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EFFECT OF SYNTHETIC ESTROGENS AND ESTROGEN–PROGESTIN COMBINATIONS ON THE HEPATIC MICROSOMAL ENZYME SYSTEM*

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Abstract—Pretreatment of male rats with mestranol or ethynyl estradiol 10 min prior to the administration of pentobarbital had no effect on the duration of pentobarbital-induced sleep. Although the estrogens are alternate substrates for the microsomal enzyme system which metabolizes pentobarbital, the concentrations used in this study did not affect pentobarbital metabolism. Chronic pretreatment with mestranol or ethynyl estradiol did not induce the hepatic microsomal enzyme system, as determined by measuring the concentration of cytochrome P-450, the rate of activity of cytochrome P-450 reductase and the amount of microsomal protein/g of rat liver. However, chronic pretreatment with ethynyl estradiol or mestranol markedly decreased the daily body weight gain of the rats, but did not significantly alter the weight of their livers.

REPORTS in the literature suggest a direct correlation between the chronic use of oral contraceptives and the development of certain adverse side effects. These adverse effects include alterations in lipid metabolism[1] and carbohydrate metabolism,[2] a direct relationship between the use of oral contraceptives and death from pulmonary embolism, infarction and cerebral thrombosis,[3] the development of severe depression and frigidity,[4] the production of structural changes in the liver mitochondria,[5] and alterations of hepatic microsomal enzyme-mediated drug metabolism.[6–8]

Both endogenous and exogenous steroids are substrates for the microsomal enzyme system.[9,10] Although steroids have been shown to be competitive inhibitors of drug metabolism *in vitro*,[11,12] it has been suggested that the synthetic progestational and estrogenic steroids of the oral contraceptives are able to induce the hepatic microsomal enzyme system.[6,7] Recently, this laboratory has shown that three commonly used synthetic progestins (norethynodrel, norethindrone and ethynodiol diacetate) are not microsomal enzyme inducers, even at the relatively high steroid concentrations required for contraception in rats.[13] However, the progestins were found to be weak inhibitors of microsomal drug metabolism, apparently acting via an alternate substrate mechanism.[13]

The present study was undertaken to determine the effects of the synthetic estrogens, ethynyl estradiol and mestranol, and estrogen–progestin combinations, on the drug-metabolizing hepatic microsomal enzyme system.

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MATERIALS AND METHODS

Materials. The rats used in these studies were 110–180 g males, Sprague–Dawley strain, obtained from Charles River Breeding Laboratories (Wilmington, Mass.). These animals received commercial Purina laboratory chow and water *ad lib.* until the time of sacrifice. Ethynyl estradiol, ethynyl estradiol-3-methyl ether (mestranol), estradiol, estrone, estriol, progesterone, norethynodrel and norethindrone were obtained from Sigma Chemical Co. (St. Louis, Mo.) and ethynodiol diacetate was a gift from G. D. Searle & Co. (Chicago, Ill.).

Preparation of microsomes. The animals were sacrificed by decapitation between 8:30 and 9:00 a.m. and their livers were immediately excised, rinsed with cold 1·15% KCl containing 0·02 M Tris buffer, pH 7·4 (KCl-Tris), blotted dry, weighed and then chilled on crushed ice. All subsequent steps in preparing microsomes were performed with the tissue kept at a temperature of 0–4°.

The livers were homogenized in 3 vol. of KCl-Tris buffer with a Potter-type homogenizer having a motor-driven plastic pestle. The homogenate was centrifuged in a Servall RC-2 refrigerated centrifuge for 20 min at 10,000 *g*. The resulting supernatant was centrifuged in a Beckman L2–65B ultracentrifuge for either 60 min at 105,000 *g* or 30 min at 220,000 *g*. The supernatant was removed by aspiration after which the microsomal pellet was resuspended in 0·1 M Tris, pH 7·4, and centrifuged for 30 min at 220,000 *g*. The washed microsomes were resuspended in 0·1 M Tris buffer, pH 7·4. Microsomal protein concentration was determined by the method of Lowry *et al.*[14] using crystalline bovine serum albumin as the protein standard.

