fraction	40	0.1	1	145	6	2

Table 2. Effects of carbon monoxide on estradiol-2/4-hydroxylase activity in microsomes of rat liver and brain

Incubating conditions	Estradiol-2/4-hydroxylase activity (pmol 2-methoxyestradiol/mg microsomal protein/10 min)			
	Liver	Brain (whole)	Hypothalamus	Hippocampus
Air	1310	11.2	6.5	10.3
$N_2 + O_2$ (9:1)	1270	10.7	—	—
$CO + O_2$ (9:1)	421	5.0	3.4	4.4
% Inhibition by $CO + O_2$	68	55	48	57

carbon monoxide on estradiol-2/4-hydroxylase activity in brain and liver microsomes are summarized in Table 2. Carbon monoxide caused significant inhibition of the enzyme activity in both brain and liver microsomes, with the percentage inhibition ranging between 48% (hypothalamus) and 68% (liver).

The effects of α-naphthoflavone and metyrapone on estradiol-2/4-hydroxylase activity in hypothalamic and hippocampal microsomes were studied by assaying the enzyme activity in the presence of different amounts of each of the two P-450 inhibitors, using 30 μM estradiol as substrate. The results are presented in Fig. 3. The main findings which we derived from the inhibition curves in Fig. 3, were as follows: Both α-naphthoflavone and metyrapone inhibited estradiol-2/4-hydroxylase activity in hypothalamic and hippocampal microsomes, but α-naphthoflavone was the more potent of the two inhibitors. Concentration requirements for 50% inhibition of enzyme activity were 50–70 μM for α-naphthoflavone, and 200 μM for metyrapone. Biphasic inhibition curves, with plateaus between about 60 and 150 μM, were obtained in the case of metyrapone. The estradiol-2/4-hydroxylase activity in the hippocampus was significantly more sensitive

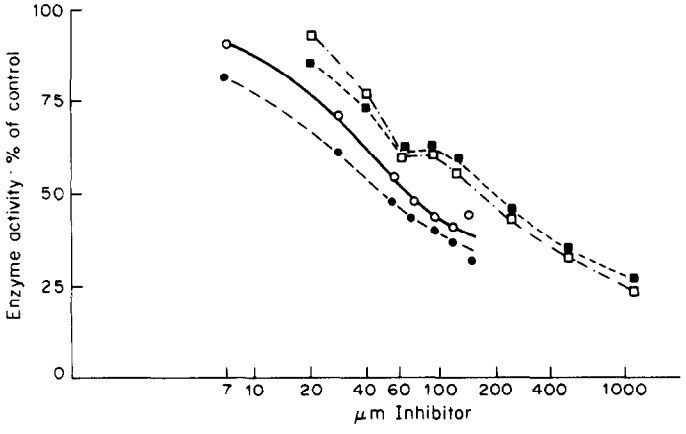

Fig. 3. Effects of α-naphthoflavone and metyrapone on estradiol-2/4-hydroxylase activity in hypothalamic and hippocampal microsomes of 50-day old female rats. Estradiol concentration used was 30 μM. Enzyme activity is expressed as the percentage of the activity (100%) present in samples containing no inhibitors. The curves from left to right represent: Hippocampal activity in presence of α-naphthoflavone (●——●); Hypothalamic activity in presence of α-naphthoflavone (○——○); Hypothalamic activity in presence of metyrapone (■---■); Hippocampal activity in presence of metyrapone (□—·—□). The sensitivity of the hippocampal enzyme activity towards α-naphthoflavone was significantly greater than that of the hypothalamus ($p < 0.01$, paired t-test).

to α-naphthoflavone inhibition than the hypothalamic activity ($P < 0.01$, paired t-test). With respect to metyrapone, however, the hippocampal and hypothalamic activities did not differ significantly in their sensitivity to inhibition.

Effects of ontogeny and sex on enzyme kinetics

To determine whether development and sex affect estradiol-2/4-hydroxylase activity in discrete brain areas of the rat, two series of experiments were done. In the first series estradiol-2/4-hydroxylase activity in the hypothalamus and hippocampus was assayed, using a single, saturating substrate concentration (50 μM estradiol), at different ages from 20 to 75 days in male and female rats. In the second series of experiments kinetic parameters (K_m and V_{max}) for estradiol-2/4-hydroxylase activity in hypothalamic and hippocampal microsomes were determined in male and female rats at three different ages between 23 and 71 days after birth.

