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BILIARY EXCRETION OF GREEN PIGMENTS PRODUCED BY NORETHINDRONE IN THE RAT

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Abstract—Bile was a major route for the excretion of green pigments produced in the liver following treatment of rats with the contraceptive steroid norethindrone (100 mg/kg, i.p.). Only very low concentrations were detected in the plasma and none in extracts of urine. No green pigments of this type were detected in control rat bile. Separation of the biliary green pigments following esterification with methanol/H_2SO_4 by HPLC or TLC showed the presence of two major components. These co-chromatographed with two of the major components present in the liver of rats given norethindrone. Biliary green pigments had a similar visible spectrum to those extracted from liver with the Soret absorption maximum in $CHCl_3$ at 417 nm. The time course for the excretion of green pigments in the bile showed maximum concentrations were reached 6 hr after dosing followed by a more gradual decline over the next 18 hr. The total concentration of green pigments excreted into the bile in the 24 hr following dosing of rats with norethindrone was greater than the initial concentration of cytochrome P-450 in the liver. Pretreatment of rats with cycloheximide (1.5 mg/kg) prior to norethindrone reduced the concentration of green pigments in the bile over an 8 hr period by about 40% but caused no accumulation of these compounds in the liver. This suggested protein synthesis may be necessary for the continued formation of green pigments. When rats were dosed with [^{59}Fe]$FeCl_3$ prior to norethindrone, biliary extracts contained ^{59}Fe radioactivity. This was significantly higher than in control rats given only ^{59}Fe. TLC of green pigments from the bile of norethindrone dosed rats, esterified under neutral pH conditions, showed ^{59}Fe radioactivity associated with a poorly defined green component (R_f 0.23) which did not fluoresce red under u.v. light. Results suggested that green pigments in the bile still contained chelated iron. Green pigments excreted into the bile following dosing with norethindrone were found in the faeces, the majority within 48 hr of dosing. The amounts excreted suggested they were largely resistant to attack of enzymes from the gut and gut flora.

The administration of the contraceptive steroid norethindrone to rats causes a time-dependent decrease in the concentration of cytochrome P-450 in the liver. Studies *in vitro* showed that this was brought about by metabolic activation of the ethynyl substituent of the steroid by an enzyme system having many of the characteristics of the NADPH-dependent microsomal mixed function oxidases [1, 2]. Destruction of the haem moiety of cytochrome P-450 by norethindrone and other ethynyl-substituted compounds is not accompanied by an increased bilirubin formation but by the appearance of abnormal green coloured pigments in the liver [1–3]. Such green pigments represent a 1:1 covalent adduct between the steroid and the protoporphyrin 1X ring of haem [4], the site of alkylation being one of the tetrapyrrole nitrogen atoms [5, 6]. Studies using ^{59}Fe suggest that green pigments within the liver retain their iron, although this iron is readily removed from the adduct under acidic workup conditions [7].

The biochemical effects of green pigments on the liver and other organs are not known. Related green porphyrins containing an *N*-methyl substituent on the protoporphyrin IX ring, such as are formed following the administration of 3,5-diethoxycarbonyl-1,4-dihydrocollidine or griseofulvin, are potent inhibitors of protohaem ferrolyase [8]. However higher molecular weight substituents appear to be less effective inhibitors of this enzyme [8, 9].

Following the administration of norethindrone to rats, there is an accumulation of green pigments in the liver reaching a maximum 4 to 8 hr after dosing but declining to low concentrations by 24 hr [7]. Studies by Back *et al.* [10] showed that bile is a major route for the excretion of norethindrone in rats, 76% of the dose being eliminated by this route within 4 hr of dosing. The nature and the routes of elimination of green pigments from the liver are unknown. It is this latter aspect which has now been examined and the results presented in this paper.

MATERIALS AND METHODS

Chemicals. Norethindrone acetate was from the Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). Cycloheximide was from Aldrich Chemical Co. Ltd. (Gillingham, Dorset, U.K.). [^{59}Fe]$FeCl_3$ (sp. act. 7.4 mCi/mg iron) was obtained from the Radiochemical Centre (Amersham, Bucks, U.K.). The bilirubin assay kits were from the Boehringer Chemical Co. Ltd. (Lewes, Sussex, U.K.; Cat. No. 123927).

