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INTERACTION BETWEEN OESTROGENS AND NEUROTRANSMITTERS AT THE HYPOPHYSIAL-HYPOTHALAMIC LEVEL

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SUMMARY

When 2-hydroxylated oestrogens are incubated with the 150,000 *g* supernatant of rat brain in the presence of *S*-adenosylmethionine, the isomeric 2- and 3- monomethyl ethers are formed. The enzymic methylation of 2-hydroxyoestradiol-17β and 2-hydroxy-17α-ethinyloestradiol-17β in brain tissue is catalysed by a *S*-adenosylmethionine: catechol *O*-methyltransferase (EC 2.1.1.6) which exhibits similar kinetics as the catechol *O*-methyltransferase of human liver. The K_m values were 19 μM for 2-hydroxyoestradiol-17β and 36 μM for 2-hydroxy-17α-ethinyloestradiol-17β; the K_m values for catechol amines were about 10 times higher and amounted to 320 μM for adrenaline, 270 μM for noradrenaline and 240 μM for dopamine. These findings indicated that the methylation of catechol amines by the brain catechol *O*-methyltransferase may be inhibited by catechol oestrogens. Accordingly, the effect of 2-hydroxyoestradiol-17β and 2-hydroxy-17α-ethinyloestradiol-17β on the methylation of adrenaline, noradrenaline and dopamine has been studied.

The following K_i values were found: 8·4 μM for adrenaline, 9·4 μM for noradrenaline and 6·8 μM for dopamine with 2-hydroxyoestradiol-17β as inhibitor; the corresponding values with 2-hydroxy-17α-ethinyloestradiol-17β as inhibitor were 4·5 μM for adrenaline, 6·8 μM for noradrenaline and 3·8 μM for dopamine. As for the inhibitory effect of 2-hydroxylated oestrogens, no significant differences were observed in the thalamic, hypothalamic, medullar and hypophysial areas, respectively.

INTRODUCTION

It is now well established that enzymic hydroxylation of phenolic steroids at C-atom 2 is an important reaction in the intermediary metabolism of oestrogens in mammals [1, 2]. C-2 hydroxylation has been shown to occur in a variety of organs, particularly in liver (cf. [3]). The 2-hydroxylated oestrogens have a catechol structure and are further metabolized by methylation of the phenolic hydroxyl groups at C-atoms 2 and 3. This reaction is catalysed by a *S*-adenosylmethionine: catechol *O*-methyltransferase (EC 2.1.1.6) [4, 5]. Recently, Fishman reported that tritiated oestradiol-17β is converted to the corresponding 2-hydroxylated derivative by hypothalamic tissue of intact and of ovariectomized rats at a rate of 0·3% and 1·3%, respectively; no conversion was found in the cerebral cortex or in the anterior pituitary [6].

In previous experiments from our laboratory, it has been shown that, *in vitro*, enzymic methylation of catecholamines by the *S*-adenosylmethionine: catechol *O*-methyltransferase (catechol *O*-methyltransferase; COMT) from liver tissue of various species is strongly inhibited by 2-hydroxylated oestrogens [7–10]. This inhibition is a competitive one; with adrenaline as substrate, the K_i values for 2-hydroxyoestrone and 2-hydroxyoestradiol-17β were found to be 8·5 μM and 7·0 μM, respectively [9]. It has been repeatedly suggested that the results of these studies, carried out with a highly purified COMT from human liver, may be of clinical relevance with respect to the regulation of blood pressure under normal and pathological conditions [9, 10].

The recent finding by Fishman[6] that 2-hydroxylation of oestrogens also takes place in certain areas of brain led us to the investigation of the following question: Do 2-hydroxylated oestrogens inhibit the enzymic methylation of catecholamines by brain COMT? In the present paper, an attempt has been made to elucidate this question.