The results of the first series of experiments are shown in Fig. 4. While the estradiol-2/4-hydroxylase activity in the hypothalamus increased between 20 and 75 days of age, the hippocampal activity did not appear to change significantly. No significant sex differences in activity were observed at any stage in the hypothalamus or hippocampus. The activity in the hippocampus was significantly greater than in the hypothalamus throughout the period of development studied.

The effects of development and sex on the kinetic parameters of estradiol-2/4-hydroxylase activity in the hypothalamus and hippocampus are presented in Table 3. The most important finding which emerges from these data is that the K_m of estradiol-2/4-hydroxylase in the female hypothalamus showed a significant increase from 14 to 47 μM between 23 days and 71 days of age, while it remained constant at about 29 μM in male rats. This is shown in Fig. 5. The K_m and V_{max} values for the hippocampal enzyme showed no significant sex differences or ontogenetic changes, but were in general substantially

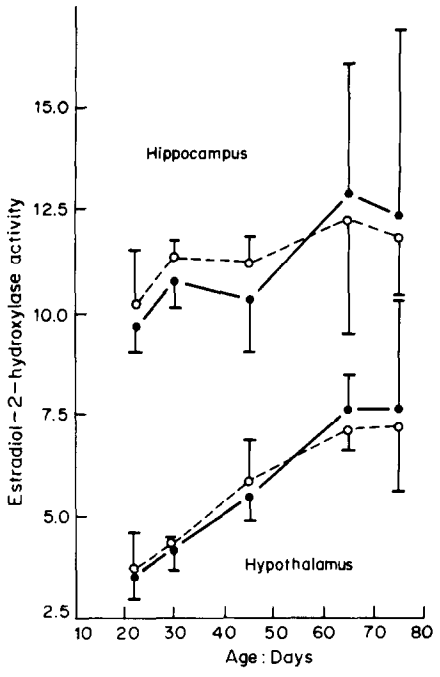

Fig. 4. Effects of age on estradiol-2/4-hydroxylase activity in hypothalamic and hippocampal microsomes of male and female rats. Values shown are means of 2–3 experiments. Bars at 22, 45 and 75 days represent ±SD, and at 30 and 65 days the range of 2 experiments. Enzyme activity was assayed at pH 8.0, using 100 μg microsomal protein and 50 μM estradiol. Activity shown is pmol 2-methoxyestrogen formed/mg microsomal protein/10 min. Solid lines represent activity in males and dotted lines in females.

greater than the K_m and V_{max} values of the hypothalamic estradiol-2/4-hydroxylase. Representative Lineweaver–Burk plots of enzyme activity in female hypothalamus and hippocampus at 23 and 71 days of age are shown in Fig. 6.

DISCUSSION

It has recently been suggested that *in vitro* measurements of estradiol-2/4-hydroxylase activity in the

Table 3. Ontogenetic changes and sex differences in kinetic parameters of estradiol-2/4-hydroxylase activity in microsomes of rat hypothalamus and hippocampus

			Kinetic parameters measured			
			K_m (μM estradiol)		V_{max} (pmol methoxyestradiol/mg microsomal protein/10 min)	
Brain areas	Age (days)		Male	Female	Male	Female
Hypothalamus	23 ± 2	$N = 3$	29.2 ± 4.71	13.6 ± 2.54	9.3 ± 1.27	6.9 ± 1.93
	49 ± 5	$N = 2$	29.7 (24.3–35.2)	19.7 (17.8–21.6)	12.8 (11.4–14.2)	10.1 (10.0–10.1)
	71 ± 4	$N = 3$	27.3 ± 2.24	46.9 ± 16.76	11.1 ± 1.19	15.3 ± 2.66
Hippocampus	23 ± 2	$N = 2$	69.2 (49.5–88.9)	95.1 (76.9–113.3)	32.1 (27.4–36.8)	43.4 (34.3–52.5)
	49 ± 5	$N = 2$	53.4 (44.3–62.7)	68.7 (38.9–98.4)	28.3 (27.2–29.4)	34.7 (22.1–47.2)
	71 ± 4	$N = 2$	116.0 ($N = 1$)	104.3 (60.5–148)	49.3 ($N = 1$)	43.6 (29.9–57.3)

Values shown are means ± SD (range in case of 2 experiments) of the number (N) of experiments indicated.