Analytical methods. All spectral studies were performed in an Aminco–Chance recording spectrophotometer. Cytochrome P-450 concentrations were determined in the split beam mode, using the dithionite difference technique[15] rather than the more common carbon monoxide difference method, to allow for any hemoglobin contamination in the washed hepatic microsomes. The cytochrome P-450 concentrations, expressed as nmoles/mg of microsomal protein, were determined using the difference in absorbance between 450 and 490 nm and an extinction coefficient of 100 mM^{-1} cm^{-1}.[15] The substrate-induced difference spectra were obtained with non-reduced microsomes using 2 mg microsomal protein/ml. Steroids were added to the sample cuvette in 10 μl ethanol and an equal amount of ethanol was added to the reference cuvette. The difference in absorbance was measured between the peak at 390 nm and the trough at 424 nm. The spectral dissociation constants (K_s) were obtained from double-reciprocal plots, using five substrate concentrations.

Cytochrome P-450 reductase activity was determined by a method similar to those described by Gigon *et al.*[16] and Fouts and Pohl.[17] A suspension of washed microsomes was diluted with 0·1 M Tris to a final protein concentration of 2 mg/ml. The diluted microsomal suspension was bubbled continuously with carbon monoxide which had been passed through a deoxygenating solution composed of 0·5% sodium dithionite and 0·05% sodium anthraquinone-2-sulfonate in 0·1 N NaOH. After bubbling the suspension with CO for at least 7 min, 3-ml aliquots of the CO-saturated microsomes were pipetted into Aminco anerobic cells, the stopcock plunger assembly containing 60 mM NADPH was attached to the cuvette and CO was then flushed through the cuvette for 3 min. The cuvette was then sealed, placed in the Aminco–Chance spectrophotometer and the difference in absorption between 450 and 490 nm was determined at 26°, using the dual-wavelength mode. The chart speed was 1 in/sec.

Steroid pretreatment. Ethynyl estradiol (0·1 mg/kg or 0·5 mg/kg), mestranol (0·2 mg/kg or 1·0 mg/kg) norethynodrel, norethindrone, ethynodiol diacetate (each progestin 10 mg/kg or 50 mg/kg), or a combination of synthetic estrogen and progestin (10 mg/kg or 50 mg/kg) were administered in sesame oil through an oral intubation needle. The lower dose of each estrogen has been shown to be the oral antifertility dose for rats.[18]

In the acute studies, the estrogen, progestin or estrogen–progestin combination was administered to groups of ten rats, 10 min prior to the intraperitoneal injection of sodium pentobarbital (30 mg/kg). A second (control) group of rats was given sesame oil 10 min before the barbiturate. Barbiturate-induced sleep time was measured as the period between the loss and regaining of the righting reflex.

In the chronic studies, the steroids or vehicle were administered to groups of eight rats, once daily between 9:00 and 10:30 a.m. for 4 days. Twenty-four hr after the last dose, the animals either received sodium pentobarbital, and the duration of the barbiturate-induced sleep was measured, or were sacrificed and washed microsomal pellets were prepared from their livers. These microsomal enzyme preparations were used for the spectral studies.

RESULTS

Pretreatment with mestranol or ethynyl estradiol 10 min prior to the administration of sodium pentobarbital did not significantly alter pentobarbital-induced sleep time. Pretreatment with norethindrone (50 mg/kg) or norethynodrel (10 mg/kg and 50 mg/kg) increased pentobarbital sleep time, as shown in Table 1. However, when the estrogen and progestin are given in combination, only the combination containing norethynodrel caused a significant alteration in pentobarbital-induced sleep (Table 2).

