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EFFECTS OF NORETHYNODREL AND PROGESTERONE ON HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYME SYSTEMS*

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Abstract—Two progestational steroids, norethynodrel and progesterone, have been compared in their effects on hepatic drug-metabolizing enzyme systems *in vitro*. The steroids were added directly to incubation mixtures containing 9000 *g* supernatant fractions of rat liver homogenates, appropriate cofactors, and drug substrates. Norethynodrel and progesterone at 10^{-5} and 10^{-4} M concentrations markedly inhibited the side-chain oxidation of hexobarbital, ring hydroxylation of zoxazolamine, and *p*-hydroxylation of aniline. To a lesser degree they also inhibited the ring hydroxylation of 3,4-benzpyrene and the N-demethylation of aminopyrine. They did not appreciably affect the reduction of the nitro radical of *p*-nitrobenzoic acid, the reduction of the azo linkage of neoprontosil, the *o*-demethylation of codeine, or the metabolism of chlorpromazine.

When animals were pretreated with these steroids, only the metabolisms of zoxazolamine and hexobarbital were affected *in vitro*. Both agents were capable of inhibiting the metabolism *in vitro* of these drug substrates when administered 1 or 2 hr prior to sacrifice of the animals. Norethynodrel stimulated the metabolism of hexobarbital when administered between 18 and 48 hr before sacrifice. Hexobarbital metabolism was also stimulated after subacute (daily for 3 days) and chronic (daily for 3 weeks) pretreatment with norethynodrel. Pretreatment with norethynodrel produced a stimulation of zoxazolamine metabolism when administered between 18 and 36 hr prior to sacrifice of the animals. Stimulation was also observed after chronic administration of norethynodrel. Pretreatment with progesterone produced no stimulation of hexobarbital or zoxazolamine metabolism.

VARIOUS progestational steroids have been widely employed as oral contraceptive agents. Several authors[1–4] have indicated that certain oral contraceptives are capable of inducing hepatic damage. Other investigators,[5–11] however, believe these steroids cause no liver damage.

Kuntzman *et al.*[12] have suggested that the same hepatic microsomal enzyme systems may be involved in the metabolism of both drugs and steroid hormones. Tephly and Mannering[13] reasoned that if such were the case, the steroids should competitively inhibit the metabolism of drugs. Their results indicated that estradiol, testosterone, androsterone, diethylstilbestrol, and hydrocortisone were all capable of competitively inhibiting the N-demethylation of ethyl morphine *in vitro*. Trivus[14] found that the stimulation of NADPH oxidation *in vitro* by drug substrates could also occur when testosterone or estradiol 17-B was employed. Substrate competition also occurred in the presence of either drug or steroid.

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Kuntzman and Jacobson[15] and Conney and Schneidman[16] have shown that administration of drugs can alter the metabolism of progesterone by hepatic enzyme systems. The work of DuBois and Kinoshita[17] suggests that pretreatment of female rats with progesterone may induce the synthesis of a hepatic microsomal enzyme system which is involved in the metabolism of O,O-diethyl O-(4-methylthio-*m*-tolyl)phosphorothioate (DMP). It therefore seemed logical to investigate the effects of progestational agents on other hepatic microsomal drug-metabolizing enzyme systems. The purpose of the present investigation was to determine the effects of a widely employed oral progestational agent (norethynodrel) on a number of such systems *in vitro*. The effects were compared with the naturally occurring progestational hormone progesterone. Effects of Enovid, which is a combination of norethynodrel and the estrogenic agent mestranol, were also studied.

METHODS

Unfasted male Holtzman rats weighing 80 to 100 g were maintained on regular Purina lab chow and water *ad libitum* until they were killed by cervical dislocation before the experiments. The livers were quickly removed, placed immediately on ice, blotted, and weighed. To each gram of liver was added 2 ml of ice cold 1·15% KCl solution; the mixture was then homogenized in the cold with a Potter homogenizer having a Teflon pestle. This homogenate was centrifuged at 1–3° at 9000 *g* for 20 min. Studies were carried out on the 9000 *g* supernatant fractions.

