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DESTRUCTION OF CYTOCHROME P-450 AND FORMATION OF GREEN PIGMENTS BY CONTRACEPTIVE STEROIDS IN RAT HEPATOCYTE SUSPENSIONS

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Abstract—The contraceptive steroid norethindrone caused a rapid time and dose-dependent loss of cytochrome P-450 from rat hepatocytes in suspension cultures. Up to 30% of this cytochrome was lost in the first 5 min of incubation; longer incubations resulted in little further loss even though not all the steroid was metabolised and the cells remained viable. Such cultures were used to investigate the formation of *N*-alkylated porphyrins (green pigments) which could be extracted from cell incubation mixtures following exposure to norethindrone and separation by HPLC or TLC. The number of *N*-alkylated porphyrins formed was dependent both on the time of incubation and the concentration of steroid. After 1 min, 1 major green pigment (GP1) was resolved using either high (0.3 mM) or low (0.03 mM) norethindrone concentrations. With longer incubation times (60 min), at high steroid concentrations, only one additional polar adduct (GP2) was formed. At lower steroid levels, 3 more polar components (GP2, 3 and 4) were seen. As judged by HPLC or TLC, GP1 corresponds to the pigment formed in microsomal preparations incubated with norethindrone *in vitro*, while GP2, 3 and 4 correspond to the pigments extracted from the livers of rats administered this steroid *in vivo*.

Pretreatment of rats with either phenobarbitone or 3-methylcholanthrene induced cytochrome P-450s. Relative to controls, phenobarbitone pretreatment also resulted in a greater accumulation of green pigments in hepatocytes incubated with norethindrone, the more polar forms of green pigments (GP3 and 4), showing a disproportionate increase in concentration.

The mixed function oxidase inhibitor SKF 525-A or high concentrations of steroid not containing an ethynyl function, e.g. norethandrolone, when added to cell cultures containing norethindrone, preferentially inhibited the formation of GP3 and 4.

When purified green pigments were added to cell incubation mixtures in the absence of norethindrone, no interconversion of one form to another could be demonstrated.

The results suggest that the more polar norethindrone–protoporphyrin IX adducts (GP2, 3 and 4) arise as a result of metabolic modification of norethindrone rather than the protoporphyrin IX moiety, either prior to or after activation of the ethynyl function. The formation of several green pigment components in hepatocyte suspensions was not unique to norethindrone but occurred with a number of other 17-ethynyl-substituted steroids.

Norethindrone (17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one or norethisterone), a widely used progestational component of the contraceptive pill has the 17β-hydroxy-17α-ethynyl substituent characteristic of many orally active contraceptive steroids. In the rat, norethindrone causes a time and dose dependent decrease in hepatic cytochrome P-450 content both *in vivo* and in microsomal systems *in vitro* [1]. The ethynyl function was implicated in this process since neither the parent steroid progesterone nor a 17α-ethyl substituted analogue, norethandrolone (17α-ethyl-17β-hydroxy-19-norandrost-4-en-3-one), caused any significant decrease [1]. A number of structure activity studies have confirmed the ability of the ethynyl function to destroy cytochrome P-450 [2–4]. Using a microsomal system *in vitro*, NADPH and oxygen were required for such loss. This reaction was inhibited by carbon monoxide [1]. These properties are typical of the microsomal mixed function oxidases [5]. Catalytic turnover of cytochrome P-450 in its own destruction has been implicated. Phenobarbital (PB)-inducible forms of cytochrome P-450 are preferentially destroyed by norethindrone [1, 6].

The loss of cytochrome P-450 is accompanied by the production of abnormal haem breakdown products, green pigments [2]. Such green pigments fluoresce red under u.v. light and have an aetio-type absorption spectra characteristic of *N*-alkylated porphyrins [6]. Green pigments formed after norethindrone administration to rats are due to a covalent 1:1 association between the steroid and the protoporphyrin IX ring of haem [7], although their exact structural identity is not known. They have been resolved, after esterification, by TLC and HPLC [2, 6, 7, 8].

