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Decreased Liver Cytochrome *P*-450 in Rats Caused by Norethindrone or Ethynyloestradiol

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1. 19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol (ethynyloestradiol) or 17β-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one (norethindrone) but not 17α-ethyl-17β-hydroxy-19-norandrost-4-en-3-one (norethandrolone) caused a time-dependent loss of cytochrome *P*-450 when incubated *in vitro* with rat liver microsomal fractions and NADPH-generating systems. 2. The enzyme system catalysing the norethindrone-mediated loss of cytochrome *P*-450 had many characteristics of the microsomal mixed-function oxidases. It required NADPH and air, and was inhibited by CO. However, it was unaffected by 1 mM-compound SKF 525A. 3. In microsomal fractions from phenobarbitone-pretreated rats the norethindrone-mediated loss of cytochrome *P*-450 was increased relative to controls. The norethindrone-mediated cytochrome *P*-450 loss was less pronounced when the animals were pretreated with 3β-hydroxy-pregn-5-en-2-one 16α-carbonitrile (pregnenolone 16α-carbonitrile). Pretreatment with 3-methylcholanthrene rendered the animals resistant to the norethindrone effect. 4. Administration *in vivo* [100 mg/kg, intraperitoneally] of norethindrone or ethinyl oestradiol also produced a time-dependent loss of liver cytochrome *P*-450. Norethandrolone had a similar, though much less-marked, effect. All three steroids lead to an induction of 5-aminolaevulinate synthase and an accumulation of porphyrins in the liver. 5. The loss of cytochrome *P*-450 and the accumulation of porphyrins in the liver 2h after the administration of norethindrone to female rats was similar to that seen in males. 6. Rats pretreated with phenobarbitone and given norethindrone or ethynyloestradiol (100 mg/kg, intraperitoneally) formed green pigments in their livers. These had characteristics similar to the green pigments produced in the livers of rats after the administration of 2-allyl-2-isopropylacetamide. No green pigments could be extracted from the livers of control rats or those given norethandrolone, oestradiol or progesterone.

Steroids such as 17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one (norethindrone) or 19-nor-17α-pregna-1,3,5(10)-trien-20-yne-3,17-diol (ethynyloestradiol), constituents of some oral contraceptives, bind covalently to rat liver microsomal fractions. For covalent binding to occur, these steroids must be metabolized by mixed-function oxidases to more reactive intermediates (Bolt & Kappus, 1974; Kappus & Remmer, 1975). In the case of oestrogens, including ethynyloestradiol, it has been suggested that metabolic activation takes place by sequential 2-hydroxylation and oxidation to the semiquinone derivative (Marks & Hecker, 1969; Nelson *et al.*, 1976). For norethindrone, a different mechanism has been proposed, involving a 4β,5β-epoxy intermediate (Cook *et al.*, 1974; Kappus & Remmer, 1975).

Administration of these steroids effects numerous biochemical changes, but none has been specifically related to the action of one of the types of active metabolite described above. For example, repeated administration of ethynyloestradiol to rats results in a marked decrease in liver cytochrome *P*-450. This has been attributed to a cholestatic effect of the steroid (Mackinnon & Simon, 1975). It has been suggested that the use of oral contraceptives is a factor leading to the onset of porphyria cutanea tarda in humans (Behm & Unger, 1974; Haberman *et al.*, 1975). Moreover, steroid hormones, which are potent inducers of 5-aminolaevulinate synthase in human foetal tissue (Congote *et al.*, 1974), have been implicated as teratogens by way of perturbing the

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normal regulation of haem synthesis during development (Cole & Cole, 1976).

We decided to investigate whether the destruction of cytochrome *P*-450 could be effected by the action of an active metabolite of ethinyl oestradiol, norethindrone or the ethyl-substituted analogue, 17α-ethyl-17β-hydroxy-19-norandrost-4-en-3-one (norethandrolone). Loss of cytochrome *P*-450 might be expected to result in a corresponding induction of 5-aminolaevulinate synthase and an accumulation of porphyrins in the liver, similar to that observed after the administration to rats of allyl-substituted compounds such as 2-allyl-2-isopropylacetamide (De Matteis, 1971). The results of such investigations are given in the present paper.

Experimental

Chemicals

Norethindrone acetate, ethynyloestradiol, oestradiol and progesterone were from Sigma Chemical Co., St. Louis, MO, U.S.A. Norethandrolone and 3β-hydroxy-pregn-5-en-20-one 16α-carbonitrile (pregnonolone 16α-carbonitrile) were generously donated by Searle Chemicals, Chicago, IL, U.S.A. and the Upjohn Co., Kalamazoo, MI, U.S.A. respectively. The purity of norethindrone, ethynyloestradiol and norethandrolone was ascertained by t.l.c. Each steroid migrated as a single spot on silica-gel plates (silica-gel 60; layer thickness 0.25mm; E. Merck, Darmstadt, Germany); a chloroform/acetone (19:1, v/v) solvent system was used, and spots were detected with a concentrated-H_2SO_4 spray (Wald, 1965). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NAD, ATP and NADP were from P–L Biochemicals, Milwaukee, WI, U.S.A. Trioctanoin was from Eastman Organic Chemicals, Rochester, NY, U.S.A. All remaining chemicals were Analysed Reagent grade from the Baker Chemical Co., Hayward, CA, U.S.A., unless mentioned otherwise.

