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AFFINITY OF ETHYNYL-ESTRADIOL AND MESTRANOL FOR THE UTERINE ESTROGEN RECEPTOR AND FOR THE MICROSOMAL MIXED FUNCTION OXIDASE OF THE LIVER

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SUMMARY

Ethynyl-estradiol binds to rabbit uterine supernatant ($K_D = 1{\cdot}5 \times 10^{-9}$ M) but mestranol shows no such affinity. This observation supports the concept that mestranol can act as an estrogenic compound only after demethylation to ethynyl-estradiol, this step being performed by the microsomal mixed function oxidase of the liver.

On incubation with rat liver microsomal fractions and a NADPH-regenerating system, the apparent K_M of mestranol was $3{\cdot}1 \times 10^{-5}$ M.

The demethylation of mestranol was non-competitively inhibited by ethynyl-estradiol ($K_i = 5{\cdot}4 \times 10^{-4}$ M).

Some progestational compounds (chlormadinon acetate, lynestrenol, norethynodrel, progesterone and ethynodioldiacetate) also inhibited the reaction. A competitive inhibition type ($K_i = 7{\cdot}2 \times 10^{-5}$ M) was confirmed for norethynodrel.

INTRODUCTION

ORALLY active estrogenic compounds used today in contraceptive formulations include ethynyl-estradiol and its 3-methylether, mestranol.*

Considering that ethynyl-estradiol is bound to uterine tissue of rats after administration of mestranol [1], we suggest that mestranol must be demethylated in order to act as hormonally active ethynyl-estradiol at the target tissue. This demethylation is catalyzed by the microsomal mixed function oxidase of the liver [2–4]. The ethynyl-estradiol formed during this reaction may leave the liver cell and be available in the general circulation as free compound [5].

Furthermore, ethynyl-estradiol is degraded to more polar products by the mixed function oxidase of the liver. The affinity of ethynyl-estradiol and mestranol for the estrogen receptor protein of the target tissues and their affinity for the microsomal mixed function oxidase of the liver influence the effectiveness and transformation of both compounds.

The aim of this work is to compare the interaction of ethynyl-estradiol and mestranol with both these macromolecular systems, on which the behaviour of ethynyl-estradiol and mestranol may be based.

Furthermore hydroxylations by the mixed function oxidase in the liver might

*The following trivial names have been used: Ethynyl-estradiol: 17α-ethynyl-1,3,5(10)-estratriene-3,17β-diol, Mestranol: 17α-ethynyl-3-methoxy-1,3,5(10)-estratrien-17β-ol, Lynestrenol: 17α-ethynyl-4-estren-17β-ol, Norethisterone: 17α-ethynyl-17β-hydroxy-4-estren-3-one, Norethynodrel: 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one, Chlormadinon-acetate: 17α-acetoxy-6β-chlor-4,6-pregnadiene-3,20-dione, Ethynodiol-diacetate: 3β,17β-diacetoxy-17α-ethynyl-4-estren, Megestrol-acetate: 17α-acetoxy-6β-methyl-4,6-pregnadiene-3,20-dione.

be inhibited by any other compound which can be bound to this enzymatic system. The extent of the inhibition depends on the affinity and concentration of the compound at the binding site of the enzyme. Therefore we examined progestational compounds, which accompany mestranol in commercial contraceptive formulations for their ability to inhibit the microsomal demethylation of mestranol *in vitro* in order to find out whether estrogens or progestogens might interfere with each other during their metabolism. Answering this question could contribute to solving a widely discussed problem, because O'Malley *et al.*[31] reported a decrease of antipyrin metabolism in women taking oral contraceptives.

EXPERIMENTAL

Materials

[6,7-^{3}H]-Estradiol (S.A. 40 Ci/mmol) was obtained from New England Nuclear Chemicals, Boston, Mass., and [6,7-^{3}H]-ethynyl-estradiol (S.A. radioactivity 2·72 Ci/mmol) was kindly donated by Schering AG, Berlin. A part of this material was methylated according to Nocke[6] to yield [6,7-^{3}H]-mestranol. [Methoxy-^{3}H]-mestranol (S.A. 55 mCi/mmol) was prepared by methylation of cold ethynylestradiol with [^{3}H]-methyliodide[2].