In rats treated with norethindrone, three major green pigments could be separated whose ratio varied according to the dose and length of exposure to norethindrone. Using a microsomal system, only a single (different) green pigment was resolved [6]. It was suggested that metabolic transformation of green pigments might be occurring to account for

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these results [6]. In contrast, Ortiz de Montellano *et al.* [7] resolved two green pigments by HPLC after treatment of rats *in vivo* with norethindrone and proposed that they represented different isomeric forms [4].

The present study investigates if rat hepatocytes in short term culture represent a better model for the study of the metabolic activation of norethindrone with regard to green pigment formation than do hepatic microsomal systems *in vitro* and addresses the question of whether different green pigments represent isomeric forms or arise as a result of metabolic modification.

MATERIALS AND METHODS

Chemicals. The following sources were used: norethindrone, 17α-ethynyl oestradiol, norethynodrel, aryl sulphatase, type H2 (*Helix pomatia*) and type B1 (bovine liver) β-glucuronidase, Sigma Chemical Co. (Poole, Dorset). 5α-Androstan-17α-ethynyl-17β-ol-3-one, 5α-Estran-17α-ethynyl-17β-ol-3-one, and norethandrolone, Steraloids Ltd (Croydon, Surrey). Lynestrenol, Koch-Light Labs (Colnbrook, Bucks); [6,7-^{3}H] norethindrone (specific activity 55 Ci/mmol), New England Nuclear (Hants, U.K.). SKF 525-A was a generous gift from F. De Matteis of the MRC Toxicology Unit, Carshalton, Surrey, U.K.

Treatment of rats. Male rats (180–220 g) were of the inbred Fischer F.344/N strain. Where indicated, animals were pretreated with 0.1% (w/v) sodium phenobarbitone (PB) in the drinking water for 7 days or injected i.p. with 3-methylcholanthrene (3-MC), 20 mg/kg body weight delivered in trioctanoin daily for 3 days prior to hepatocyte or microsomal preparations. In some experiments, phenobarbitone-pretreated rats were injected i.p. with norethindrone dissolved in trioctanoylglycerol (100 mg/kg body weight). Animals were killed 15 min or 2 hr after dosing and green pigments were extracted from the excised livers as previously described [1].

Hepatocyte preparation and culture. Hepatocytes were prepared by collagenase perfusion of rat livers using a modified method of Berry and Friend [9] as described by Paine and Legg [10]. Hepatocytes were finally suspended in Williams Medium E containing 5% (v/v) foetal calf serum, 1% (w/v) 1-glutamine and 50 μg/ml gentamycin (complete WME). The number and viability of cells was determined by exclusion of 0.1% (w/v) trypan blue in a haemocytometer. Cell preparations with a viability less than 80% were discarded.

Freshly prepared hepatocytes were incubated at a density of 1×10^6 cells/ml in complete WME at 37° in a shaking water bath. Norethindrone or other steroids were added in propylene glycol, final volume <1% (v/v). Incubation mixes of 10 or 25 ml were placed in glass conical flasks of 100 or 250 ml, respectively. Initial experiments indicated that no difference in green pigment formation was seen if incubation mixtures were gassed with 95% O_2:5% CO_2 (v/v) or exposed to air. All incubations were carried out in the dark.

Reactions were terminated by decanting the contents of the flask into plastic centrifuge tubes on ice and rapidly pelleting the cells by centrifugation (1000 *g* for 1 min).

Cytochrome P-450 determination. The cytochrome P-450 content of hepatocyte pellets following either incubation with steroid or propylene glycol was estimated according to the method of Omura and Sato [11] as described by Paine *et al.* [12].

Microsomal preparation and estimation of green pigments. Preparation of microsomes and incubation mixtures were as described by White [2]. Green pigments were isolated from hepatocyte pellets, microsomal incubation mixes and the excised liver of rats treated with norethindrone *in vivo* as described previously [2].

Separation of esterified green pigments was carried out by HPLC [6]. Pigment peaks were located in the HPLC eluents by absorption at 417 nm and by fluorescence using a Kratos FS-970 fluorimeter coupled to the outlet of the absorbance detector. Excitation wavelength was 417 nm, emission filter was >550 nm.