Treatment of animals

Male Sprague–Dawley rats (140–150g) were used unless otherwise specified. These were fed on Purina Lab Chow pellets and had free access to water. Phenobarbitone sodium [80mg/kg, intraperitoneally (i.p.)] was given daily in 0.1–0.2ml of water for 3 days. Animals were killed 24h after the last dose. 3-Methylcholanthrene dissolved in trioctanoin was given at 20mg/kg (i.p.) daily for 3 days. Animals were killed 24h after the last dose. Pregnenolone 16α-carbonitrile was suspended in trioctanoin and sonicated for 3min at 80% power (Biosonic IV ultrasonic disintegrator, VWR Scientific, Los Angeles, CA, U.S.A.). Rats were given 75mg/kg (i.p.) twice daily for 3 days and killed 18h after the last dose. Norethindrone, ethynyloestradiol and norethandrolone were dissolved in trioctanoin, and were given in 0.2–0.3ml at a dose of 100mg/kg (i.p.) between 08:00 and 09:00h. Control animals received trioctanoin only. Animals were starved 24h before dosing and killed at various times as indicated.

Preparation of liver microsomal fraction and determination of cytochromes P-450 and b_5

The livers of rats killed by decapitation were quickly removed, rinsed in ice-cold 1.15% (w/v) KCl, blotted dry, weighed, and 10% (w/v) homogenates were prepared in 0.25M-sucrose. After centrifugation at 10000*g* for 20min, 4vol. of 12.5mM-sucrose in 8mM-$CaCl_2$ (Ca^{2+}/sucrose) was added to the supernatant (Kamath & Rubin, 1972). The microsomal fraction was precipitated by centrifugation at 10000*g* for 10min, washed in Ca^{2+}/sucrose and was resuspended in 0.25M-sucrose so that 1ml of microsomal suspension was equivalent to 0.5g of liver wet wt. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Microsomal fractions were used on the day of preparation. Cytochrome b_5 was determined from the NADH reduced-versus-oxidized difference spectra by using a millimolar absorption coefficient of 185litre·mmol^{-1}·cm^{-1} (Omura & Sato, 1964*a*). Cytochrome *P*-450 was determined from the CO–dithionite-reduced versus dithionite-reduced difference spectra by using a millimolar absorption coefficient of 91litre·mmol^{-1}·cm^{-1} (Omura & Sato, 1964*b*).

Assay for steroid-mediated destruction of cytochrome P-450 in vitro

Because of the insolubility of the steroid in aqueous media, suspensions of these compounds, nominally 1.5mM, were prepared by ultrasonic dispersion in 1.15% KCl for 3min at full power (Biosonik IV ultrasonic disintegrator). Reaction mixtures of final volume 3ml normally contained: steroid, 1.5μmol; NADP, 1.3μmol; glucose 6-phosphate, 16.4μmol; glucose 6-phosphate dehydrogenase, 1 unit; EDTA, 5μmol; $MgCl_2$, 10μmol; potassium phosphate buffer, pH7.4, 200μmol. After equilibration at 37°C, the reaction was started with 0.5ml of microsomal suspension. After incubation in a shaking water bath (100 strokes/min) in air for various times as indicated, the flasks were placed on an ice/salt mixture and the cytochrome *P*-450 content was determined as described above. Control mixtures contained no steroid. In some experiments, NADP was omitted or replaced by NAD (1.3μmol).

Liver porphyrins

These were determined as described by Abbritti & De Matteis (1971). To 0.5ml of 10% (w/v) liver homogenate in 0.25M-sucrose was added 4.5ml of ice-cold 1M-$HClO_4$/methanol (1:1, v/v). After mixing and centrifuging (3000*g* for 20min), the

porphyrin content in the supernatant was determined in an Aminco–Bowman fluorimeter (excitation wavelength 400nm, emission wavelength 600nm) with a protoporphyrin-IX standard.