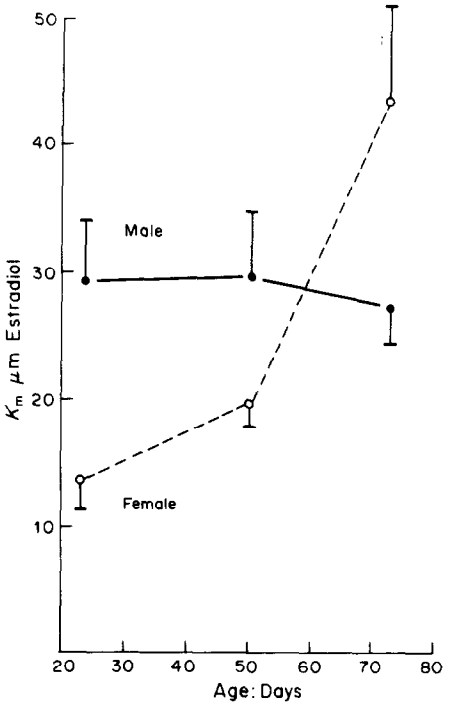

Fig. 5. Effects of age on apparent K_m of estradiol-2/4-hydroxylases in hypothalamus of male and female rats. Values shown are means of 3 experiments for each age group, except at 49 days where only 2 experiments were done. Bars denote ± SD, except at 49 days (= range). The K_m values were determined from Lineweaver–Burk plots (Fig. 6) and detailed data are shown in Table 3.

brain are prone to errors due to multiple, non-specific reactions which can hydroxylate estradiol in the C-2 and C-4 positions [13]. Several factors, such as ionic iron and lipid peroxidases, were cited as non-specific sources of catecholestrogen formation. Since the estradiol-2/4-hydroxylase activity in brain tissue is very low, it was argued that non-specific product formation might contribute substantially to the total activity measured. Particular care would therefore be required to distinguish between physiologically relevant enzymatic activity and *in vitro* artifactual activity (non-specific catecholestrogen formation).

Under the assay conditions used in the present study non-specific estradiol-2/4-hydroxylase activity did not constitute a significant problem, as judged by the relatively low blank values. The activities in "non-tissue" blanks, and in blank samples containing heat-inactivated brain microsomes, were similar and did not exceed 10% of the total activity measured in freshly prepared brain microsomes. Similar low blank values have also been reported by previous workers who used the radioenzymatic assay [12]. This contrasts sharply with the high blank values reported for the tritiated water assay [13, 14]. A noteworthy point is that Fishman and Norton [13] observed greater tritiated water formation in the case of heated brain microsomes than in freshly prepared microsomes. They stated that heating, and other membrane disrupting processes, can be expected to increase the availability of ionic iron and lipid peroxides and therefore to increase non-specific catecholestrogen formation.

The fact that the radio-enzymatic assay yielded significantly lower blank values than the tritiated water assay, indicates that the importance of non-specific product formation as a general source of error in estradiol-2/4-hydroxylase assays, may have been overrated. It now seems very likely that most of the tritiated water measured in blank samples of this assay, does not represent catecholestrogen formation [14–15]. Hersey *et al.*[14] found that 85–90% of the total amount of tritiated water formed from [2-^{3}H]-estradiol by rabbit hypothalamic tissue could not be ascribed to estrogen-2-hydroxylase activity. In a more recent study [15] it was shown that catecholestrogens can form tritiated water by reactions not involving hydroxylation. The authors of this study therefore concluded that methods for the measurement of catecholestrogen formation, which are based on the generation of tritiated water, are only valid if subsequent reactions can be eliminated. The contention that non-specific factors may contribute significantly to catecholestrogen formation *in vitro* is therefore not entirely valid, since it is based largely on high blank values obtained in the tritiated water assay [13]. Our present results indicate that