Studies of effects of pretreatment with progesterone and norethynodrel. Progesterone (preg-4-ene-3,20-dione) and norethynodrel (17α-ethynyl-17-hydroxy-ester-5(10)-en-3-one) were suspended in aqeous 1% carboxymethylcellulose. Progesterone was administered only by intraperitoneal injection at several time intervals prior to the sacrifice of the animals. Norethynodrel was given both intraperitoneally and orally. The oral route was also used since this is the most common therapeutic route. In addition, oral administrations of Enovid (Searle Laboratories) in 1% carboxymethylcellulose were also given at several time intervals prior to sacrifice (Enovid contains a mixture of norethynodrel with 1·5% mestranol which is ethynylestradiol 3-methyl ether). Acute, subacute, and chronic studies were carried out. In the acute studies, animals were given a single administration of 50 mg drug/kg at intervals ranging from 1 to 48 hr before sacrifice. In subacute studies, animals were given the drug once daily for 3 days on a dosage schedule of 50 mg/kg/day. The animals were sacrificed the day after the last injection. In chronic studies, animals were given 50 mg drug/kg once daily for 3 weeks. They were also sacrificed the day after the last injection. Controls were given a similar volume of carboxymethylcellulose.

Studies of the effects of additions of progesterone and norethynodrel in vitro. Progesterone and norethynodrel were dissolved in a suitable volume of reagent grade acetone and added to the incubation mixture such that the effects of 10^{-5} and 10^{-4} M (final concentrations) could be observed. The volume of acetone added to the incubation mixture was 0·1 ml. An equal volume of acetone was added to control incubates. All assays were performed in triplicate; a pooled homogenate of 9000 *g* supernatant fraction was used. Determination of the nature of inhibition was accomplished by plotting reaction velocities against the ratios of the reaction velocities over the substrate concentration (v vs. v/s). Two inhibitor concentrations and four substrate concentra-

tions were employed in the determination of the nature of inhibition of the hexobarbital and zoxazolamine metabolic pathways by progesterone and norethynodrel.

Assay procedures. The following metabolic pathways were studied. Side-chain oxidation of hexobarbital (determined by the method of Cooper and Brodie[18]); reduction of the aromatic nitro group of *p*-nitrobenzoic acid (Fouts and Brodie[19]); ring sulfur oxidation of chlorpromazine (Salzman and Brodie[20]); determination of morphine by a colorimetric method of Snell and Snell[21] as an estimate of the O-dealkylation of codeine; N-dealkylation of aminopyrine (La Due *et al.*[22]); aromatic ring *p*-hydroxylation of aniline (method of Gillette as described by Dixon *et al.*[23]); reductive cleavage of the azo linkage of neoprotosil to sulfanilamide (Fouts *et al.*[24]); ring oxidation of zoxazolamine and polycyclic aromatic ring hydroxylation of 3,4-benzpyrene (methods described by Juchau *et al.*[25]). The results of the studies on additions *in vitro* of norethynodrel or progesterone were expressed as μmoles substrate metabolized per incubate in 15 min. Other results were expressed as μmoles substrate metabolized/g 9000 *g* supernatant nitrogen in 30 min. Conditions of incubation, cofactors, and concentrations thereof were the same as those reported by McLuen and Fouts[26] except that incubation times were reduced to 15 min for additions *in vitro* of norethynodrel or progesterone and 30 min for all other incubations, and that the final concentration of NADP was $9{\cdot}4 \times 10^{-4}$ M. Substrate concentration in μmoles/5 ml incubate were: 3·0 μmoles hexobarbital, 40 μmoles aminopyrine, 13·2 μmoles *p*-nitrobenzoic acid, 7·8 μmoles neoprontosil, 10·2 μmoles codeine, 10·0 μmoles aniline, 1·0 μmole chlorpromazine, 3·0 μmoles zoxazolamine, and 0·6 μmole 3,4-benzpyrene. Nitrogen determinations were made on the supernatant fractions by a modified Kjeldahl method described by Juchau *et al.*[25]

Statistical evaluation of the data. Comparison of hepatic microsomal enzymic activity of homogenates from norethynodrel-, progesterone-, and Enovid-treated rats to that of the carboxymethylcellulose-treated controls were made by Student's *t* test. The level of significance chosen was $P < 0{\cdot}05$.

RESULTS

Effects of pretreatment of rats with progesterone and norethynodrel

Pretreatment of rats with 50 mg progesterone or norethynodrel/kg significantly affected the metabolism *in vitro* of only hexobarbital and zoxazolamine. Other metabolic pathways were not significantly altered, regardless of the pretreatment interval or duration.

The effects of intraperitoneal progesterone and norethynodrel administration on hexobarbital metabolism are shown in Fig. 1. The results indicated that both progestational steroids are capable of initially inhibiting hexobarbital side-chain oxidation as measured *in vitro*. Norethynodrel may have been somewhat more potent than progesterone in this regard. A type of biphasic effect was observed with both compounds. Norethynodrel was able significantly to stimulate hexobarbital metabolism between 18 and 48 hr after oral or intraperitoneal administration. This stimulatory effect was also observed in subacute and chronic studies. The inhibitory effect of progesterone on hexobarbital metabolism seemed to disappear 2 hr after i.p. injection and reappear between 24 and 36 hr. No significant enhancement of hexobaribtal side-chain oxidation was observed after i.p. administration of progesterone.