Mitochondrial 5-aminolaevulinate synthase

Rat liver mitochondria were prepared from 10% (w/v) homogenates in ice-cold 0.25M-sucrose containing 0.1mM-EDTA (Schneider & Hogeboon, 1950). The washed mitochondrial pellet was resuspended in sucrose/EDTA so that 1ml of suspension corresponded to 1g wet wt. of liver. The procedure used to measure 5-aminolaevulinate synthase activity was essentially that described by Narisawa & Kikuchi (1966). Incubation mixtures of 2ml contained: ATP, 5μmol; CoA, 0.05μmol; pyridoxal phosphate, 0.2μmol; $MgCl_2$, 5μmol; mercaptoethanol, 5μmol; sodium succinate, 60μmol; glycine, 100μmol; potassium phosphate buffer, pH7.0, 100μmol. After equilibration at 37°C, 0.5ml of the mitochondrial suspension was added. The reaction was stopped after incubation for 30min in a shaking water bath (100 strokes/min) in air, with 0.5ml of ice-cold 12.5% (w/v) trichloroacetic acid. The mixture was centrifuged at 10000*g* for 15min, and the 5-aminolaevulinate determined in the supernatant by means of a modified Ehrlich reagent (Mauzerall & Granick. 1956) after conversion into an Ehrlich-reactive pyrrole and column fractionation on Dowex AG-1 X8 resin (acetate form). Blank values ($t = 0$min) were obtained by adding trichloroacetic acid to the assay mixture kept on ice before the addition of the mitochondria.

Isolation of green pigments from the liver

This was carried out as described by De Matteis & Unseld (1976) and Unseld & De Matteis (1976). Portions of liver were homogenized in ice-cold 5% (v/v) H_2SO_4 in methanol (20ml/g wet wt. of liver). After being left for 17h at 4°C in the dark, the methylated products were extracted with chloroform, dried in a stream of N_2 at 37°C and then separated on silica-gel thin-layer plates (silica gel 60; layer thickness 0.25mm) with a chloroform/kerosene/methanol (20:5:3, by vol.) solvent system. In some instances the green bands were scraped off and the pigments eluted with chloroform/methanol (4:1, v/v). The extract was dried in a stream of N_2 at 37°C, the residue dissolved in chloroform and its spectrum recorded.

Results

Comparison between the effects of norethindrone, ethynyloestradiol and norethindrone on cytochrome P-450 in vitro

Incubation *in vitro* of norethindrone or ethynyloestradiol with rat liver microsomal fractions and an NADPH-generating system causes a time-dependent loss of cytochrome *P*-450 (Fig. 1). The results are expressed relative to control mixtures containing no steroid, to compensate for the small amount of destruction of cytochrome *P*-450 caused by NADPH-catalysed lipid peroxidation (De Matteis & Sparks, 1973). Incubation of the microsomal fraction with these steroids effects neither a shift in the absorption maxima of cytochrome *P*-450 nor the production of a *P*-420 component. NADPH (20μmol) can replace NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase in the reaction mixture. Unlike norethindrone and ethynyloestradiol, norethandrolone or oestradiol causes little or no destriction of cytochrome *P*-450.

Effect of pretreatment of rats with inducers of mixed-function oxidases on the steroid-mediated loss of cytochrome P-450 in vitro

The effects of some inducers of mixed-function-oxidase activities on the rate of loss of cytochrome *P*-450 mediated by norethindrone *in vitro* are shown in Fig. 2. Pretreatment of rats with phenobarbitone results in a marked stimulation of cytochrome *P*-450 breakdown, whereas the methylcholanthrene-induced cytochrome *P*-448 appears to be unaffected by incu-

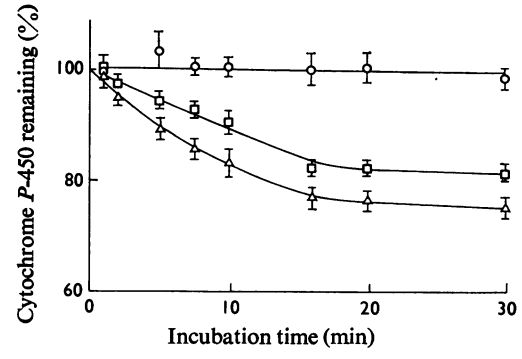

Fig. 1. *Loss of cytochrome P-450 in vitro after incubation of rat liver microsomal fraction with norethindrone, ethynyloestradiol or norethandrolone*

Results are expressed as the percentage of cytochrome *P*-450 remaining relative to control incubation mixtures containing a NADPH-generating system but no steroid. Points represent the mean (±S.E.) for four determinations. Details of the incubation mixture are given in the Experimental section. Flasks were shaken in air at 37°C for the times indicated. They were then placed on a salt/ice mixture and the cytochrome *P*-450 content was determined from the CO–dithionite-reduced difference spectrum. ○, Norethandrolone; □, ethynyloestradiol; △, norethindrone. The final concentration of each steroid was nominally 0.5mM. Each flask contained 8nmol of cytochrome *P*-450.

bation with this steroid. Pregnenolone 16α-carbonitrile was also investigated because this steroid causes a marked increase in the covalent binding *in vitro* of 2-hydroxy oestrogens to microsomal preparations (Nelson *et al.*, 1976). However, after pretreatment of rats with this inducer, norethindrone produces a rate of breakdown of cytochrome *P*-450 similar to that seen in microsomal preparations from control animals.