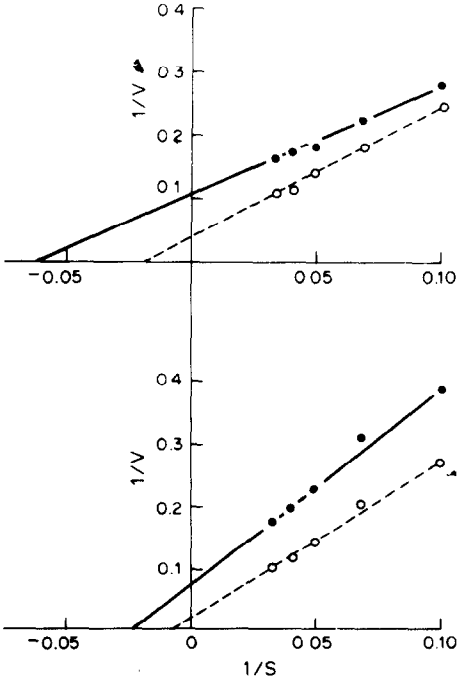

Fig. 6. Representative Lineweaver–Burk plots of estradiol-2/4-hydroxylase activity in hypothalami and hippocampi of female rats at different ages. Upper part of figure (23 days of age); lower part (71 days of age). Solid lines represent hypothalamic activity and dotted lines hippocampal activity. $S = \mu M$ estradiol; V = pmol 2-methoxyestradiol/mg microsomal protein/10 min.

COMT is the only readily discernible source of non-specific estradiol-2/4-hydroxylase activity in the radio-enzymatic assay. This problem can, however, be overcome by using sufficiently pure COMT [12] and appropriate blanks. We therefore conclude that the radio-enzymatic assay is a reliable and valid method for measuring estradiol-2/4-hydroxylase activity in brain tissue.

In the present study we have provided evidence that the estradiol-2/4-hydroxylase activity in rat hypothalamus and hippocampus is enzymatically mediated, since it exhibited cofactor and substrate saturation. The microsomal location, requirement for NADPH, and inhibition by CO, α-naphthoflavone and metyrapone, provide substantial evidence that estradiol-2/4-hydroxylase activity in the brain is mediated by microsomal P-450 isozymes, and fully confirm earlier findings [1, 3, 16, 17] in this respect.

The observation that both α-naphthoflavone and metyrapone inhibited estradiol-2/4-hydroxylase activity in rat hypothalamus and hippocampus, strongly suggests that different forms of microsomal P-450 may catalyze catecholestrogen formation in the brain. Metyrapone is a relatively specific inhibitor of those forms of P-450 which are inducible by phenobarbitone in the liver, while α-naphthoflavone specifically inhibits forms of P-450 (P448) normally inducible by aromatic hydrocarbons such as 3-methylcholanthrene [18]. Previous workers have suggested that multiple P-450 isozymes may be involved in catecholestrogen formation [3] and direct evidence for this possibility has been found in the case of mouse liver [4]. To our knowledge the present findings constitute the first evidence that multiple P-450 isozymes may be involved in catecholestrogen formation in the brain. Of perhaps even greater significance is the fact that the estradiol-2/4-hydroxylase activities in the hypothalamus and hippocampus appeared to differ qualitatively as evidenced by the greater sensitivity to α-naphthoflavone and the higher K_m values of the hippocampal enzyme. The possibility that qualitative differences between the hypothalamic and hippocampal activities might be partly due to differences in microsomal membrane composition cannot, however, be ruled out by the present findings.

While more evidence is obviously required, our tentative conclusion that multiple P-450 isozymes in the brain may be responsible for *in vitro* catecholestrogen formation is consistent with current concepts about these multisubstrate monooxygenases. There is now overwhelming evidence that the expression of individual microsomal P-450 isozymes may vary independently between different tissues and as a function of age, sex, species and extraneous factors, such as drug induction [5–7].

We can see no *a priori* reason why multiple forms of P-450 should not also be independently expressed in functionally different brain regions. The present evidence for qualitative differences between estradiol-2/4-hydroxylase activity in the hypothalamus and hippocampus certainly favours such a possibility.