Non-radioactive steroids were supplied by Organon, Oss, Holland (lynestrenol, mestranol); Schering AG, Berlin (progesterone, norethisterone); Gideon Richter, Budapest (norethynodrel); Merck, Darmstadt (ethynyl-estradiol, chlormadinon-acetate); Boehringer, Mannheim (ethynodiol-diacetate); Novo, Kopenhagen (megestrol-acetate).

Binding experiments

To determine the dissociation constant (K_D) of the ethynyl-estradiol-receptor complex in relation to mestranol binding, we applied the method of Mešter *et al.*[7] However, the relatively low specific activity of our tritiated ethynyl-estradiol and mestranol preparations necessitated a slight modification. For the incubation of the tritiated estrogen with the receptor solution the amount of the incubated mixture was increased from 0·2 ml, as originally described by Mešter, to 2·0 ml in order to increase the radioactivity for liquid scintillation measurement. Thus, for the individual assay 0·2–5·0 pmol estrogen in 1·0 ml buffer was used. The Scatchard-plot[8] was constructed for the binding experiments as described by Mešter *et al.*[7].

Kinetic assay

[Methoxy-^{3}H]-mestranol was incubated with liver microsomal fractions of male, phenobarbital-pre-treated rats as previously described[3]. To estimate the initial reaction velocity (v_i), different substrate concentrations were chosen and aliquots of the reaction medium were poured into ice cold 7·5% (w/v) trichloroacetic acid (TCA) at incubation times of 0–5 min.

This method of using radioactive labelling guaranteed sufficient sensitivity. We applied the double scintillator technique, which has been described by Whyman[9] and by Hess *et al.*[10] for the determination of lipophilic drugs and their hydrophilic metabolites.

During the demethylation of the methoxy-labelled mestranol cold ethynyl-

estradiol, tritium labelled formaldehyde and tritiated water are formed as products [11]:

$$R\text{-}OCH_3^* + O_2 + NADPH + H^+ \rightarrow R\text{-}OH + CH_2^*O + H_2^*O + NADP^+.$$

These labelled products unlike the labelled substrate are not soluble in toluene, a condition required for the application of the double scintillator method.

After centrifugation, 0·5 ml of the TCA-supernatant was transferred to a scintillation vial with 10 ml of the water-miscible Bray's scintillator [12] and also to a vial with 10 ml toluene scintillator (composition: 4 g PPO + 0·1 g POPOP in 1 l toluene). The latter scintillator takes up the unaltered mestranol after equilibration of both phases while polar products remain in the aqueous layer and are not measured.

From the difference of the measurements, corrected for quenching, the turnover of the enzymatic reaction can be calculated.

Affinity of ethynyl-estradiol for the microsomal mixed function oxidase

In order to determine the apparent K_M for the microsomal oxidation of ethynyl-estradiol, incubations have been carried out with [6,7-^{3}H] ethynyl-estradiol as substrate.

In these experiments difficulty was encountered in attempting to isolate an individual product, although most of the ethynyl-estradiol disappeared during incubation.

Besides unaltered ethynyl-estradiol and 2-hydroxy-ethynyl-estradiol, we detected considerable amounts of polar metabolites [32], which could not be isolated. Furthermore, some radioactivity was associated with the microsomal protein.

Instead of the K_M-determination, we aimed at the determination of the K_I-value of ethynyl-estradiol on the mestranol demethylation, because this also may serve as a measure of the affinity of ethynyl-estradiol for the microsomal mixed function oxidase.

For this estimation, inhibitor concentrations (ethynyl-estradiol) of 1×10^{-4}, 2×10^{-4} and 5×10^{-4} M were chosen. A Lineweaver–Burk-plot has been constructed from the initial velocity data (v_i).

Inhibition experiments with progestational compounds

The inhibition of the microsomal demethylation of mestranol by progestational compounds was performed in a similar manner using equimolar concentrations (1×10^{-4} M) of substrate (mestranol) and inhibitor (progestogen). The incubation was stopped after 15 min and the turnover was measured as described above.