Animals and bile duct cannulation. Male Fischer F.344 rats (190–210 g) were used. These were anaesthetised with diethyl ether and the common bile duct cannulated using a 40 cm length of PE10 polythene catheter. Following a 30 min recovery period, the rats were placed in individual restraining cages and the bile was collected in the dark in polythene tubes packed in solid CO_2. Fractions were

collected at 2 hr intervals for 24 hr using a modified fraction collector. During the experiment, the core body temperature of the rats was monitored with a thermocouple inserted 60 to 80 mm up the anus and was maintained at 37 ± 0.5° using a heating lamp. Animals had free access to food and to water.

Dosing procedures. Norethindrone acetate, dissolved in trioctanoin was administered i.p. (100 mg/kg) 45 min after the completion of the surgical procedures. Control animals received trioctanoin only. In some instances, cycloheximide dissolved in 0.14 M saline was administered at a dose of 1.5 mg/kg i.p. 15 min prior to norethindrone. [^{59}Fe]$FeCl_3$ was given at a dose of 50 μCi/kg i.p. 17 hr before norethindrone.

Extraction and separation of green pigments. (a) *Bile.* Portions of bile (0.3 ml) were mixed with 10 ml ice-cold 5% (v/v) H_2SO_4 in methanol. After 18 hr at 4° in the dark, the esterified products were centrifuged (3000 *g* for 5 min at 4°). The supernatant was decanted and the precipitate washed with 5 ml of methanol and recentrifuged. The combined supernatants were extracted into $CHCl_3$ which was washed, dried and concentrated to dryness under N_2 as described previously [1]. The residue was made up in $CHCl_3$ (0.3 ml) and subjected either to TLC or HPLC as described below.

(b) *Plasma.* Rats were anaesthetised with diethyl ether and blood was collected from the descending dorsal aorta into plastic heparinised tubes by means of a PE-50 polythene cannula. Following centrifugation (3000 *g* for 15 min at 4°), the plasma was removed and 2 ml was added to 40 ml ice-cold 5% H_2SO_4 in methanol. This was then treated in the same way as described above.

(c) *Liver and faeces.* The livers of rats killed by decapitation were perfused *in situ* with 0.14 M NaCl through the hepatic portal vein. Portions of liver (1 g) were homogenised in 20 ml of ice-cold 5% H_2SO_4 in methanol using an Ultraturrax homogeniser and treated as described for bile. Faecal samples were collected at 24-hr intervals in containers packed in solid CO_2 from rats in individual metabolism cages. These were weighed and homogenised (1 g in 20 ml ice-cold 5% H_2SO_4 in methanol) and processed as described above.

Esterification of bile or faecal extracts with trimethyloxonium tetrafluoroborate gel permeation chromatography. Faeces (1 g) were homogenised in 10 ml 1.15% KCl using an Ultraturrax homogeniser. The faecal suspension or bile (10 ml) was extracted with an equal volume of toluene and the upper toluene phase discarded. The pH value of the aqueous phase was adjusted to 2.0 with 4 M HCl and was extracted with an equal volume of butanone at 0°. Centrifugation (3000 *g* for 5 min at 4°) was used to separate the aqueous and butanone phases. Two further extractions with butanone were carried out and the combined butanone phases esterified with trimethyloxonium tetrafluoroborate at a nominal pH value of 7.4 as described previously [7]. The esterified extracts were subjected to silica gel TLC using a $CHCl_3$–methanol (90:10 v/v) solvent system. Where rats had been dosed with [^{59}Fe]$FeCl_3$, 0.5 cm bands of silica were scraped off the plates and assayed for ^{59}Fe radioactivity using a Packard Model 5330 autogamma counter. In some instances the bands were scraped off and eluted with $CHCl_3$–methanol (1:1 v/v). Gel permeation chromatography was carried out using a 60 × 0.7 cm poly(styrene-divinylbenzene) column (PL gel 5 μM 100 A, Polymer Labs Ltd., Salop, U.K.). $CHCl_3$–methanol (95:5 v/v) was used as the solvent at a flow rate of 1 ml min^{-1}. Absorbance was determined at 412 nm.

TLC of green pigments. Extracts of liver, bile or faeces esterified with methanol/H_2SO_4, were chromatographed on silica gel TLC plates layer thickness 0.25 mm (E. Merck, Darmstadt, W. Germany) using a $CHCl_3$–kerosene–methanol (65:15:9 v/v) solvent system.

HPLC of green pigments. Green pigments were separated on a 25 × 0.47 cm silica gel column (Machery Nagel Nucleosil 50:5, Camlab Ltd., Cambridge, U.K.) using an isocratic solvent system of cyclohexane–chloroform–methanol (3:2:1 v/v) containing 0.2% (v/v) acetic acid. Solvent flow using a Waters pump (6000A) was 1.5 ml min^{-1}. Absorbances were measured at 417 nm and peak areas were determined using a Pye-Unicam DP88 integrator. Samples (20 μl) were injected with a Wisp (Waters Assoc. Ltd.) autoinjector. Riboflavin (retention time 13.8 min) was used as an internal standard. Very different retention times were obtained for unknown reasons using silica gels from other manufacturers. Green pigment concentrations were estimated using an extinction coefficient of 106 litre mmole^{-1} cm^{-1} [7].