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EXPERIMENTAL

Steroids

2-Hydroxyoestradiol-17β (1,3,5(10)-oestratriene-2,3, 17β-triol) was supplied by Schering (Berlin, Germany); 2-hydroxy-17α-ethinyloestradiol-17β (17α-ethinyl-1,3,5 (10)-oestratriene-2,3,17β-triol) was generously donated by Professor R. Knuppen of Lübeck, Germany. 2-Methoxyoestradiol-17β (2-methoxy-1,3,5(10-oestratriene-3,17β-diol) and 2-hydroxyoestradiol-17β 3-methyl ether (3-methoxy-1,3,5(10)-oestratriene-2,17β-diol) were prepared as described by Fishman *et al.*[11].

Catechol amines

Adrenaline (3,4-dihydroxy-phenyl-ethanolamine N-methyl), noradrenaline (3,4-dihydroxy-phenyl-ethanol amine) and dopamine (3,4-dihydroxy-phenyl-ethyl amine) were purchased from Fluka (Buchs, Switzerland). The purity of all substrates was checked by paper and thin-layer chromatography.

Chemicals

S-adenosyl-L-[*Me*-^{14}C]methionine (specific radioactivity 60·4 mCi/mmol) was obtained from the Radiochemical Centre (Amersham, U.K.); *S*-adenosyl-L-methionine was purchased from Boehringer Mannheim (Mannheim, Germany). The *S*-adenosyl-L-[*Me*-^{14}C] methionine, dissolved in sulphuric acid (pH 3·5–4·0), was diluted to a specific activity of 0·4 nCi/nmol by the addition of crystalline *S*-adenosyl-L-methionine.

All other reagents (A.R. grade when available) and solvents (distilled before use if necessary) were obtained from Merck (Darmstadt, Germany). The following buffer was used: 0·06 M phosphate buffer, pH 8·0. Unisolve 1 (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) was used as scintillation fluid.

Animals and preparation of tissue

The animals were adult female rats of an inbred Wistar strain (mus rattus, Brunntal bei München, Germany), weighing 150–200 g. Rats were killed by decapitation. The brains were rapidly removed, cleansed of adhering blood clots, and, in addition to the hypophysis, three brain regions (hypothalamus, thalamus and medulla) dissected according to the procedure, described by McEwen and Pfaff[12]. The tissue was homogenized with 2 parts (w/v) of 0·25 M sucrose in a Potter-Elvehjem homogenizer for 1 min. The homogenate was centrifuged for 30 min at 12,000*g*. The supernatant was carefully decanted and centrifuged at 150,000*g* for 60 min to sediment the microsomes.

Determination of protein

The protein concentration of the 150,000*g* fractions (cytosol) was determined by the method of Lowry *et al.*[13]; protein concentrations were expressed in mg/ml and were read from a standard curve, obtained with bovine serum albumin.

Incubation procedure

100 μl of the 150,000*g* supernatant from rat brain, containing approx. 1 mg protein, was incubated with varying amounts (5–100 μM) 2-hydroxylated oestrogens or catechol amines, respectively, 100 μM *S*-adenosyl-L-[*Me*-^{14}C]methionine, 6 mM $MgCl_2$ and 10 mM cysteine hydrochloride, neutralized, in 0·06 M phosphate buffer, pH 8·0, in a total volume of 0·5 ml. Incubations were carried out for 2 h at 37° under air. Control incubations contained S-adenosyl-L-[*Me*-^{14}C] methionine and buffer (reagent blank).

Extraction and measurement of radioactivity

The incubation mixtures were extracted with 2 ml of ether/chloroform (3:1, v/v), the ether/chloroform extracts evaporated to dryness and the residues dissolved in a small volume of methanol. Ten millilitres of scintillation fluid (Unisolve 1) was added. A Nuclear Chicago liquid scintillation spectrometer (Mark II) was used for the measurements of radioactivity. In control experiments, it was found that only negligible amounts of radioactive *S*-adenosylmethionine were extracted by ether/chloroform from the aqueous incubation mixtures.

Thin-layer chromatography

Commercially prepared (Merck, Darmstadt, Germany) 20 × 20 cm plates, coated with silica gel G (layer thickness 0·25 cm), were used after being washed overnight with chloroform/methanol (9:1, v/v), The chromatograms were developed at room temperature with 99:1 chloroform/methanol (v/v). The plates were dried in air and the reference steroids located by Folin-Ciocalteu reagent. The radioactive metabolites were scraped off and eluted with methanol; radioactivity was measured in Unisolve 1.