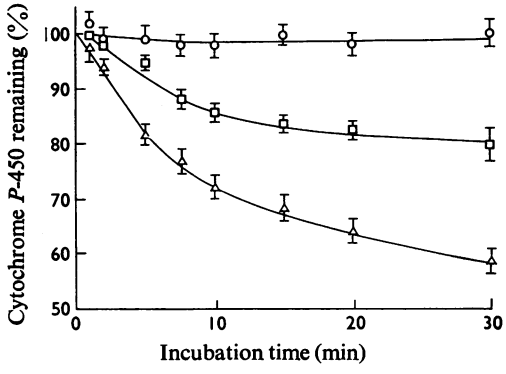

Fig. 2. *Effect of pretreating rats with phenobarbitone, pregnenolone 16α-carbonitrile or 3-methylcholanthrene on norethindrone-mediated loss of cytochrome P-450 in vitro*
Incubation mixtures contained rat liver microsomal fraction and a NADPH-generating system as described in the Experimental section. Results are expressed as the percentage of cytochrome *P*-450 remaining relative to control incubation mixtures containing an NADPH-generating system but no steroid. Points represent the mean (±S.E.) for four determinations. The norethindrone concentration was nominally 0.5mM. The concentrations of cytochrome *P*-450 (or *P*-448) in the induced rat livers were: phenobarbitone, 54.7±2.3nmol/g; 3-methylcholanthrene, 23.2±2.9nmol/g; pregnenolone 16α-carbonitrile, 32.1±3.9nmol/g. The reaction mixtures contained microsomal fraction with a cytochrome *P*-450 content of 20, 8 and 10nmol for phenobarbitone-, methylcholanthrene- and pregnenolone 16α-carbonitrile-pretreated animals respectively. Microsomal preparations were from rats pretreated with: ○, 3-methylcholanthrene; □, pregnenolone 16α-carbonitrile; △, phenobarbitone.

Characterization of the enzyme system responsible for norethindrone-mediated loss of cytochrome P-450

The steroid-mediated destruction of cytochrome *P*-450 has been further characterized (Table 1). The enzyme system involved has many of the characteristics of the microsomal mixed-function oxidases; it requires air, NADPH and is inhibited by CO. The classical mixed-function oxidase inhibitor SKF 525A (Fouts & Brodie, 1955) is without effect up to a concentration of 1mM as is the epoxide hydratase inhibitor 1,2-epoxy-3,3,3-trichloropropane (Oesch, 1971). Glutathione (1mM) also does not affect the destruction of cytochrome *P*-450 by norethindrone, although it significantly decreases the covalent binding of this steroid to microsomal fractions (Kappus & Remmer, 1975).

Dependence of cytochrome P-450 loss on norethindrone concentration in vitro

Fig. 3. shows the effect of changing the nominal norethindrone concentration on the loss *in vitro* of cytochrome *P*-450. Microsomal fractions from phenobarbitone-pretreated rats are used in this experiment to obtain a reasonable loss of cytochrome *P*-450 over a fairly short incubation time. Relatively high concentrations of norethindrone are required to saturate the enzyme system, even though at concentrations greater than 0.1mM the norethindrone exists as a suspension before the addition of the microsomal fraction.

Effects on cytochrome P-450 and b_5 in vivo

Since loss *in vitro* of cytochrome *P*-450 appears to be caused by metabolites of norethindrone and ethynyloestradiol, we also studied the effects of the

Table 1. *Norethindrone-mediated loss of cytochrome P-450 in vitro: cofactor requirements and effect of inhibitors*
Liver microsomal fraction from phenobarbitone-pretreated male rats was incubated for 15min at 37°C with norethindrone (0.5mM) and, except where indicated below, a NADPH-generating system as described in the Experimental section. The cytochrome *P*-450 content is expressed relative to controls incubated for the same time in the absence of steroid. Values are means (±S.E.) for four experiments.

Incubation media	Cytochrome *P*-450 remaining (%)
Complete system incubated in air	69.1±4.6
+1mM-Compound SKF 525A	65.6±2.3
+1mM reduced glutathione	71.3±3.9
+0.5mM-1,2-epoxy-3,3,3-trichloropropane	69.4±3.2
Complete system incubated under N_2	94.1±3.0
incubated under CO/O_2 (4:1)	98.0±4.2
less NADPH	110.4±2.6
with NADH replacing NADPH	107.5±1.9