Estimation of cytochrome P-450. The livers of rats killed by decapitation were perfused *in situ* with 0.14 M NaCl through the hepatic portal vein to remove haemoglobin. 10% (w/v) homogenates were prepared in ice-cold 0.25 M sucrose. Cytochrome P-450 was measured by suspending 1 ml of homogenate in 5 ml 0.1 M phosphate buffer, pH 7.4 and recording the CO-reduced less the CO-oxidised difference spectrum [11]. An extinction coefficient of 91 litre mmol^{-1}cm^{-1} was used [12].

Bilirubin determinations. Bilirubin was estimated by the diazotized sulphanilic acid procedure using a Boehringer kit and the procedures described therein. Absorbances were measured at 578 nm in a Pye-Unicam SP-500 spectrophotometer.

RESULTS

Bile and bilirubin excretion. Figure 1 shows the volumes of bile excreted by the rats in each 2-hr fraction slowly decreased over the 24-hr period. Bile volumes were consistently lower in norethindrone dosed animals than in controls, confirming earlier observations on the cholestatic effects of this steroid [13]. A similar picture was seen with respect to bilirubin excretion where the concentration of bilirubin in the bile of the norethindrone dosed rats was lower than in the controls (Fig. 2). Pretreatment of rats with cycloheximide (1.5 mg/kg) prior to norethindrone did not significantly reduce either bile flow or bilirubin production, relative to animals given only norethindrone, at least during the first 8 hr after dosing. Significant reductions in both these parameters were observed however at times greater than 8 hr or at earlier times with larger doses

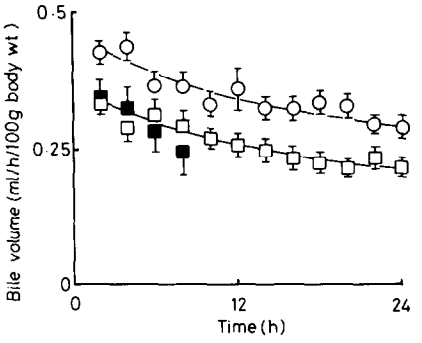

Fig. 1. Volumes of bile excreted in control and norethindrone dosed rats. Results are expressed as volume of bile excreted/hr obtained from fractions collected at 2-hr intervals. ○, controls; □, rats given 100 mg/kg norethindrone intraperitoneally; ■, cycloheximide (1.5 mg/kg i.p.) administered 15 min prior to dosing with norethindrone (100 mg/kg i.p.). Results represent the mean ± S.E.M. of 4 experiments.

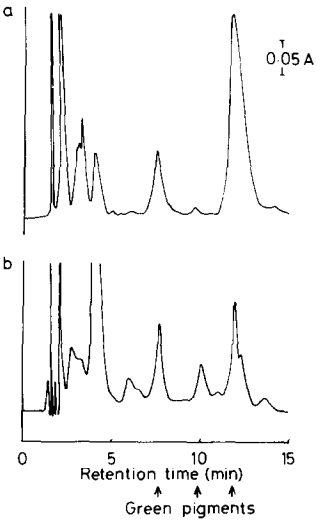

Fig. 3. Green pigments separated by HPLC from extracts of liver and bile. Samples of liver or of bile were esterified with methanol–H_2SO_4 as described in Materials and Methods. Following extraction into $CHCl_3$ and concentration, 20 μl were injected onto a silica gel column. Ordinates represent absorbance recorded at 417 nm. (a) Bile. (b) Liver.

(5 mg/kg) of cycloheximide (I. N. H. White, unpublished results).

Comparison between green pigments in bile and in liver. Extracts of bile from norethindrone dosed rats, esterified with methanol–H_2SO_4 showed two major green pigments components following HPLC (retention times 7.8 and 11.6 min, Fig. 3a). These were not detectable in extracts from the bile of control rats. These two pigments had the same retention times and co-chromatographed with two of the three major liver green pigments (Fig. 3b). The third major green pigment in the liver (retention time 10.4 min) appeared to be present only in trace amounts in the bile extracts.