The effects of intraperitoneally administered progesterone and norethynodrel on zoxazolamine metabolism are indicated in Fig. 2. A marked similarity appeared to exist between the effects of these compounds on hexobarbital and zoxazolamine oxidations. Pretreatment with norethynodrel again caused an initial inhibition of

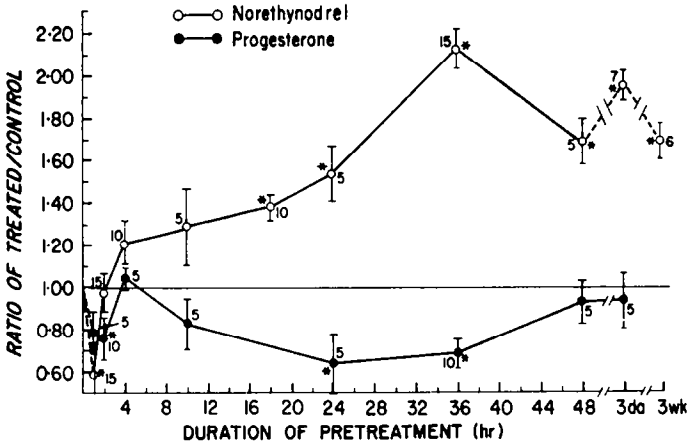

FIG. 1. Effects of intraperitoneal pretreatment of rats with norethynodrel and progesterone on the metabolism of hexobarbital *in vitro*. Each point represents the mean ratio of treated animals/control animals. Numbers represent the number of animals employed in each group for each determination. Asterisks indicate that means of treated groups were significantly different from means of control groups ($P < 0.05$). Where abscissa values are in hours, a single dose of norethynodrel or progesterone was given at time zero. Where abscissa values are in days or weeks, multiple doses of norethynodrel or progesterone were given: one dose every day for 3 days or for 3 weeks.

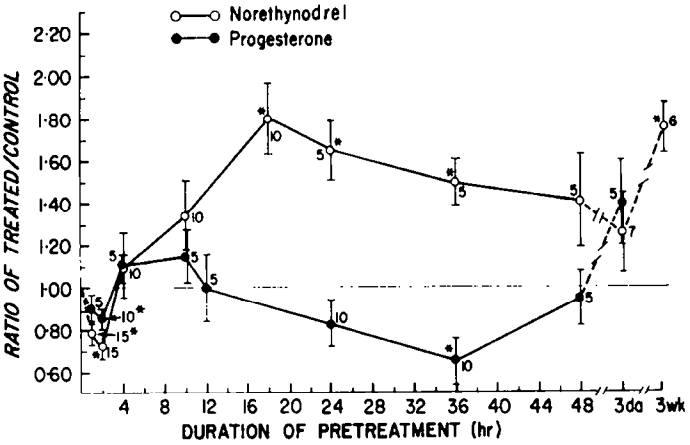

FIG. 2. Effects of intraperitoneal pretreatment of rats with norethynodrel and progesterone on the metabolism of zoxazolamine *in vitro*. Conditions as in Fig. 1.

zoxazolamine metabolism which may have persisted for a somewhat longer interval than that seen with hexobarbital as substrate. Zoxazolamine metabolism was also significantly enhanced 18 hr after oral or intraperitoneal administration of norethynodrel. However, the magnitude of stimulation did not appear to be as great as that

seen with hexobarbital metabolism, and the peak effect seemed to be somewhat earlier. Effects of pretreatment with progesterone on zoxazolamine metabolism were also similar to those observed with hexobarbital metabolism except that significant inhibition was observed only at 2 and 36 hr after i.p. injections.

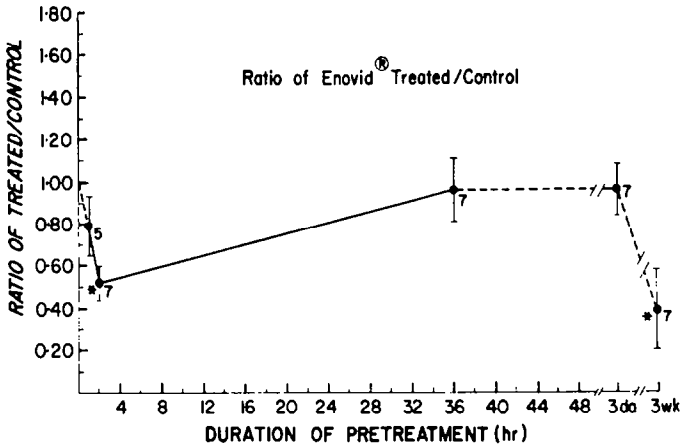

FIG. 3. Effects of oral pretreatment of rats with Enovid on the metabolism of hexobarbital *in vitro*. Conditions as in Fig. 1.