Treatment of green pigments with sulphatase or glucuronidase. Esterified green pigments from hepatocyte incubation mixtures were purified by Biobeads SX-2 column chromatography [6] followed by TLC. Individual green pigment components (4 nmol) in methanol (50 μl) were incubated in the dark for 2, 4 or 21 hr at 37° in 0.2 M acetate buffer pH 5.0 (2 ml) containing either arylsulphatase (600 Sigma units) together with D-saccharo-1,4-lactone (20 mM), or β-glucuronidase (15,000 Sigma units). Green pigments were then re-esterified with methanol/H_2SO_4 and subjected to HPLC or TLC as described above.

Norethindrone estimation. The amount of norethindrone remaining in hepatocyte incubations was determined by HPLC. To aliquots (0.2 ml) of hepatocyte suspension, incubated with [6,7-^{3}H] norethindrone (0.03 or 0.3 mM) for various lengths of time, were added 0.4 ml of ice-cold acetonitrile in 1 ml plastic Eppendorf tubes. The samples were mixed and centrifuged (10,000 *g* for 1 min) to pellet protein. Aliquots (40 μl) of the supernatants were resolved by HPLC using a C_{18} reverse phase column (Magnusphere, 5 μm C-18, Magnus Scientific, Aylesbury, Bucks, 25 × 0.47 cm) and a solvent system of CH_3CN/H_2O (7:3, v/v) at a flow rate of 1 ml/min. Norethindrone was detected by its absorbance at 235 nm. Fractions (0.5 ml) were collected from the detector outlet. The peak corresponding to norethindrone was collected and after addition of scintillant (4.5 ml, Ready-solv CP, Beckman) radioactivity was determined by scintillation counting.

Other assays. Proteins were estimated according to the method of Lowry *et al.* [13] using a bovine serum albumin standard. The release of lactate dehydrogenase (LDH EC 1.1.1.27) into media during hepatocyte incubations was measured according to the procedure described by Bergmeyer and Bernt [14].

RESULTS

Destruction of cytochrome P-450 by norethindrone

The amounts of cytochrome P-450 destroyed with time by norethindrone (0.3 mM) in hepatocyte suspensions from control, phenobarbitone (PB) or 3-

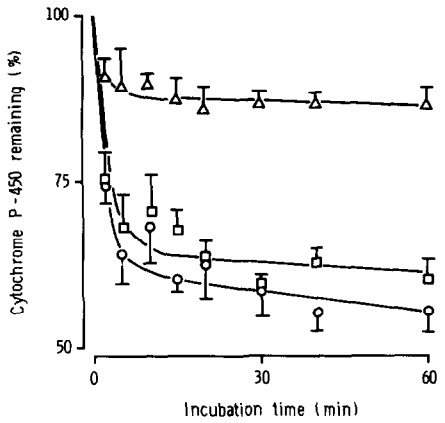

Fig. 1. Destruction of cytochrome P-450 by norethindrone (0.3 mM) in hepatocyte suspensions. Cells were from control (□), PB (○) or 3-MC (△) rats (2.5×10^7 cells in 25 ml complete WME) after incubation for various lengths of time. The initial amounts of cytochrome P-450 present in the hepatocyte preparations were 326 ± 13, 1327 ± 45 and 849 ± 27 pmol/10^6 cells for control, PB and 3-MC cultures respectively. Protein concentrations were: 2.28 ± 0.08, 2.76 ± 0.13 and 2.90 ± 0.06 mg/10^6 cells for control, PB and 3-MC hepatocytes, respectively (mean ± S.E.M. for 4 determinations).

methylcholanthrene (3-MC) pretreated rats are shown in Fig. 1. In each case there was a very rapid destruction of this cytochrome in the first 5 min followed by little further loss. Less than 2.5% of the cytochrome P-450 was lost after 1 hr in similar cultures treated only with propylene glycol solvent, and the viability of the hepatocytes in all 3 norethindrone treated cultures was >75% as assessed by trypan blue exclusion. Lactate dehydrogenase released into the media after 60 min in solvent-treated hepatocyte cultures was 330 ± 12, 362 ± 15 and 482 ± 20 U/L, while after norethindrone treatment these values increased to 511 ± 30, 568 ± 34 and 747 ± 52 U/L in control, PB and 3-MC hepatocytes respectively (mean ± S.E.M., 4 experiments). Destruction of cytochrome P-450 was dose dependent reaching a maximum at norethindrone concentrations of 0.2–0.3 mM when about 40% of the total is destroyed (Fig. 2).