There is substantial evidence that the brain contains microsomal P-450 [16, 19–23], but the biological function of these monooxygenases in neurons is not known. The results of the present study provide indirect evidence for a possible physiological role for at least one type of P-450-catalyzed reaction. In this context the age-related increase in the K_m of hypothalamic estradiol-2/4-hydroxylases in female rats appears to be particularly significant. Since this change in K_m was opposite to that previously observed in the liver of female rats [8] and was not observed in the male hypothalamus, or in the hippocampus of either sex, it may possibly reflect a specific, age-related physiological requirement of the hypothalamus in female rats. What this putative requirement is, must for the moment remain speculative. Assuming that estradiol-2/4-hydroxylases act to terminate the biological effects of estradiol, a protective role could be envisaged for them during the stage when the rat brain undergoes sexual differentiation. Exposure of neonatal rats to excessive amounts of estradiol disrupts the normal sexual differentiation of the brain, leading to sterility and male sex behaviour in the female [24]. Different mechanisms, including binding by α-feto-protein, may protect the immature rat brain against over-exposure to estradiol [24, 25]. It is therefore conceivable that estradiol-2/4-hydroxylases may provide an additional protective measure, particularly in central target areas such as the hypothalamus. Very recent work by Brown *et al.*[26] offers additional and independent support for this possibility. They suggested that the increased estradiol-2/4-hydroxylase activity, which they observed in female rat brain after 10 days of age, may explain the attenuated response to androgens adminstered at this time, i.e. protection against androgenization.

In the present study we confirmed our previous observation [9] that the estradiol-2/4-hydroxylase activity in rat hippocampus is significantly greater than in the hypothalamus. This is of some interest since previous studies have indicated that the hypothalamus has the highest catecholestrogen-forming activity in the brain [27–31]. The reasons for these conflicting results are not clear, but some of the previous findings may be questionable on methodological grounds. A more important question, however, is why the hypothalamus, being a major target area for estradiol, should exhibit a smaller *in vitro* capacity to hydroxylate estradiol than the hippocampus. At present we can only speculate about the reasons for this apparent anomaly. A likely possibility is that the *in vitro* estradiol-2/4-hydroxylase activity measured in hippocampal microsomes reflects the participation of relatively non-specific forms of P-450 due to the wide substrate specificity of these monooxygenases [4–5]. It is possible that the hippocampus, being an important target area for corticosteroids [32], may contain P-450 isozymes that

are induced by, and specifically metabolize these steroids under physiological *in vivo* conditions. Of possible interest in this context is the recent finding that dexamethasone and other glucocorticoids induce a specific form of P-450 in rat liver [33]. Under *in vitro* conditions, utilizing non-physiological estradiol concentrations, such putative corticosteroid-inducible, or corticosteroid-metabolizing P-450 isozymes in the hippocampus may well be able to hydroxylate estradiol, but in a relatively non-specific manner. The high K_m values observed in the case of the hippocampus tends to supprt this possibility. An analogous situation is seen in the case of rat liver *in vitro*, where adult male liver exhibits a 5- to 10-fold higher capacity than that of females to form catecholestrogens [8, 12], despite the fact that male rats do not have to cope with the same circulating estradiol levels as females. Another example is the significant increase of hepatic estradiol-2/4-hydroxylase activity caused by phenobarbitone treatment. Unpublished studies in our laboratory indicate that virtually all of the phenobarbitone-induced estradiol-2/4-hydroxylase activity in female rat liver is due to a form(s) of P-450 which is very sensitive to metyrapone inhibition and is not affected by α-naphthoflavone, while the constitutive activity (untreated female rats) is much more sensitive to α-naphthoflavone than to metyrapone inhibition.

In summary then the findings of the present study may have important general implications. Firstly, they indicate that there are qualitative differences in P-450 isozymes between functionally different brain regions. Secondly, they indicate that the P-450 isozymes, or spectra of P-450 isozymes, in different parts of the brain may be independently affected by biological factors, such as age and sex, thus giving indirect support to the notion that microsomal P-450 may play a physiological role in the brain, which might vary between different brain areas.

Finally, we suggest that the participation of multiple forms of P-450 in catecholestrogen biosynthesis lends a new perspective to our view of estradiol-2/4-hydroxylases, but at the same time poses an important practical problem in characterizing them. The essential problem is that, while more than one P-450 isozyme may catalyze catecholestrogen formation *in vitro*, not all of these isozymes may be physiologically relevant in the intact animal. In characterizing catecholestrogen-forming enzymes it may therefore be of crucial importance to distinguish between physiologically "relevant" and "non-relevant" P-450 isozymes which are able to hydroxylate estrogens in the 2- and 4-positions of the A ring.

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