Effects of Enovid (given orally) on hexobarbital metabolism are shown in Fig. 3. At 36 hr after oral administration of this preparation, no significant effect on hexobarbital metabolism was noted. This contrasts with a more than twofold increase in metabolism of hexobarbital observed when animals were treated orally or intraperitoneally with norethynodrel alone. Chronic administration of Enovid also resulted in a significant inhibition of hexobarbital side-chain oxidation, as opposed to a significant enhancement produced by norethynodrel alone.

Effects of additions of progesterone and norethynodrel in vitro

Several experiments were run in which norethynodrel or progesterone was introduced directly into incubation mixtures. In these experiments the incubation mixtures consisted of 9000 *g* supernatant from pooled homogenates of rat livers, phosphate buffer, cofactors, and drug substrate as described previously. Norethynodrel or progesterone at 10^{-5} M and 10^{-4} M final concentrations was added to the incubation mixtures, and the effect on the metabolism of several drug substrates was measured. In the measurement of the inhibitory effects of these progestational compounds on hexobarbital, zoxazolamine, aniline, and 3,4-benzpyrene metabolism, substrate concentrations were also varied. The results of these studies are shown in Table 1.

The metabolic pathways involved in the oxidation of hexobarbital and zoxalamine appeared to be most sensitive to the inhibitory effects of these progestational agents. This finding is in agreement with results obtained in the pretreatment studies, in which it was shown that only hexobarbital and zoxazolamine metabolism could be significantly affected by pretreatment of intact animals with norethynodrel or progesterone.

Treatment of animals with progesterone (50 mg/kg) can cause inhibition of zoxazolamine metabolism (Fig. 2). However, when progesterone is directly added to a 9000 *g* supernatant fraction, the metabolism of zoxazolamine *in vitro* is not greatly affected (Table 1).

TABLE 1. EFFECTS OF VARYING SUBSTRATE AND PROGESTATIONAL INHIBITOR CONCENTRATIONS ON HEPATIC MICROSOMAL METABOLISM OF DRUG SUBSTRATES

Substrate	Principal metabolic reaction	Substrate concentration* (M)	% Inhibition by			
			Norethynodrel		Progesterone	
			10^{-4} M	10^{-5} M	10^{-4} M	10^{-5} M
Hexobarbital	Side-chain oxidation	6×10^{-4}	69	39	74	41
Hexobarbital		$1{\cdot}2 \times 10^{-3}$	66	29	70	30
Zoxazolamine	Ring hydroxylation	6×10^{-4}	84	38	58	17
Zoxazolamine		$1{\cdot}2 \times 10^{-3}$	72	30	32	17
Aniline	Ring *p*-hydroxylation	$1{\cdot}0 \times 10^{-3}$	80	21	72	54
Aniline		$2{\cdot}0 \times 10^{-3}$	51	14	49	20
3,4-Benzpyrene	Ring hydroxylation	$6{\cdot}0 \times 10^{-5}$	21	16	35	5
3,4-Benzpyrene		$1{\cdot}2 \times 10^{-4}$	9	6	28	17
Aminopyrine	N-Dealkylation	8×10^{-3}	31	8	27	6
p-Nitrobenzoic acid	Reduction of nitro group	$2{\cdot}6 \times 10^{-3}$	4	0	5	2
Neoprontosil	Reduction of azo linkage	$1{\cdot}6 \times 10^{-3}$	0	10	0	0
Chlorpromazine	Sulfoxidation	$2{\cdot}0 \times 10^{-4}$	0	0	3	0
Codeine	O-Dealkylation	$2{\cdot}0 \times 10^{-3}$	0	0	0	0

* Final molar concentration of substrate which was added to the incubation mixture. Assays were run in triplicate.

The only other metabolic pathways that were significantly affected by progesterone and norethynodrel when these were directly added *in vitro*, were those that metabolized aniline, 3,4-benzpyrene, and aminopyrine. These pathways were not affected by pretreatment of animals with the progestational agents (50 mg/kg).