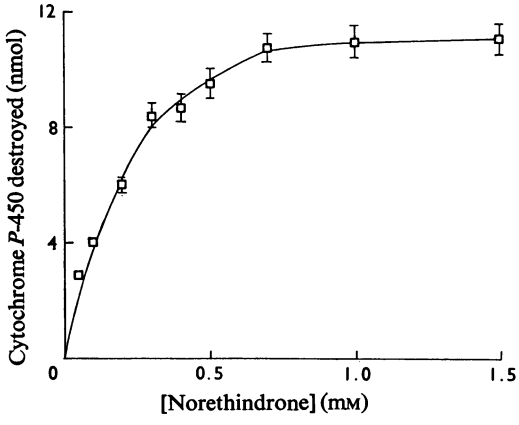

Fig. 3. *Effect of nominal norethindrone concentrations on the loss of cytochrome P-450 in vitro*

Points represent the mean (±S.E.) for four experiments. Incubation mixtures of final volume 3 ml were as described in the Experimental section. Flasks were shaken for 15 min (100 strokes/min) in air at 37°C, then placed on ice and the cytochrome *P*-450 content was determined. The microsomal fraction used was from phenobarbitone-pretreated rats. Incubation mixtures initially contained 20 nmol of cytochrome *P*-450.

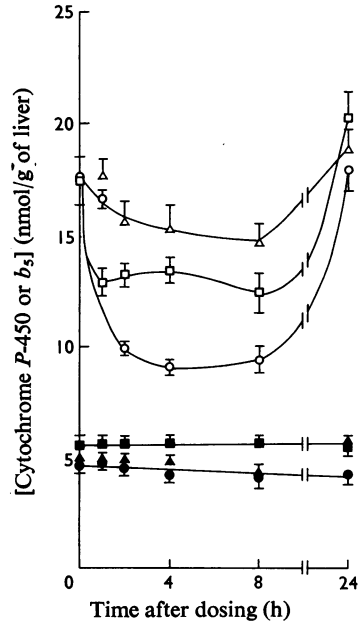

Fig. 4. *Effect of a single dose of norethindrone, ethynyloestradiol or norethandrolone (100 mg/kg) on the loss of hepatic cytochromes P-450 or b_5 with time*

Rats were starved 24 h before dosing. At various times as indicated, animals were killed and the cytochrome *P*-450 (open symbols) and cytochrome b_5 (closed symbols) content in the liver microsomal fraction determined as described in the Experimental section. Each point represents the mean (±S.E.) for four experiments. ▲, △, Norethandrolone; ■, □, ethynyloestradiol; ●, ○, norethindrone.

steroid on this cytochrome *in vivo*. A marked decrease in the amount of cytochrome *P*-450 to about 52% of control values is seen 4 h after a single intraperitoneal injection of norethindrone (100 mg/kg) (Fig. 4). Cytochrome b_5 concentrations are only little affected by this steroid. Relative to control animals, there is no significant change in liver weight (expressed as a percentage of the body weight) or in the protein content of the microsomal preparations during the 24 h duration of these experiments. However, between 2 and 8 h after dosing, the microsomal pellets appear noticeably paler in colour than those of the controls. The loss of cytochrome *P*-450 after ethynyloestradiol administration is somewhat less than that caused by norethindrone, whereas the effect of norethandrolone is considerably less marked.

Effects on the accumulation of porphyrins in the liver

The loss of cytochrome *P*-450 in the norethindrone-dosed rats is paralleled by an increase in the accumulation of porphyrins in the liver, reaching a maximum 4 h after dosing (Fig. 5). Similar (although smaller) increases in porphyrin concentration are seen after the administration of ethynyloestradiol or norethandrolone, both reaching a maximum 2 h after dosing.

Induction of 5-aminolaevulinate synthase

Since 5-aminolaevulinate synthase is believed to be the rate-limiting enzyme in porphyrin synthesis, we measured the activity of this enzyme in hepatic mitochondrial preparations at various times after the administration of the steroids to rats. Fig. 6 shows that, within 2 h of dosing, norethindrone causes a 3.2-fold induction of 5-aminolaevulinate synthase activity; norethandrolone or ethynyloestradiol cause smaller increases in activity. It is surprising, however, that ethynyloestradiol is less effective in inducing hepatic 5-aminolaevulinate synthase than norethandrolone, since the former steroid is more effective in causing both the loss of cytochrome *P*-450 and increasing the amounts of porphyrins in the liver. The reason for this apparent anomaly is not known.

Comparison of the effects of norethindrone in male and female rats

The rate of loss *in vitro* of cytochrome *P*-450 with time in microsomal preparations from female rats closely parallels that of control male rats (results not shown). Comparable results are seen for microsomal fractions from female and male rats with respect to

cytochrome *P*-450 destruction *in vivo* (Table 2). The accumulation of porphyrins in the liver *in vivo*, 2h after the administration of norethindrone, is also similar in both male and female rats. The induction of 5-aminolaevulinate synthase in the livers of female rats, although significant, is slightly less than that in males.