The kinetics of inhibition were determined for norethynodrel as inhibiting agent in the same concentration (1×10^{-4} M). For these experiments a Lineweaver–Burk-plot has also been applied.

RESULTS

Binding of ethynyl-estradiol and mestranol to the receptor site of rabbit uterine supernatant

The binding behaviour of ethynyl-estradiol and mestranol is demonstrated in the Scatchard-plot of Fig. 1.

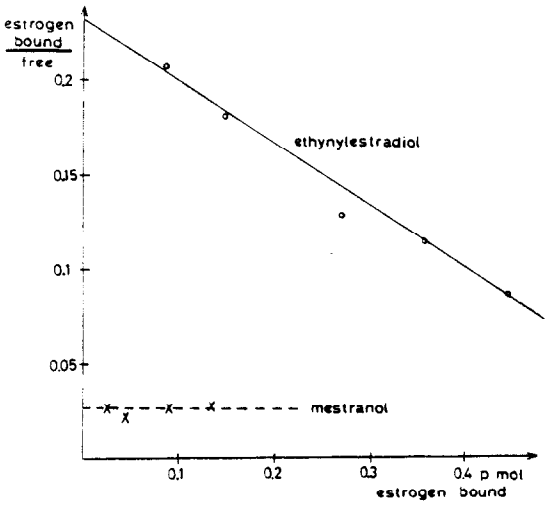

Fig. 1. Scatchard-plot [8] of the binding characteristics of [6,7-^{3}H]-ethynyl-estradiol and [6,7-^{3}H]-mestranol to the estrogen receptor in rabbit uterine supernatant. From the slope of the ethynyl-estradiol curve, a K_D of $1{\cdot}5 \times 10^{-9}$ M was calculated. Mestranol has no affinity to the receptor.

From the slope of the ethynyl-estradiol curve we calculated a K_D of $1{\cdot}5 \times 10^{-9}$ M, while the mestranol curve was parallel to the abscissa, indicating no measurable affinity for the specific estrogen-binding receptor protein of the uterus.

Hence it must be concluded that mestranol can act as an estrogen only after demethylation to ethynyl-estradiol.

Apparent K_M of the mestranol demethylation

Figure 2 shows a Lineweaver–Burk-plot of the microsomal mestranol demethylation. Further incubations at different protein concentrations gave similar results. The K_M-value was $3{\cdot}1 \times 10^{-5}$ M, the V_{max} 2 n mol/mg microsomal protein/min.

Affinity of ethynyl-estradiol for the microsomal mixed function oxidase

The product of the demethylation step, ethynyl-estradiol, acts as an inhibitor of the microsomal demethylation (Fig. 2). The K_i of this inhibition was determined as a measure of the affinity of ethynyl-estradiol for the microsomal mixed function oxidase, because we failed to determine the K_M of ethynyl-estradiol for methodological reasons. Using ethynyl-estradiol concentrations of 1×10^{-4}, 2×10^{-4} and 5×10^{-4} M, only in this latter concentration could an inhibition be seen from the v_i-data. As shown in Fig. 2, the type of inhibition is purely non-competitive.

From the Lineweaver–Burk-plot of Fig. 2 we calculated a K_i for ethynyl-estradiol on the mestranol demethylation of $5{\cdot}4 \times 10^{-4}$ M using the formula of Dixon and Webb [13], the intercept on the ordinate of the non-competitively inhibited reaction being $(1 + [i]/K_i)/V_{max}$.

The type of inhibition is in agreement with the results of Lee and Chen [4], who also found a non-competitive inhibition of the microsomal O-demethylation of *p*-nitro-anisole by ethynyl-estradiol.