TABLE 1. EFFECT OF ACUTE STEROID PRETREATMENT ON PENTOBARBITAL-INDUCED SLEEP*

Drug pretreatment	Dose (mg/kg)	Sleep duration (min ± S. E.	P
Sesame oil		84 ± 2·9	
Ethynodiol	10	89 ± 3·9	NS†
Ethynodiol diacetate	50	84 ± 4·1	NS
Norethindrone	10	91 ± 4·5	NS
Norethindrone	50	145 ± 12·7	< 0·01
Norethynodrel	10	134 ± 6·4	< 0·01
Norethynodrel	50	201 ± 13·6	< 0·01

* Ten rats per group. Steroids were administered orally 10 min prior to the intraperitoneal administration of sodium pentobarbital (30 mg/kg).

† NS = not significant.

Four-day pretreatment with the two synthetic estrogens did not cause induction or impairment of the hepatic microsomal enzyme system as determined by measuring pentobarbital sleep time, microsomal protein concentration, concentration of cytochrome P-450 and activity of P-450 reductase. None of the values obtained from the estrogen-pretreated or estrogen–progestin-pretreated animals differed significantly from those of the control rats.

TABLE 2. EFFECT OF ACUTE PROGESTIN–ESTROGEN PRETREATMENT ON PENTOBARBITAL-INDUCED SLEEP

Progestin	Dose (mg/kg)	Estrogen	Dose (mg/kg)	Sleep time ± S. E.	P
Sesame oil				79·0 ± 6·5	
Norethynodrel	50	Ethynyl estradiol	0·5	132·9 ± 11·2	< 0·01
Norethynodrel	50	Mestranol	1·0	101·9 ± 7·6	< 0·05
Norethindrone	50	Ethynyl estradiol	0·5	84·4 ± 5·6	< 0·6
Norethindrone	50	Mestranol	1·0	65·7 ± 3·6	< 0·4
Ethynodiol diacetate	50	Ethynyl estradiol	0·5	64·4 ± 6·6	< 0·2

Ethynyl estradiol, mestranol and the three endogenous estrogens (estradiol-17β, estrone and estriol) were shown to be type I substrates by the formation of a type I difference spectra when added to hepatic microsomal enzyme preparations. All five estrogens had identical A_{max} and A_{min}. Their spectral dissociation constants (K_s) followed closely the order of relative lipophilicity of the estrogens (Table 3).

TABLE 3. SPECTRAL VALUES*

Steroid	A_{max}	A_{min}	ΔA_{max}	K_s (μM)
Estriol	390	425	0·027	32
Estrone	390	425	0·049	12
Estradiol	390	425	0·051	8
Ethynyl estradiol	390	425	0·027	5
Mestranol	390	425	0·029	4

* A double-reciprocal graph and six steroid concentrations were used to determine the K_s and ΔA_{max} for each estrogen. The ΔA was calculated from the difference in absorbance between the peak at 390 nm and the trough at 425 nm. A microsomal enzyme preparation of 2 mg protein/ml in 0·1 M Tris buffer, pH 7·4, was used for the spectral studies listed above. The steroids were dissolved in ethanol. An equal amount of ethanol was always added to the reference cuvette.

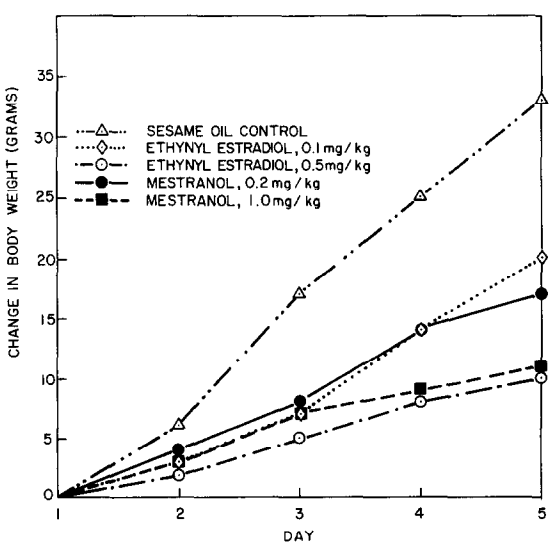

FIG. 1. Eight rats were given either ethynyl estradiol, mestranol or sesame oil (vehicle) by oral intubation, once daily for 4 days. The steroids inhibited the daily body weight gain.