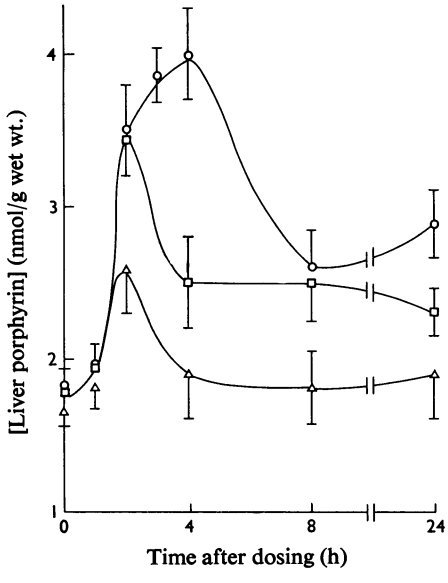

Fig. 5. *Effects with time of a single dose of norethindrone, ethynyloestradiol or norethandrolone on the amount of porphyrins in the liver*

The rats were starved 24h before being dosed (100mg/kg, i.p.). At the various times indicated the animals were killed and the porphyrin content of the liver was determined as protoporphyrin-IX as described in the Experimental section. Each point represents the mean (±S.E.) for four experiments. ○, Norethindrone; □, ethynyloestradiol; △, norethandrolone.

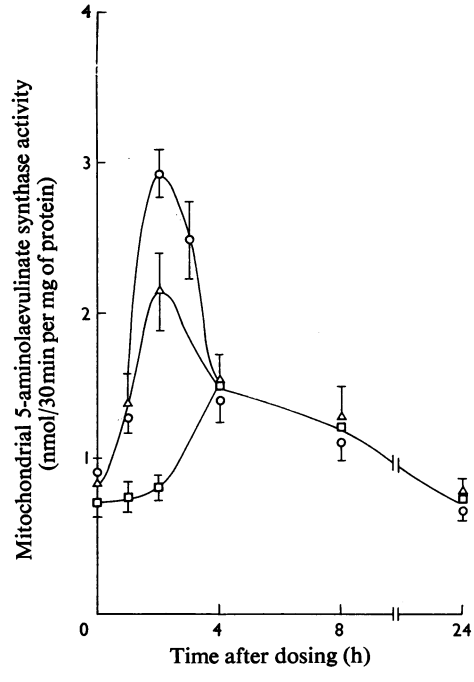

Fig. 6. *Effects with time of a single dose of norethindrone, ethynyloestradiol or norethandrolone on hepatic mitochondrial 5-aminolaevulinate synthase*

Animals were starved 24h before being dosed (100mg/kg, i.p.) with steroid. At various times after dosing, animals were killed and the livers removed. The mitochondrial 5-aminolaevulinate synthase was determined as described in the Experimental section. Each point represents the mean (±S.E.) for four determinations. ○, Norethindrone; □, ethynyloestradiol; △, norethandrolone.

Table 2. *Comparison between male and female rats: effects of a single dose of norethindrone on liver cytochromes b_5 and P-450, 5-aminolaevulinate synthase and the accumulation of porphyrins*

Rats were starved for 24h, then given norethindrone (100mg/kg, i.p.) dissolved in trioctanoin. Control animals received trioctanoin only. Animals were killed 2h after dosing, and the various parameters indicated below were determined as described in the Experimental section. Results represent the mean (±S.E.) for four experiments.

	Sex	Control	Treated	Significance*
Cytochrome *P*-450 (nmol/g wet wt.)	Male	17.4±0.8	9.8±0.4	$P<0.001$
	Female	13.9±1.0	6.9±0.4	$P<0.001$
Cytochrome b_5 (nmol/g wet wt.)	Male	4.9±0.5	4.4±0.5	N.S.
	Female	5.5±0.4	4.2±0.4	N.S.
Liver porphyrins (nmol/g wet wt.)	Male	1.8±0.03	3.2±0.2	$P<0.001$
	Female	2.7±0.2	4.7±0.4	$P<0.01$
5-Aminolaevulinate synthase	Male	0.93±0.09	2.96±0.30	$P<0.001$
(nmol/30min per mg of mitochondrial protein)	Female	1.37±0.12	2.12±0.19	$P<0.01$

* Probability of significance of difference between control and treated animals; N.S., not significant.

Extraction of green pigments from the livers of rats dosed with norethindrone or ethynyloestradiol

Green pigments can be extracted from the livers of phenobarbitone-pretreated male rats given either norethindrone or ethynyloestradiol (100mg/kg, i.p.). Rats are killed 4h after dosing with the steroid, and the livers homogenized in a methanol/H_2SO_4 mixture as described in the Experimental section. The methylated products are extracted with chloroform and chromatographed on silica-gel thin-layer plates. Three major green bands are seen. These have R_F values between 0.49 and 0.59 and migrate just behind the red–brown dimethylhaem derivative. In addition, there are a number of less-well-defined green bands with R_F values between 0.2 and 0.35. Each of these green pigments fluoresce red under u.v. light. The spectrum of the pooled three major components show a Soret band at 417nm with smaller peaks at 512, 547 and 590nm. Further characterization of these derivatives has not been attempted. No green pigments are seen in liver extracts from control rats or those dosed with norethandrolone, progesterone or oestradiol (100mg/kg, i.p.).