Silica gel TLC of bile extracts, esterified with methanol–H_2SO_4, also showed two major green coloured bands (R_f 0.45 and 0.37). These had similar R_f values to two of the major liver pigments. When prepared in this way, both the bile green pigment bands fluoresced red under u.v. light.

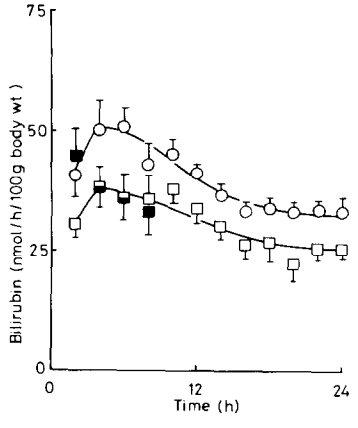

Fig. 2. Excretion of bilirubin in the bile of control and norethindrone dosed rats. Animals were given norethindrone (100 mg/kg i.p.) and bile was collected at 2-hr intervals. ○, controls; □, norethindrone dosed; ■, cycloheximide (1.5 mg/kg, i.p.) given 15 min prior to norethindrone. Results represent the mean ± S.E.M. of 4 experiments.

Absorption spectra in $CHCl_3$ of the bile green pigments esterified with methanol–H_2SO_4 and purified by TLC and HPLC showed both to have a similar aetio-type spectrum, Soret λ max 417 nm with minor peaks 512, 546, 590 and 646 nm. This spectrum closely resembled that previously reported for the liver green pigments [4, 7].

On the basis of similarities in their chromatographic behaviour on the TLC and HPLC (R_f values and retention times) and their fluorescence and absorption spectra, it was assumed that the identity of the two major green pigments extracted from the bile was the same as two of the major green pigments extracted from the liver of norethindrone dosed rats. Although the precise chemical identity of these green pigments has not been determined, evidence of Ortiz de Montellano *et al.* [3, 4] has shown that green pigments are formed as a result of covalent binding of the steroid to the protoporphyrin IX ring of haem in a 1:1 molar ratio. Assuming such a 1:1 adduct is formed, the use of radioactively labelled norethindrone of known specific activity has enabled an estimate to be made of the molar absorption coefficient of the purified green pigments [7]. This value (106,000 litre $mole^{-1}cm^{-1}$ at 417 nm in $CHCl_3$) was used as the basis for the estimation of the concentrations of green pigments in the bile.

Time course for the excretion of green pigments into bile. Figure 4 shows the effects of time on green pigment concentrations in the bile of rats given norethindrone. Concentrations rose rapidly, reaching a maximum 6 hr after dosing followed by a slower decline over the next 18 hr. The total (cumulative) concentration of green pigments excreted from the liver in 24 hr in the bile was 277 nmoles/100 g body

Table 1. Effects of cycloheximide on the norethindrone mediated destruction of hepatic cytochrome P-450 and the accumulation of green pigments determined 8 hr after dosing in bile duct cannulated rats

Treatment*	Cytochrome P-450 (nmoles/liver/100 g body wt†)	Green pigments (nmoles/liver/ 100 g, body wt†)
Controls	202 ± 7	0
Cycloheximide	168 ± 3	0
Norethindrone	116 ± 7	69 ± 1
Cycloheximide + norethindrone	107 ± 3	49 ± 8

* Cycloheximide dissolved in 0.14 M NaCl was administered at a dose of 1.5 mg/kg i.p. Norethindrone acetate dissolved in trioctanoin was given at a dose of 100 mg/kg i.p. Controls received trioctanoin vehicle only. When administered together cycloheximide was given 15 min prior to norethindrone.

† Results represent the mean ± S.E.M. of 4 experiments.

wt. This was greater than the total concentration of hepatic cytochrome P-450, estimated from liver homogenates (202 nmoles/liver/100 g body wt, Table 1).

The role of iron in biliary green pigments. When iron in the liver of rats was prelabelled with ^{59}Fe by the administration of [^{59}Fe]$FeCl_3$, radioactive label was released into the bile both in control and norethindrone dosed rats (Fig. 5). The amount released in the 24 hr following norethindrone treatment (39,316 ± 5495 cpm, mean ± S.E., 4 experiments) was considerably greater than that in the controls (15,632 ± 2384 cpm, $P < 0.01$). Maximum levels of radioactivity in the norethindrone rats were reached 6 hr after dosing and generally mirrored the time course of green pigment excretion into the bile.