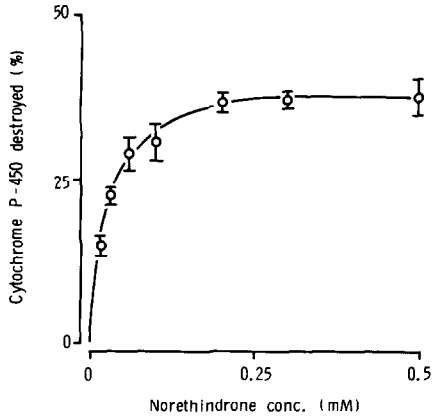

Fig. 2. Effect of norethindrone concentrations on the destruction of cytochrome P-450 in hepatocyte suspensions. Liver cells from phenobarbitone pretreated rats were incubated for 5 min at 37°. The amount of cytochrome P-450 in cultures incubated in the absence of norethindrone for 5 min was 1345 ± 34 pmol/10^6 cells. (Results are the mean ± S.E.M. for 5 determinations.)

Formation of green pigments due to norethindrone

In hepatocyte incubation mixtures, no green pigments could be extracted in the absence of norethindrone (Fig. 3a). On incubation for 60 min with 0.03 mM norethindrone, 4 major green pigments were resolved by HPLC, with retention times of 6.2, 6.9, 10 and 11 min, respectively (Fig. 3b). These components have been called GP1, 2, 3 and 4, respectively. All 4 pigments purified by TLC from

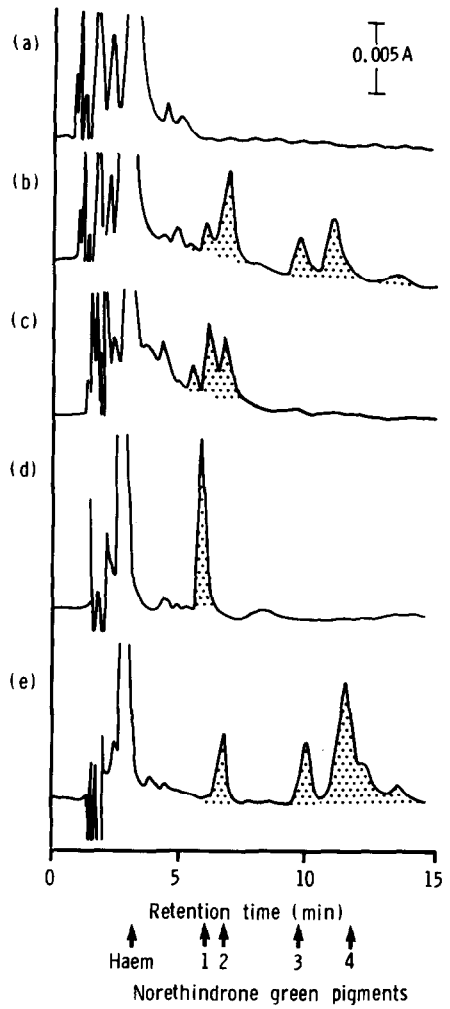

Fig. 3. Silica gel HPLC traces of green pigments. Hepatocytes from phenobarbitone pretreated rats were used. Pigments were extracted from cells incubated: (a) with propylene glycol vehicle for 60 min; (b) norethindrone (0.03 mM) for 60 min; and (c) norethindrone (0.3 mM) for 60 min. (d) Represents an extract from rat liver microsomes incubated with norethindrone (0.03 mM) for 2 min. (e) Whole liver extract from a rat dosed 100 mg/kg, i.p. 2 hr earlier with norethindrone. Ordinates represent relative absorbance at 417 nm. Green pigment peaks are shown shaded black.