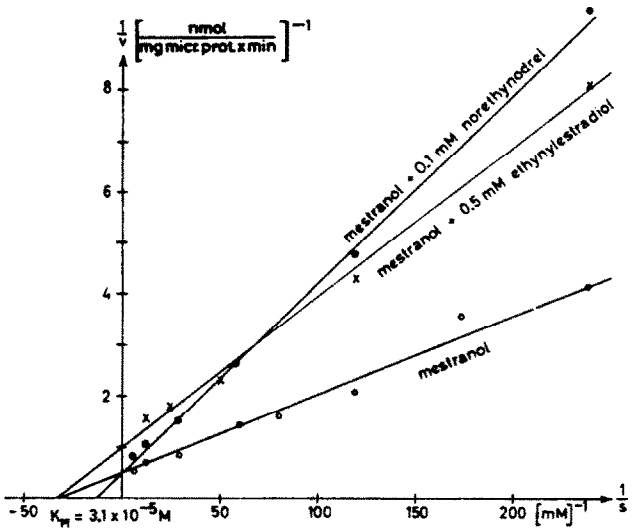

Fig. 2. Lineweaver-Burk-plot of the demethylation of mestranol, effected by liver microsomes from phenobarbital-pretreated male rats. O——O——O mestranol demethylation without inhibitor; X——X——X mestranol demethylation in presence of 0·5 mM ethynyl-estradiol; ●——●——● mestranol demethylation in presence of 0·1 mM norethynodrel. The experiments shown have been carried out at a microsomal protein concentration of 2 mg/ml. Norethynodrel inhibits the reaction competitively, ethynyl-estradiol non-competitively.

However, under the conditions described, a real product inhibition of the mestranol demethylation does not take place because the high concentration of the product ethynyl-estradiol, which is essential for an inhibition, is never reached. Only after 15 min incubation with the high 0·1 mM equimolar concentration of both ethynyl-estradiol and mestranol did a 28% inhibition of the demethylation appear (Table 1).

Inhibition studies with progestational compounds

The inhibition of mestranol demethylation by progestational drugs is demon-

Table 1. Inhibition of the demethylation of mestranol by liver microsomes from phenobarbital-pre-treated rats. The incubation time was 15 min. Mestranol (substrate) and inhibitor were present in equimolar concentration of 0·1 mM

		Demethylation rate nmole/mg micr. prot./15 min*	% inhibition
without progestogen		9·5	0%
with	megestrol-acetate	9·3	2%
	norethisterone	9·0	5%
	chlormadinon-acetate	7·1	22%
	lynestrenol	6·5	32%
	norethynodrel	5·4	43%
	progesterone	4·8	50%
	ethynodiol-diacetate	4·3	55%
	ethynyl-estradiol	6·8	28%

*The turnover of the reaction has been calculated as nmole of mestranol demethylated by 1 mg of microsomal protein during incubation time of 15 min.

strated in Table 1. Only norethisterone and megestrol-acetate do not inhibit this reaction. All other compounds examined acted as inhibitors, particularly ethynodiol-diacetate, progesterone and norethynodrel. Both latter steroids have already been found by Juchau and Fouts [14] to be strong inhibitors of the microsomal mixed function oxidation, as in the side-chain oxidation of hexobarbital, the ring hydroxylation of zoxazolamine and the *p*-hydroxylation of aniline.

Norethynodrel showed a purely competitive inhibition of the mestranol demethylation in our kinetic experiment (see Fig. 2). From our data in Fig. 2 a K_I-value of $7{\cdot}2 \times 10^{-5}$ M was calculated, indicating a considerable affinity of this progestational compound for the microsomal mixed function oxidase.

This type of inhibition agrees with the findings of Tephly and Mannering [15], that other neutral steroids, i.e. testosterone, androstenedione, progesterone and cortisol, also exhibit a competitive inhibition on the microsomal mixed function oxidation of hexobarbital.

DISCUSSION

As previously mentioned, the action of mestranol and ethynyl-estradiol is based on the affinity of ethynyl-estradiol for the estrogen receptor protein in the target tissues as well as on the affinity of mestranol for the microsomal drug oxidizing enzyme system.

Our experiments relating to the binding of ethynyl-estradiol by the estrogen receptor of the rabbit uterus gave a K_D-value of $1{\cdot}5 \times 10^{-9}$ M while mestranol was not bound.

This is in agreement with the results of Korenman [16], who determined the relative binding of ethynyl-estradiol and mestranol indirectly by competition with the [^{3}H]-estradiol-receptor complex measuring the displacement of [^{3}H]-estradiol.

This suggests that mestranol is totally ineffective as an estrogenic compound, unless converted to ethynyl-estradiol in the liver.