RESULTS

When 2-hydroxyoestradiol-17β was incubated with the COMT preparation from rat brain (150,000*g* supernatant) in the presence of *S*-adenosylmethionine, methylation of the substrate was observed. To find whether both the isomeric 2- and 3-monomethyl ethers of 2-hydroxyoestradiol-17β had been formed, the extract from the incubation mixtures were sub-

jected to thin-layer chromatography. Two radioactive metabolites were detected which correspond in mobility to authentic 2-methoxyoestradiol-17β and 2-hydroxyoestradiol-17β 3-methyl ether, respectively. This indicates that the 2-hydroxyl group as well as the 3-hydroxyl group of 2-hydroxyoestradiol-17β is methylated by brain COMT. The two monomethyl ethers were formed in a ratio of approximately 1·4. In all further experiments, the total rate of methylation was measured, without separation of the isomeric ethers, by counting directly the radioactivity, extracted from the aqueous incubation mixtures.

The rate of methylation of 2-hydroxyoestradiol-17β and 2-hydroxy-17α-ethinyloestradiol-17β by the COMT preparation from rat brain was proportional to the amount of protein incubated. A linear relationship between the time of incubation and the rate of methylation was found for the first three hours. There was no sharp pH maximum but rather a plateau between pH 7 and 8; this finding is in contrast to the results obtained with the purified COMT from human liver, where two distinct maxima were observed [5]. There appears to be an absolute requirement for magnesium ions. At a substrate concentration of 50 μM of 2-hydroxyoestradiol-17β, methylation was maximal at a $MgCl_2$ concentration of 3 mM; further increase in Mg^{2+} concentration reduced the activity of COMT from rat brain. The activating effect of magnesium ions at lower concentrations may be due to its essential role in forming a quaternary complex between the various reactants (cf.[14]).

The dependence of reaction velocity on the concentration of substrate was studied with both 2-hydroxyoestradiol-17β and 2-hydroxy-17α-ethinyloestradiol-17β (Fig. 1). The curve of the rate of methylation of 2-hydroxyoestradiol-17β reached a plateau at 40 μM, followed by a slight decrease above a substrate concentration of 70 μM. Similarly, with 2-hydroxy-17α-ethinyloestradiol-17β as substrate, a maximum of methylation was observed at about 30 μM, followed by a slight decrease above 40 μM. Thus, methylation of 2-hydroxylated oestrogens by brain COMT, although dependent on substrate concentration, does not fully obey simple Michaelis–Menten kinetics. It may be concluded that the slight decrease in the rate of methylation is due to product inhibition, as has previously been shown for the COMT from human liver [5]. The K_m value for 2-hydroxyoestradiol-17β was 19 μM and that for 2-hydroxy-17α-ethinyloestradiol-17β was 36 μM; the K_m value for *S*-adenosylmethionine, in the presence of 2-hydroxyoestradiol-17β, was found to be 31 μM.

From numerous studies, it is well known that the COMT is primarily involved in the methylation of catecholamines; it seemed, therefore, of interest to compare the K_m values for 2-hydroxyoestrogens with those for adrenaline, noradrenaline and dopamine. The Michaelis–Menten constants for the catecholamines were about ten times higher than those for the catechol oestrogens; they were 320 μM for adrenaline, 270 μM for noradrenaline and 240 μM for dopamine. This indicates that 2-hydroxyoestrogens have a higher affinity for the enzyme than have adrenaline, noradrenaline and dopamine; furthermore, it seems not unreasonable to assume that the enzymic methylation of catecholamines by brain COMT may be inhibited by 2-hydroxylated oestrogens. Accordingly, the interaction between the two classes of hormones was studied.