Table 1. Green pigment formation in hepatocytes from control, phenobarbitone or 3-methylcholanthrene pretreated rats

Hepatocytes	Green pigment [pmol/10^6 cells]*					
	Minor	1	2	3	4	Total
Control	46 ± 4	22 ± 2	61 ± 3	10 ± 2	20 ± 2	160 ± 13
Phenobarbitone	82 ± 6	40 ± 6	78 ± 7	58 ± 6	126 ± 6	384 ± 31
3-Methylcholanthrene	36 ± 2	15 ± 1	53 ± 5	4 ± 1	5 ± 1	112 ± 7

* Hepatocytes incubated with 0.03 mM norethindrone for 60 min. Results represent the mean ± S.E.M. of 4 experiments.

hepatocyte incubation mixtures (R_F values 0.49, 0.41, 0.32 and 0.30 respectively) had aetio-type spectra in $CHCl_3$ similar to those reported previously [6]. Incubation of hepatocytes with a higher concentration of norethindrone (0.3 mM), which causes maximal destruction of cytochrome P-450, resulted in the formation of only 2 major green pigments (Fig. 3c). Their retention times on HPLC and R_F values on TLC corresponded to GP1 and 2. Using similar HPLC and TLC criteria, the present results suggest that GP1 corresponds to the green pigment component extracted from microsomal mixtures incubated for short time periods with norethindrone and a NADPH generating system *in vitro* (Fig. 3d) while GP2, 3 and 4 correspond to the three major green pigments extracted from the livers of rats given norethindrone *in vivo* (Fig. 3e).

In addition to the major green pigments extracted from hepatocyte incubation mixtures, a number of minor components were generally resolved. These mostly had retention times on HPLC less than that of GP1. Taken together such minor peaks often represented a substantial proportion of the total formed (Table 1).

Little or no green pigment could be extracted from the incubation media following precipitation of the cells by centrifugation, irrespective of the concentration of norethindrone or time of incubation.

Time course for green pigment formation

Figures 4(a) and (b) show time courses for the formation of green pigments in hepatocytes from phenobarbitone pretreated rats incubated with 0.03 or 0.3 mM norethindrone, respectively. At both steroid concentrations, GP1 is formed rapidly during the first 5 min and decreases thereafter. With 0.3 mM norethindrone, this is followed by the appearance of only GP2, (<30 pmol/10^6 cells GP3 and 4 being detected at any time). With 0.03 mM steroid all 4 green pigments appear. Assuming all the green pigments have the same extinction coefficients [2], at both norethindrone concentrations after the first 5 min, the overall concentration of green pigment changes little with time. The ratios of the total concentration of green pigment accumulated in the cells to the loss in the concentration of cytochrome P-450 remains at about 1.2, independent of the time of incubation and steroid concentration (Table 2).

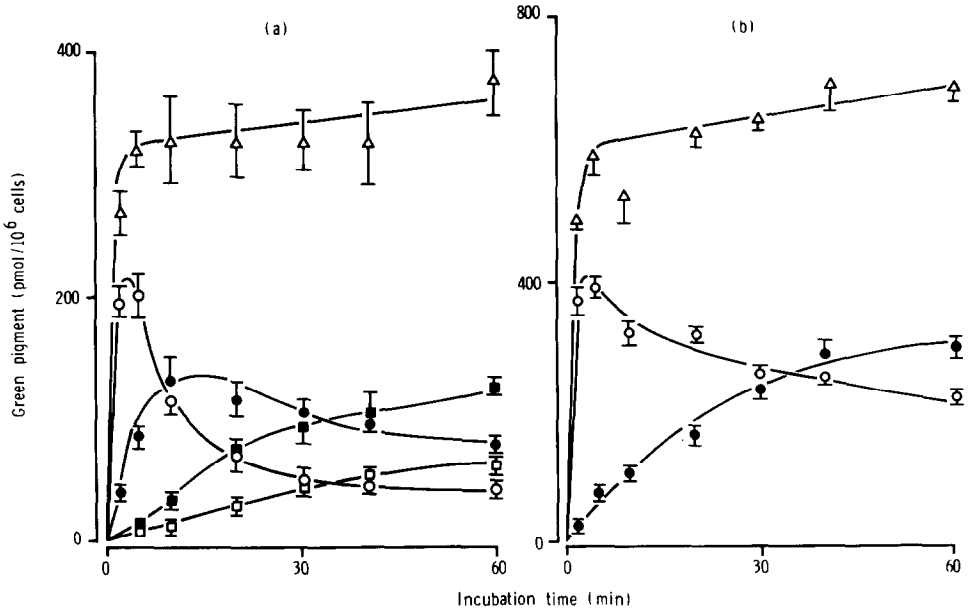

Fig. 4. Time course for green pigment formation in hepatocytes from phenobarbitone pretreated rats incubated with 0.03 mM norethindrone (a) or 0.3 mM norethindrone (b). ○, green pigment 1; ●, green pigment 2; ■, green pigment 3; □, green pigment 4; △, total green pigment (including minor components). (Results are mean ± S.E.M. for 5 determinations).