Pretreatment with ethynyl estradiol or mestranol results in a decrease in body weight gain, as shown in Fig. 1. The decreased weight gain appeared to be dose related. It has previously been shown that the synthetic progestins also decrease body weight gain.[13] Figure 2 shows the additive effect of an estrogen–progestin combination on daily body weight gain.

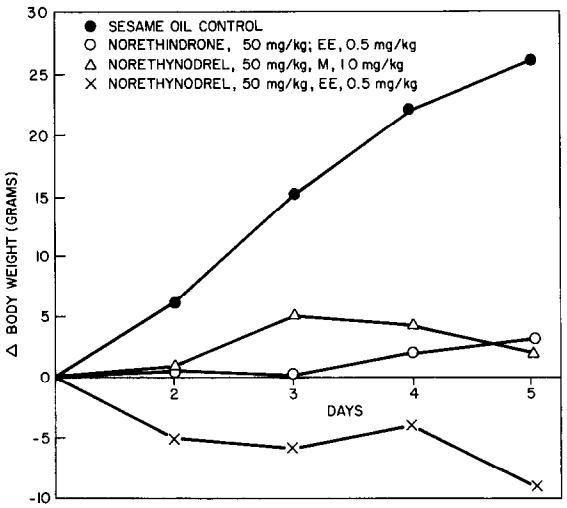

FIG. 2. Groups of eight rats were given estrogen–progestin combinations. This figure clearly shows the additive effect of the progestin to the estrogen-mediated decrease in daily body weight gain, shown in Fig. 1.

DISCUSSION

The administration of mestranol or ethynyl estradiol at five times their antifertility dose in rats did not alter the duration of pentobarbital-induced sleep. However, pretreatment with either norethynodrel or norethindrone significantly increased pentobarbital-induced sleep time. When estrogen–progestin combinations were used, only the pairs containing norethynodrel increased the duration of barbiturate-induced sleep. Chronic pretreatment with the synthetic estrogen or estrogen–progestin combination had no apparent effect on the microsomal enzyme system. There was no measurable difference between the control rats and those pretreated with steroid with respect to the concentration of cytochrome P-450, the rate of reduction of P-450 by P-450 reductase or mg microsomal protein/g of liver.

The literature contains a great many papers listing the spectral dissociation constants (K_s) for a variety of compounds. In general, there appears to be a direct correlation between the K_s value and the lipid solubility of the compound. In a previous study using progestins, this laboratory found that the more lipophilic the steroid, the lower (numerically) the K_s value.[13] To more clearly show the relationship between the lipid solubility of a group of compounds and their apparent spectral dissociation constants, we included estrone and estriol when determining the K_s values for mestranol, ethynyl estradiol and estradiol-17β. As shown in Table 3, in which the steroids are listed in their order of increasing lipophilicity, the K_s values decrease numerically with increasing lipid solubility, indicating that the K_s value may actually

be a measurement of a drug's ability to penetrate the endoplasmic reticulum and thus reach the binding site for a type I spectral response.

The decrease in body weight gain in the rats pretreated with either ethynyl estradiol or mestranol appeared to be dose related, as the higher dose of both steroids resulted in a more marked inhibition of weight gain. Although this type of decrease in daily body weight gain in rats has been reported for synthetic progestins[13,19] and for estrogen–progestin combinations,[20] no one has reported this alteration as a result of estrogen treatment. It is of interest that, although the estrogens affected body weight gain, they had no effect on the weight of the livers in the experimental animals.

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