When *adrenaline* was incubated with the COMT preparation from rat brain in the presence of increasing amounts of 2-hydroxyoestradiol-17β, an

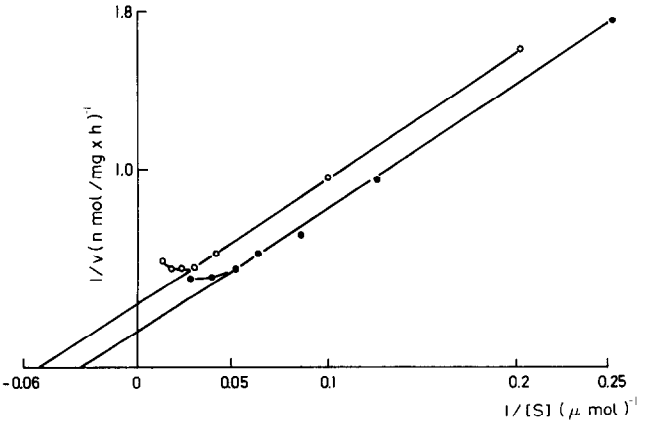

Fig. 1. Dependence of reaction velocity (rate of methylation) on the concentration of 2-hydroxyoestradiol-17β (○---○) and 2-hydroxy-17α-ethinyloestradiol-17β (●—●), respectively. 0·1 ml portions of the cysteine-activated enzyme preparation (containing 1 mg protein) were incubated with increasing amounts of 2-hydroxyoestrogens in the presence of 50 nmol *S*-adenosyl-L-[*Me*-^{14}C]methionine (50 nCi) and 3 μmol $MgCl_2$ in phosphate buffer, pH 8·0.

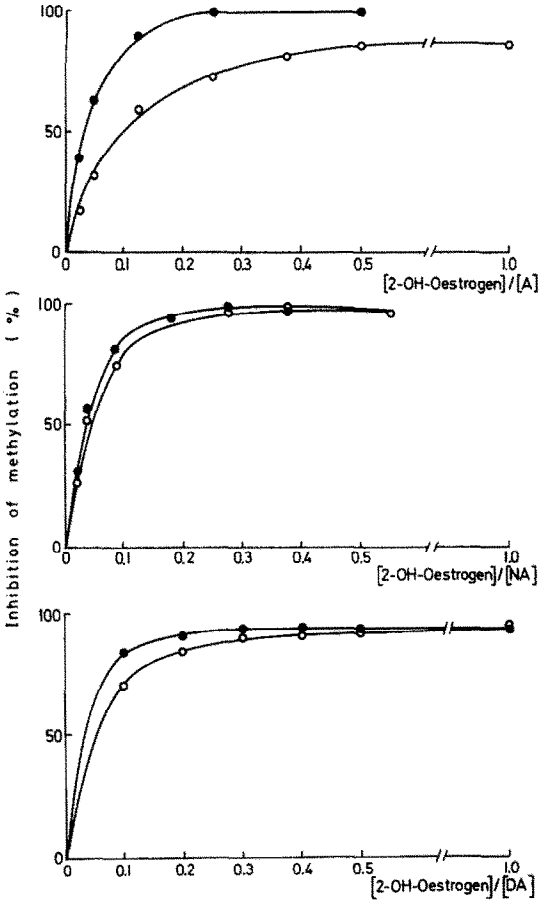

Fig. 2. Inhibition of the methylation of adrenaline (A), noradrenaline (NA) and dopamine (DA), respectively, by increasing amounts of 2-hydroxyoestradiol-17β (○—○) and 2-hydroxy-17α-ethinyloestradiol-17β (●—●), respectively. 0·1 ml portions of the cysteine-activated enzyme preparation (containing 1 mg protein) were incubated with 150 nmol adrenaline, noradrenaline or dopamine, 50 nmol *S*-adenosyl-L-[*Me*^{14}C]methionine (50 nCi) and 3 μmol $MgCl_2$ in phosphate buffer, pH 8·0, in the presence of increasing amounts (5–150 nmol) of 2-hydroxyoestradiol-17β or 2-hydroxy-17α-ethinyloestradiol-17β.