Table 2. Effects of norethindrone on cytochrome P-450 and the accumulation of green pigments in hepatocytes from control, phenobarbitone, and 3-methylcholanthrene-pretreated rats

Pretreatment	Initial cytochrome P-450 concentration [pmol/10^6 cells]	Cytochrome P-450 destroyed* [pmol/10^6 cells]	Green pigment accumulated* [pmol/10^6 cells]	Green pigment accumulated/ Cytochrome P-450 destroyed
None	326 ± 13	133 ± 5	166 ± 12	1.3
Phenobarbitone	1327 ± 45	589 ± 23	698 ± 22	1.2
3-Methylcholanthrene	849 ± 27	115 ± 3	138 ± 10	1.2

* Hepatocytes incubated with 0.3 mM norethindrone for 60 min.
Results represent the mean ± S.E.M. of 5 experiments.

Comparison of green pigments formed in hepatocytes from control, phenobarbitone or 3-methylcholanthrene pretreated rats

Table 2 also shows the effect of either PB or 3-MC pretreatment of rats on green pigment formation. PB pretreatment increased green pigment formation approximately 4-fold whilst 3-MC pretreatment had little effect.

The pattern of green pigments formed, as assessed by HPLC, was similar in hepatocytes from control, phenobarbitone and 3-methylcholanthrene pretreated rats after incubation with 0.03 mM norethindrone. However, phenobarbitone pretreatment disproportionately increased the accumulation of the more polar green pigments (GP3 and 4) (Table 1).

Concentration of norethindrone in hepatocyte incubation mixtures

Figure 5 shows that in hepatocyte incubation mixtures from PB-induced rats, norethindrone concentration rapidly decreases with time when 0.03 mM steroid is used. With 0.3 mM norethindrone, approximately 50% still remains after 60 min incubation.

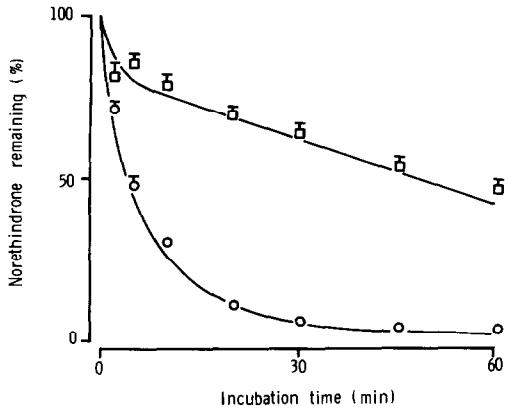

Fig. 5. Effects of time of incubation on the concentration of norethindrone remaining in hepatocyte suspensions. Hepatocytes from phenobarbitone pretreated rats were incubated with 0.3 mM (□——□) or 0.03 mM (○——○) norethindrone for various times as indicated. Concentrations of steroid in the incubation mixtures were estimated by measurement of radioactivity in the norethindrone peak following reverse phase HPLC. Results represent the mean ± S.E.M. for 3 determinations. Where error bars are not shown, they are within the symbol.

Effects of SKF 525-A on green pigment accumulation in hepatocytes

Hepatocytes from phenobarbitone pretreated rats were incubated with norethindrone (0.03 mM) in the presence or absence of the mixed function oxidase inhibitor, SKF 525-A (10 μM). The presence of this compound at time zero, reduced the total green pigments accumulated after 1 hr from 380 to 132 pmol/10^6 cells (mean, 2 experiments). In the SKF 525-A-treated cells, no GP3 or 4 could be detected following HPLC or TLC of such cell extracts. When SKF 525-A was added to hepatocytes after 2 min incubation with norethindrone (0.03 mM), i.e. after the formation of GP1 and some GP2, no subsequent formation of GP3 or 4 could be detected after a further 60 min incubation.