While not bound to the estrogen receptor protein, mestranol shows a remarkably high affinity for the microsomal mixed function oxidase, expressed by the considerably low K_M-value of $3{\cdot}1 \times 10^{-5}$ M. Moreover, ethynyl-estradiol can also readily bind to the mixed function oxidase, as indicated by the K_I of $5{\cdot}4 \times 10^{-4}$ M on the mestranol demethylation.

If we assume, that the kinetics of the mestranol demethylation strictly follow true Michaelis–Menten-conditions, the K_M of mestranol ($3{\cdot}1 \times 10^{-5}$ M) presents its ability to combine with the microsomal mixed function oxidase and to form a complex with this enzyme.

In addition, the K_I of ethynyl-estradiol ($5{\cdot}4 \times 10^{-4}$ M) represents the affinity of this compound for the same enzyme system. Comparing these figures, the demethylation of mestranol is apparently not inhibited by the product ethynyl-estradiol under physiological conditions. The lower affinity of ethynyl-estradiol may explain our findings that the bulk of ethynyl-estradiol formed by the demethylation of mestranol is not immediately further transformed, but can be isolated from the microsomal incubation *in vitro* [3].

In vivo, the ethynyl-estradiol so formed can leave the liver cell and is available to the general circulation [5] where it acts as an estrogen. Mestranol is one of the few examples of drugs which become effective after their conversion in the liver by the hydroxylating system.

The inhibition of *p*-nitro-anisole demethylation with ethynyl-estradiol and mestranol has been studied by Lee and Chen [4] Mestranol showed a competitive

and ethynyl-estradiol a non-competitive inhibition, in agreement with our results on the non-competitive inhibition of the mestranol demethylation by ethynyl-estradiol.

These data suggest, that the O-demethylation of mestranol and *p*-nitro-anisole by the microsomal mixed function oxidase is comparable. This agrees with the K_M-value of *p*-nitro-anisole ($1{\cdot}5 \times 10^{-5}$ M) as determined by Ackermann and Heinrich [17] being in the same range as our K_M of mestranol ($3{\cdot}1 \times 10^{-5}$ M). Other authors [18, 4], who found a considerably higher K_M for *p*-nitro-anisole, had added nicotinamide to their incubations and this is a competitive inhibitor of the mixed function oxidase [19–21]. Therefore, in these earlier communications the real K_M was not obtained.

Inhibition of the mestranol demethylation would lead to decreased biological activity of mestranol.

Our results clearly demonstrate that certain progestogens inhibit mestranol demethylation under *in vitro* conditions, in agreement with findings of Watanabe [23] on the inhibition of the estrone hydroxylation by 17α-ethynyl-19-nor-steroids *in vitro*.

Some of the compounds examined are substrates for the mixed function oxidase, i.e. progesterone [24, 25], norethynodrel [26, 27] norethisterone [26] and lynestrenol, which is 3-hydroxylated to norethisterone, as described by Okada *et al.* [28, 29] and later also by Mazaheri *et al.* [30].

From this point of view, the competitive inhibition on the mestranol demethylation, which we have confirmed for norethynodrel, could be assumed also for these other progestogens.

Concerning the conditions *in vivo*, however, one has to take into account the relatively low daily doses of estrogens (50–100 μg) and progestogens (250 μg–4 mg) used in human contraceptive preparations, which make it unlikely that during therapy much interference by the progestational component with the mestranol demethylation would occur, although it is well known that steroids in *high* concentrations act as inhibitors of drug hydroxylation catalyzed by the mixed function oxidase as described by Jori *et al.* [22]. They found a norethynodrel dose of 50 mg/kg necessary to elicit an increase of the phenobarbital sleeping time in rats, when it was given orally 2 h prior to the experiment.

It can be theoretically estimated from our results that an inhibition of mestranol demethylation by progestational component should never exceed about 0·1%. The statement of O'Malley *et al.* [31] that "in very low doses, i.e. in doses resembling that in oral contraceptive preparations, inhibition of these enzymes occurred" should be revised. Therefore it is also not conceivable that inhibition of drug metabolizing enzyme is the reason for the decrease of antipyrin metabolism in women taking oral contraceptives which has been observed by these authors.

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