increasing inhibition of the methylation of the catecholamine occurred (Fig. 2). At a molar ratio of 0·1 for inhibitor to substrate, methylation was inhibited by 50%; at a molar ratio of unity, inhibition was 85%. 2-Hydroxy-17α-ethinyloestradiol-17β was an even more potent inhibitor than 2-hydroxyoestradiol-17β; at a ratio of 0·25 (inhibitor to substrate), a 100% inhibition of methylation of adrenaline was found (Fig. 2). When *noradrenaline* was used as substrate, the two catechol oestrogens were effective inhibitors to approximately the same degree, reaching maximal inhibition of 98% at a molar ratio of 0·3 (Fig. 2). Methylation of *dopamine* was also inhibited by catechol oestrogens; 2-hydroxyoestradiol-17β as well as the corresponding 17α-ethinyl compound had a strong inhibitory effect on the methylation of dopamine (Fig. 2). An inhibition of 50% was already found at molar ratios between 0·03 and 0·05.

To obtain information about the type of inhibition, experiments were carried out with increasing substrate concentrations and constant concentrations of inhibitors. The values were plotted according to Lineweaver and Burk[15]. As can be seen from Fig. 3,

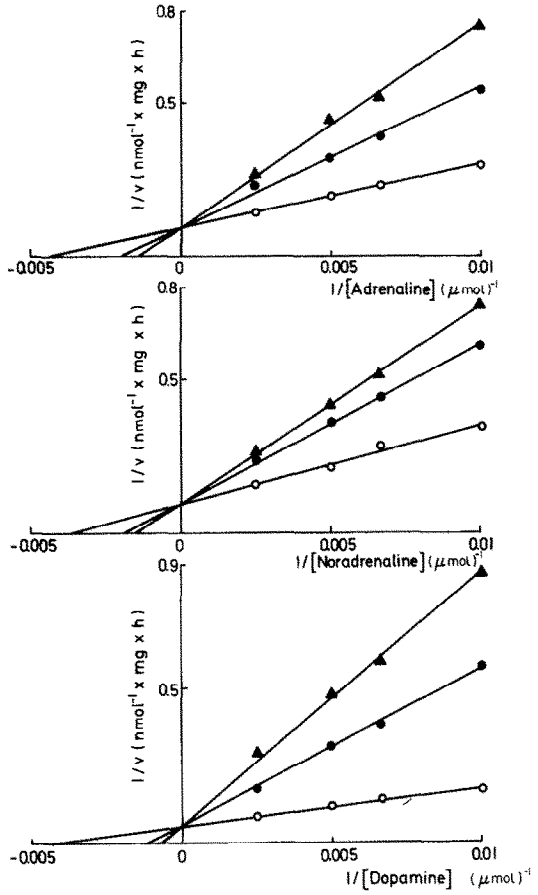

Fig. 3. Effect of 2-hydroxyoestradiol-17β and 2-hydroxy-17α-ethinyloestradiol-17β on the rate of methylation of increasing amounts of adrenaline, noradrenaline and dopamine, respectively. Addition of 2-hydroxyoestradiol-17β (●—●), addition of 2-hydroxy-17α-ethinyloestradiol-17β (▲—▲), no addition of oestrogens (○—○). 0·1 ml portions of the cysteine-activated enzyme preparation (containing 1 mg protein) were incubated with increasing amounts of catecholamines, 50 nmol *S*-adenosyl-L-[*Me*^{14}C] methionine (50 nCi) and 3 μmol $MgCl_2$ in phosphate buffer, pH 8·0, with or without addition of constant amounts of catechol oestrogens. The amounts of inhibitors were 5 nmol with adrenaline and noradrenaline as substrate, and 10 nmol with dopamine as substrate. The results are plotted according to Lineweaver and Burk[15].

Table 1. K_i values for 2-hydroxyoestradiol-17β (2–OH–E_2) and 2-hydroxy-17α-ethinyloestradiol-17β (2–OH–EE_2). Methylation of catecholamines was carried out with the catechol *O*-methyltransferase (COMT) from rat brain. The K_i values were calculated from the Lineweaver–Burk plots. For details, see Fig. 3

Substrate	Concentration of inhibitor (μM)	K_i values (μM)	
		+2–OH–E_2	+2–OH–EE_2
Adrenaline	10	8·4	4·5
Noradrenaline	10	9·4	6·8
Dopamine	20	6·8	3·8

methylation of the catecholamines by catechol oestrogens was inhibited competitively. The K_i values for the three substrates with the two inhibitors are summarized in Table 1. It is interesting to note that, in experiments with liver enzyme preparations and adrenaline as substrate, slightly higher K_i values have been reported for 2-hydroxyoestradiol-17β (18 μM) and 2-hydroxy-17α-ethinyloestradiol-17β (11 μM) [16]; again, the 17α-ethinyl compound was the stronger inhibitor.