Effects of steroids not containing an ethynyl substituent

Steroids without an ethynyl function, such as norethandrolone, when incubated with hepatocytes and norethindrone caused a dose-dependent inhibition of green pigment production, preferentially inhibiting the formation of the more polar adducts, GP3 and 4. Using hepatocytes from phenobarbitone pretreated rats incubated with 0.03 mM norethindrone and 0.27 mM norethandrolone, the concentration of GP1, 2, 3 and 4 extracted from the cells after 1 hr was 43, 105, 10 and 7 pmol/10^6 cells, respectively (mean of 2 experiments; cf. Table 2). Similar effects were observed using progesterone, testosterone or oestradiol. Inclusion of steroids lacking the 17α-ethynyl function in hepatocyte incubation mixtures on their own did not result in the loss of cytochrome P-450 or the formation of green pigments.

Absence of interconversion of green pigments

GP1 extracted from hepatocytes and purified by TLC either as the dimethyl ester or as the dicarboxylic acid following mild acid/butanone extraction [2] was added to hepatocyte incubation mixtures over a range of concentrations up to 500 pmol/10^6 cells in the absence of norethindrone. Although some destruction of the pigment occurred over 60 min incubation, no conversion at any time period to the more polar products could be detected. No transformation was achieved using other green pigments (GP2, 3 or 4) purified in a similar manner. Incubation of purified green pigments with glucuronidase or sulphatase in the absence of hepatocytes did not affect their retention times following HPLC.

Formation of green pigments by other 17α-ethynyl-substituted steroids

Green pigment formation after treatment of hepatocytes from phenobarbitone pretreated rats with low concentrations (0.03 mM) of various steroids was investigated. After 5 min incubation, with the exception of the ring A saturated 5α-estran-17α-ethynyl-17β-ol-3-one which gave 3 major green pigments, all the remaining steroids tested gave one component with different retention times following HPLC (not shown). Figure 6 shows that after 60 min, multiple green pigments were separated by HPLC of cell extracts. At this time, both lynestrenol and 5α-estran-17α-ethynyl-17β-ol-3-one gave patterns of green pigments which appeared similar, following HPLC or TLC, to those formed from norethindrone although with the latter steroid, no component with the same retention time as GP1 could be detected.

With shorter incubation times (5 min) 5α-estran-17α-ethynyl-17β-ol-3-one gave three green pigment components. One of these had the same retention time following HPLC and a similar R_F value on TLC as GP2. The remaining peaks were less polar but none with the characteristics of GP1 were detected at this time.

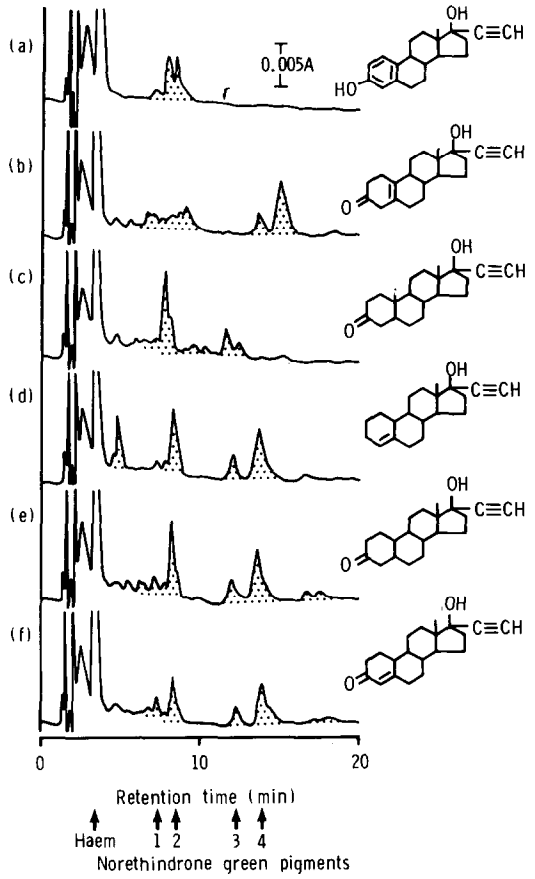

Fig. 6. HPLC traces of green pigments extracted from hepatocyte suspensions prepared from phenobarbitone pretreated rats after 60 min incubation with various steroids (0.03 mM). These were: a, ethynyl oestradiol; b, norethynodrel; c, 5α-androstan-17α-ethynyl-17β-ol-3-one; d, lynestrenol; e, 5α-estran-17α-ethynyl-17β-ol-3-one; f, norethindrone. Ordinates represent relative absorbance at 417 nm. Green pigment peaks are shown shaded.