Discussion

The present results suggested that active metabolites of norethindrone or ethynyloestradiol caused the loss of cytochrome *P*-450. The necessity for formation of active metabolites was derived from the dependency of this loss on NADPH and air, its inhibition by CO and its enhancement by pretreatment of rats with phenobarbitone. These characteristics were typical of the microsomal mixed-function oxidases (Shuster, 1966; Conney, 1967). The mixed-function-oxidase inhibitor SKF 525A did not decrease the norethindrone-mediated destruction of cytochrome *P*-450. A possible explanation for this result could be that norethindrone itself is a potent inhibitor of drug metabolism (Soyka & Deckert, 1974). If a metabolite of compound SKF 525A rather than the parent compound was ultimately responsible for inhibiting metabolism (Schenkman *et al.*, 1972), its formation could be prevented by the presence of norethindrone.

The loss of cytochrome *P*-450 both *in vitro* and *in vivo* was similarly effected by both norethindrone and ethynyloestradiol. This was surprising, as a different binding mechanism has been proposed for the interaction of each of these two steroids with the microsomal fraction (Kappus & Remmer, 1975; Marks & Hecker, 1969). Covalent binding of an active metabolite of either steroid need not necessarily be accompanied by destruction of cytochrome *P*-450. Our results indicated that there were marked differences between the two processes. The addition of glutathione to the reaction mixtures hardly affected the breakdown *in vitro* of cytochrome *P*-450 caused by norethindrone (Table 1), although it greatly decreased the covalent binding of norethindrone to the microsomal fraction (Kappus & Remmer, 1975). Pretreatment of rats with pregnenolone 16α-carbonitrile did not enhance the rate of norethindrone-mediated breakdown of cytochrome *P*-450 relative to controls. It did, however, enhance the covalent binding *in vitro* to the microsomal fraction of ethynyloestradiol and 2-hydroxyoestradiol derivatives (Nelson *et al.*, 1976). This latter result suggested that the form(s) of cytochrome *P*-450 induced by pregnenolone 16α-carbonitrile may have been more resistant to attack by the active metabolites of norethindrone. A similar argument may be made for cytochrome *P*-448 induced by pretreatment of the rats with 3-methylcholanthrene. This cytochrome was much less affected when the microsomal fraction from these rats was incubated with northindrone than when that from control rats was used (Figs. 1 and 2). A similar resistance of cytochrome *P*-448 to breakdown after incubation *in vitro* of microsomal fractions from 3-methylcholanthrene-pretreated rats with 5-allyl-5-(1-methylbutyl) barbiturate has been reported by Levin *et al.* (1973).

A further similarity between our results and those of De Matteis (1971) and Levin *et al.* (1973), who used allylic-substituted acetamides and barbiturates respectively, was the appearance of green pigments in the liver. These green pigments originate from the breakdown of liver haem (De Matteis, 1971). Judging from their migration on silica-gel t.l.c., the red fluorescence under u.v. light and the spectral characteristics, the green pigments from liver of rats dosed with norethindrone or ethynyloestradiol were similar to the green pigments resulting from 2-allyl-2-isopropylacetamide administration (De Matteis & Unseld, 1976; Unseld, 1976; Unseld & De Matteis, 1976). To our knowledge, formation of such pigments has not been reported to occur with any compounds other than allyl-substituted derivatives.

Green pigments were only seen in liver extracts from rats given steroids containing an ethynyl-(–C≡C–) substituent. This fact suggested that the ethynyl group was metabolically activated to a derivative(s) that caused destruction of liver haem. At present, we cannot identify or measure the rate of formation of this active metabolite(s); therefore we have no details on the mechanism by which the ethynyl group contributes to the loss of cytochrome *P*-450. Although the ethyl-substituted norethandrolone was apparently ineffective in causing a loss *in vitro* of cytochrome *P*-450 (Fig. 1), it caused a loss *in vivo* of the cytochrome a few hours after its administration (Fig. 4). This finding implied that metabolites other than those formed as a result of activation of the ethynyl substituent were capable to some extent

of bringing about the destruction of cytochrome *P*-450.

In addition to causing a loss of cytochrome *P*-450, treatment of rats with norethindrone, ethynyloestradiol or norethandrolone resulted in an increase in 5-aminolaevulinate synthase activity and an accumulation of porphyrins in the liver. This could be expected if these steroids lead to a loss of liver haem, since haem plays a central role in regulating the rate-limiting enzyme of porphyrin synthesis, 5-aminolaevulinate synthase (Granick, 1966).