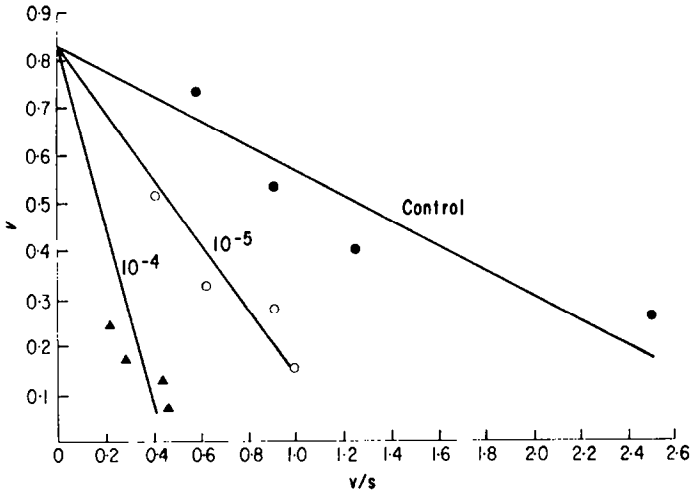

FIG. 4. Kinetics of norethynodrel inhibition of hexobarbital metabolism. Each point represents the mean of three triplicate determinations. The final micromolar concentration of hexobarbital is s; *v* is μmoles hexobarbital metabolized in 15 min.

The nature of the inhibition of the hexobarbital and zoxazolamine metabolic pathways by norethynodrel and progesterone was studied. Inhibition of hexobarbital oxidation by norethynodrel is shown in Fig 4 The plot indicates a competitive inhibition. A similar plot was obtained for inhibition of hexobarbital metabolism by progesterone. Other studies indicate that inhibition of zoxazolamine metabolism by norethynodrel may also be competitive in nature.

DISCUSSSION

The results of this study indicated that two progestational agents, norethynodrel and progesterone, are capable of producing alterations in hepatic microsomal drug-metabolizing systems as measured *in vitro*. Norethynodrel appeared to be capable of this effect even when administered orally.

It should be noted that the metabolic pathways most affected were those in which hydroxylated or oxidized products are formed. Pathways involving reduction, N-demethylation, O-demethylation, or sulfoxidation were not affected by additions of norethynodrel or progesterone *in vitro* except at very high concentrations. Oxidation of hexobarbital and hydroxylation of zoxazolamine, as measured *in vitro*, could be affected by pretreatment of rats with relatively high doses of progestational agents as well as by the direct addition *in vitro* of such agents to 9000 *g* supernatant fractions. Hydroxylations of 3,4-benzpyrene and aniline, however, did not appear to be affected by pretreatment procedures.

The nature of inhibition of drug-metabolizing enzymic pathways by norethynodrel and progesterone appeared to be competitive. This may indicate that the liver microsomal enzymes that catalyze certain aliphatic or aromatic hydroxylations of drugs and foreign compounds may also catalyze hydroxylations of progestational steroids. Evidence that such systems catalyze hydroxylations of androgenic,[27] estrogenic,[28] and glucocortical[29] steroids has already been obtained. Recent evidence also indicate that drugs that stimulate the microsomal oxidation of drugs also stimulate the microsomal hydroxylation of steroids[15, 16, 28, 30, 31] and that such effects may also be observed in man.[32, 33]

It appears that progestational compounds are capable of affecting hepatic microsomal drug metabolism as measured *in vitro* and that drugs are likewise capable of affecting hepatic metabolism of naturally occurring progestins. The manner in which these two groups (drugs vs. progestational hormones) affect each other's metabolism by the liver would seem to be by competing for the same drug-metabolizing enzymes. Whether drug metabolism would be different when drug and progestational compound were present together vs. given alone would therefore depend on the relative affinity of drug vs. progestin for the enzyme and the amount of drug and/or progestin present at the site of metabolism. It would appear unlikely therefore that progestins would block the metabolism of other drugs (such as hexobarbital or zoxazolamine) since, at least in rats, high concentrations of norethynodrel and progesterone were needed for the inhibitions observed. On the other hand, it is quite conceivable that drugs like hexobarbital might interfere with the metabolism of progestins, and this aspect should be investigated further.

Norethynodrel apparently stimulates certain drug metabolisms in hepatic microsomes, but this stimulation follows an initial inhibition of these enzymes. Thus, norethynodrel may act like certain other drug-metabolizing enzyme inhibitors (such

as SKF 525-A) which also first inhibit and then lead to enhanced hepatic microsomal enzyme activity. It is interesting that norethynodrel seems to stimulate only certain drug-metabolizing enzymes and that progesterone does not have this action. Apparently norethynodrel will be classed (as a stimulator of drug metabolism) in a different group than is phenobarbital. Whether norethynodrel acts like 3,4-benzpyrene or methyltestosterone is yet to be determined.

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