DISCUSSION

Destruction of cytochrome P-450

The destruction of cytochrome P-450 by norethindrone in control, PB and 3-MC hepatocyte cultures, as in rat liver microsomes [1], is very rapid (Fig. 1). The absence of further loss after this initial decrease was not due to gross toxicity as judged either by trypan blue exclusion or the levels of LDH released, which were small compared with those seen for other cytotoxic compounds [15, 16]. Loss of cytochrome P-450 was greater in hepatocytes from phenobarbitone pretreated rats than in those pretreated with 3-methylcholanthrene or in controls (Fig. 1), confirming earlier studies *in vivo* [2] and with microsomes *in vitro* [2, 6].

Green pigment formation

The present results show hepatocytes to be a better model for events which occur in the livers of rats given norethindrone *in vivo*, than hepatic microsomal systems incubated with this steroid *in vitro*.

It seems likely the first formed product GP1 represents the 1:1 covalent norethindrone–protoporphyrin IX adduct as proposed by Ortiz de Montellano *et al.* [7]. However, the evidence suggests that the more polar products formed *in vivo* or by hepatocytes, but not by microsomes *in vitro*, (GP2, 3 and 4) are not isomers [7] but arise as a result of some form of metabolic modification of the steroid by factors not present in the endoplasmic reticulum. The concentration of norethindrone plays an important role in the formation of the more polar adducts (GP3 and 4). As long as the concentration of parent steroid in the incubation mixture is greater than about 0.03 mM, little or none of these adducts is formed. Not only norethindrone but steroids lacking the ethynyl function prevent the formation of GP3 and 4. Many such steroids are inhibitors of mixed function oxidase enzymes [17, 18]. The mixed function oxidase inhibitor SKF 525-A also inhibits the formation of GP3 and 4.

From the time course of cytochrome P-450 destruction (Fig. 1) and green pigment formation (Fig. 4), it is apparent that the more polar green pigments are formed at a time when destruction of cytochrome P-450 is negligible. This suggests that the conversion of one green pigment form to another may be occurring, i.e. GP1 is converted to GP2 with subsequent conversion to GP3 and GP4 (Fig. 4). Attempts to show interconversion of GP1, 2, 3 and 4 directly were unsuccessful although this may have been due to an inability of the hepatocytes to take up the purified green pigments.

Green pigments formed from other ethynyl-substituted steroids

Results using ethynyl-substituted steroids other than norethindrone showed that the formation of one pigment initially, followed, on longer incubation, by the accumulation of more polar adducts, is a common phenomenon. The only exception to this rule was with ethynyl oestradiol. This result suggests that

further metabolism of the steroid ring A may be associated with the formation of the more polar green pigments. A major pathway of metabolism of norethindrone *in vivo* is reduction of ring A [19]. The ring A-reduced 5α-estran-17α-ethynyl-17β-ol-3-one gave a pattern of green pigments following HPLC similar to that formed from norethindrone itself after 60 min incubation (Fig. 6). Lynestrenol may have given a similar pattern of green pigments to norethindrone since it is metabolised to this compound *in vivo* [19].

Relevance to human situation

The amounts of norethindrone used in these studies (1×10^{-5} M to 3×10^{-4} M) were considerably greater than the plasma levels of norethindrone in women take oral contraceptives (1×10^{-8} to 5×10^{-8} M) [20]. However, norethindrone and its metabolites are concentrated in the liver [21], and it appears from pharmacokinetic data that taking repeated doses of norethindrone results in an accumulation of norethindrone and its metabolites in the plasma [22].

Exposure of rat hepatocytes in culture to norethindrone can lead to an increase in unscheduled DNA synthesis [23]. Understanding the mechanisms whereby this steroid, so routinely used by humans, undergoes metabolic activation therefore seems desirable.

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