The relatively short duration of action of the steroids with respect to effecting loss of cytochrome *P*-450 was somewhat unexpected, since although they are rapidly excreted into the bile (Hanasono & Fischer, 1974), they have a long half-life in the body owing to an extensive enterohepatic circulation (Smith, 1974; Brewster *et al.*, 1976).

No extrapolation of these results can be made to man. Differences exist in the metabolism of the steroids between species (Gerhards *et al.* 1971; Palmer *et al.*, 1969). The dose used in these experiments was very much higher than that normally used for oestrogen therapy or for contraceptive purposes. However, taken regularly over a period of years, metabolites of these steroids might predispose some individuals to the type of porphyria associated with the side-effects of these compounds or affect the unborn child.

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References

Abbritti, G. & De Matteis, F. (1971/1972) *Chem.-Biol. Interact.* **4**, 281–286

Behm, A. R. & Unger, W. P. (1974) *Can. Med. Assoc. J.* **110**, 1052–1054

Bolt, H. M. & Kappus, H. (1974) *J. Steroid Biochem.* **5**, 179–184

Brewster, D., Jones, R. S. & Symons, A. M. (1976) *Biochem. Soc. Trans.* **4**, 516–518

Cole, R. & Cole, J. (1976) *Lancet* **ii**, 640

Congote, L. F., Stern, M. D. & Solomon, S. (1974) *Biochemistry* **13**, 4255–4263

Conney, A. H. (1967) *Pharmacol Rev.* **17**, 317–366

Cook, C. E., Dickey, M. C. & Christensen, H. D. (1974) *Drug Metab. Disp.* **2**, 58–64

De Matteis, F. (1971) *Biochem. J.* **124**, 767–777

De Matteis, F. & Sparks, R. G. (1973) *FEBS Lett.* **29**, 141–144

De Matteis, F. & Unseld, A. (1976) *Biochem. Soc. Trans.* **4**, 205–209

Fouts, J. R. & Brodie, B. B. (1955) *J. Pharmacol. Exp. Ther.* **115**, 68–73

Gerhards, E., Hecker, W., Hitze, It., Nieuweboer, B. & Bellman, O. (1971) *Acta Endocrinol.* **68**, 219–248

Granick, S. (1966) *J. Biol. Chem.* **241**, 1359–1375

Haberman, H. F., Rosenberg, F. & Menon, I. A. (1975) *Can. Med. Assoc. J.* **113**, 653–655

Hanasono, G. K. & Fischer, L. J. (1974) *Drug Metab. Disp.* **2**, 159–168

Kamath, S. A. & Rubin, E. (1972) *Biochem. Biophys. Res. Commun.* **49**, 52–59

Kappus, H. & Remmer, H. (1975) *Drug Metab. Disp.* **3**, 338–344

Levin, W., Jacobson, M., Sernatinger, E. & Kuntzman, R. (1973) *Drug Metab. Disp.* **1**, 275–282

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mackinnon, M. & Simon, F. (1975) *Biochem. Pharmacol.* **24**, 748–749

Marks, F. & Hecker, E. (1969) *Biochim. Biophys. Acta* **187**, 250–265

Mauzerall, D. & Granick, S. (1956) *J. Biol. Chem.* **219**, 435–446

Narisawa, K. & Kikuchi, G. (1966) *Biochem. Biophys. Acta* **123**, 596–605

Nelson, S. D., Mitchell, J. R., Dybing, E. & Sasame, H. A. (1976) *Biochem. Biophys. Res. Commun.* **70**, 1157–1165

Oesch, F., Kaubisch, N., Jerina, D. M. & Daly, J. W. (1971) *Biochemistry* **10**, 4858–4866

Omura, T. & Sato, R. (1964*a*) *J. Biol. Chem.* **239**, 2370–2378

Omura, T. & Sato, R. (1964*b*) *J. Biol. Chem.* **239**, 2379–2385

Palmer, K. H., Feirabead, J. F., Baggett, B. & Wall, M. E. (1969) *J. Pharmacol. Exp. Ther.* **167**, 217–222

Schenkman, J. B., Wilson, B. J. & Cinti, D. L. (1972) *Biochem. Pharmacol.* **21**, 2372–2383

Schneider, W. C. & Hogeboon, G. H. (1950) *J. Biol. Chem.* **183**, 123–128

Shuster, L. (1966) *Annu. Rev. Biochem.* **33**, 571–596

Smith, R. L. (1974) *Pharmacological Models in Contraceptive Development* (*WHO Symp.* 1973), pp. 149–168

Soyka, L. F. & Deckert, F. W. (1974) *Biochem. Pharmacol.* **23**, 1629–1639

Unseld, A. (1976) Ph.D. Thesis, University of London

Unseld, A. & De Matteis, F. (1976) in *Porphyrias in Human Diseases* (Doss, M., ed.), pp. 71–75, S. Karger, Basel

Wald, G. (1965) in *Thin Layer Chromatography* (Stahl, E., ed.), pp. 249–278, Springer-Verlag, New York