The activity of COMT in various areas of rat brain has been studied by several investigators [17, 18]; only small differences were found between the pituitary and brain regions. Similar findings were observed, using noradrenaline as substrate and enzyme preparations from thalamus, hypothalamus, medulla and pituitary. As may be seen from Table 2, the specific activity of COMT was highest in the medulla, followed by hypothalamus, thalamus and pituitary. However, when the rate of methylation of noradrenaline was calculated on the basis of wet weight, a different picture emerged; the highest rate was found in the pituitary and the lowest in the thalamus. The differences in COMT activities, depending on the mode of calculation, are due to different amounts of water and soluble proteins in the various brain regions.

The inhibitory effect of 2-hydroxyoestradiol-17β and the corresponding 17α-ethinyl compound, in a molar ratio of 1:30 (inhibitor to substrate), was similar in all brain areas (Table 2). 2-Hydroxyoestradiol-17β proved to be less effective than 2-hydroxy-17α-ethinyloestradiol-17β in inhibiting methylation of noradrenaline.

DISCUSSION

The biochemical findings presented here lead to the question about the physiological significance of these experiments. At present, no definite answer can be given for the following reasons: (1) The physiological role of the brain COMT is not yet understood, (2) the actual local concentrations of oestrogens, and in particular of 2-hydroxyoestrogens, are not known and (3) no exact figures are available regarding the amount of catecholamines which escapes inactivation by reuptake in the brain. Therefore, one can only speculate.

When tritiated noradrenaline was injected into a lateral ventricel, approximately 13% of the remaining radioactivity was found in the normethanephrine fraction; 25% was associated with deaminated and methylated metabolites and 4% with deaminated fractions [19, 20]. The ratio of the methylated to the non-methylated metabolites was constant during a period of 24 h. These results indicate that the COMT is involved in the metabolism of noradrenaline in brain tissue; methylation and deamination appear to be necessary for the elimination of catecholamines from brain.

However, there can be no doubt that the actual amount of free noradrenaline which is accessible to enzymic methylation by brain COMT must be extremely small. From experiments with peripheral nerve preparations[21], it has been calculated that only 0·002% of catecholamines present in nervous tissue is liberated during a single impulse. The concentration of noradrenaline in brain tissue of rats has been found to vary from 0·2 to 2 μg/g wet weight[22, 23] with an approximate mean of 0·5 μg/g wet weight[24]; 0·002% of this figure corresponds to 10 pg/g wet weight. If approximately

Table 2. Inhibition of methylation of noradrenaline by 2-hydroxyoestradiol-17β (2–OH–E_2) and 2-hydroxy-17α-ethinyloestradiol-17β (2–OH–EE_2), using catechol *O*-methyltransferase preparations from different areas of rat brain. 80 μl portions of the enzyme preparation (150,000 ***g*** supernatant) of various rat brain areas (approx. 0·4 mg protein) were incubated with 30 nmol noradrenaline, 10 nmol *S*-adenosyl-L-[*Me*-^{14}C]methionine (10 nCi) and 0·6 μmol $MgCl_2$ in phosphate buffer, pH 8·0, with or without addition of 1 nmol 2–OH–E_2 or 1 nmol 2–OH–EE_2 (final volume 0·1 ml).

Brain area	Noradrenaline methylated (nMol/mg protein × h)	(nMol/g wet weight × h)	Inhibition of methylation (%) +2–OH–E_2	+2–OH–EE_2
Thalamus	2·13	74	24	31
Hypothalamus	2·39	102	26	32
Medulla	4·20	100	29	37
Pituitary	1·83	178	29	38

99% is inactivated by reuptake, only 1% of noradrenaline can be methylated by COMT; this corresponds to 0·1 pg noradrenaline/g wet weight.

On the other hand, after intravenous injection of tritiated oestradiol-17β, its uptake by pituitary tissue is similar to that by uterine tissue; the uptake by hypothalamus is significantly lower, probably by a factor of 10 [25]. The concentration of tritiated oestradiol-17β in hypothalamic tissue was about 2 pg/g wet weight. If oestradiol-17β is converted to 2-hydroxy-oestradiol-17β at a rate of 1% in hypothalamus, a concentration of 0·02 pg catechol oestrogen/g wet weight would result. This figure is comparable to the figure calculated for the free noradrenaline to be methylated. On the basis of these calculations, it may be concluded that an interaction between catechol oestrogens and catecholamines takes place at the hypothalamic level. It remains to be shown whether such an interaction has a modifying or regulatory effect on neurotransmission.

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DISCUSSION

Carstensen:

Estrogens have been shown to affect the brain function in many ways. Animal experiments in rats have shown that the localized electroshock seizure threshold during proestrus is increased in the lateral part of the amygdala while it is decreased in the dorsal hippocampus (P. S. Timiras: "Estrogens as organizers of CNS function" in D. H. Ford, ed., "Influence of Hormones on the Nervous system" (Karger, Basel (1971)/243)). Intraveous injection of estrogen in man may provoke epileptic seizures within 15 min (Logothetis J., Harner R., Morrell F., and Torres F.: *Neurology* **9** (1959) 352–360). Estrogen may be of interest in the human with regard to catamenial epilepsia, to menopausal distress and to premenstrual tension. Dr. Bäckström, in our laboratory, has shown that plasma estrogen levels are increased during the week before menstruation in women with premenstrual tension with anxiety but not in women with headache and water retention as their main symptoms. He also found that plasma progesterone levels were decreased at the same time (*J. steroid Biochem.* **5**, (1974) 257–260).

Breuer:

Well, I think at present, it is difficult to explain central effects of estrogens on the basis of 2-hydroxylation of estrogens. As far as 2-hydroxyoestrogens are concerned, I was told by Jack Fishman of New York that a psychiatric patient treated with 2-hydroxylated estrogens recovered after 2 or 3 days. Now, this may have been by accident but I think this is a wide field to be speculated on and this will keep us busy for the next years.

Martini:

Dr. Breuer, I find your results of an extreme interest. I have a few questions and please forgive my ignorance. The first

question is how stable are the catechol estrogens. The second question is related to the catecholamine field. The mechanism through which catecholamines are inactivated in the body is mainly through the monoamino oxidase. I wonder whether you could demonstrate any effect of the catechol estrogens on the mono-amino oxidase inactivation of catecholamines.

Breuer:

As far as the stability of the 2-hydroxy and 2-methoxy estrogens is concerned, they are not as stable as, of course, estradiol or estrone. The catechol structure is easily oxidized to give a dione, but as far as I know within the tissues at pH 7·4, 2-hydroxylated estrogens are stable. Now as far as the mono-amino oxidase is concerned, we have been thinking of measuring the effect of 2-hydroxy-estrogens on the activity of this enzyme. This is certainly the next thing we are going to do. We expect that MAO will be influenced by estrogens, but the great problem here is to see to what extent the catecholamines are inactived by the COMT and by MAO. As I indicated in my paper, we believe that only 1% of catecholamines are inactivated in brain tissue by COMT which is quite in contrast to the peripheral tissue where a large extent of the catecholamines is methylated. In fact the main excretory products in urine are methylated compounds.

Adlercreutz:

I would like to ask you if you studied the effect of estradiol and ethynyl estradiol on COMT.

Breuer:

Well, we did that in another connection when we started with the liver enzyme, and *in vivo* we did get an effect of estradiol and also of 2-methyoxyestradiol-17β; we explained this by 2-hydroxylation of oestradiol and the demethylation of 2-methoxy estradiol-17β. Again I must say, although I believe that the 2-hydroxy estrogens do have an effect, we have to show what is the actual rate of hydroxylation in brain tissue.