When bile extracts from [^{59}Fe]$FeCl_3$ pretreated rats were esterified under acidic conditions with methanol–H_2SO_4, following TLC, no ^{59}Fe radioactivity remained associated with the green pigments. If esterifications were carried out under neutral conditions with trimethyloxonium tetrafluoroborate, bile extracts from control rats showed a single band of ^{59}Fe radioactivity (R_f 0.62, Fig. 6a), while those dosed with norethindrone showed an additional slower migrating component (Fig. 6b), which appeared to be associated with a green coloured band. ^{59}Fe radioactivity remained associated with the green pigment (retention time 12.7 min), following gel permeation chromatography (Fig. 7). The absorption spectrum of the green pigment in $CHCl_3$ purified in this way showed only a Soret band at 412 nm, similar to that previously reported for green pigments isolated from liver by these procedures [7]. The faster migrating radioactive component (R_f 0.62) seen in both control and norethindrone dosed bile extracts was associated with a brown coloured band. This co-chromatographed with and had an identical visible spectrum to that of dimethylhaem. It is not known if the haem in the bile originated from the liver or as a result of the surgical procedures. Its presence was unexpected

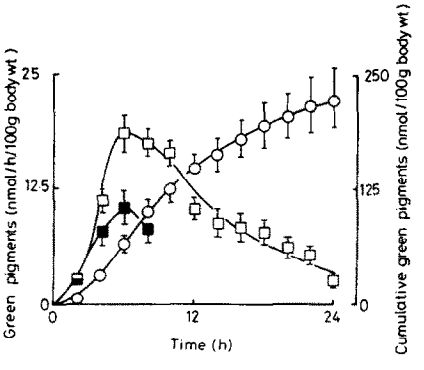

Fig. 4. Effects of time on the excretion of green pigments into bile following dosing with norethindrone. Rats were given norethindrone acetate 100 mg/kg, i.p. and bile was collected at 2-hr intervals. Samples of bile were esterified with methanol–H_2SO_4, extracted into $CHCl_3$ and the concentration of green pigments estimated by HPLC. Results represent the mean ± S.E.M. of 4 experiments. □, concentration of green pigments in each bile fraction. ■, concentration of green pigments in each bile fraction from rats that had been given cycloheximide (1.5 mg/kg i.p.) 15 min prior to norethindrone. ○, cumulative concentration of biliary green pigments.

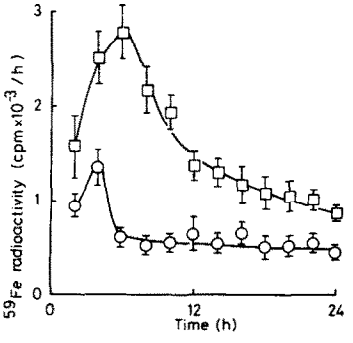

Fig. 5. Excretion of ^{59}Fe radioactivity in the bile of norethindrone dosed and control rats. Animals were given 10 μCi [^{59}Fe]$FeCl_3$ 17 hr before dosing with norethindrone acetate (100 mg/kg, i.p.) or trioctanoin vehicle (controls). Bile was collected at 2-hr intervals and the radioactivity determined. Results represent the mean ± S.E.M. of four experiments. □, norethindrone dosed; ○, controls.

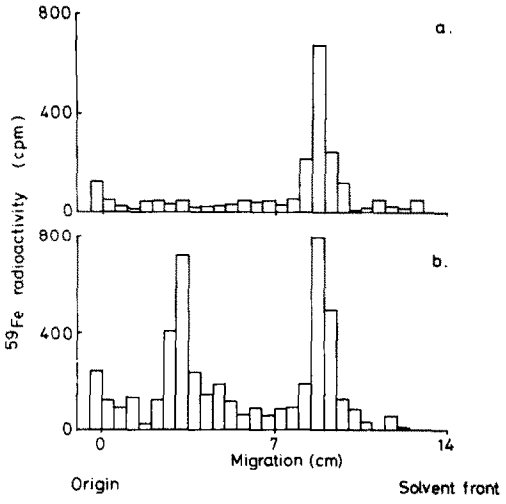

Fig. 6. ^{59}Fe radioactivity profiles of extracts of rat bile esterified with trimethyloxonium tetrafluoroborate subjected to silica gel TLC. Animals were dosed with 10 μCi $^{59}FeCl_3$ 17 hr prior to bile duct cannulation and the administration of norethindrone acetate (100 mg/kg, i.p.) or trioctanoin vehicle (controls). Bile (8-hr sample) was extracted, esterified and the esterified extracts subjected to silica gel TLC using a $CHCl_3$–methanol (90:10) solvent system. Strips (0.5 cm) were scraped off the plate and assayed for ^{59}Fe radioactivity. (a) Controls. (b) Norethindrone dosed.

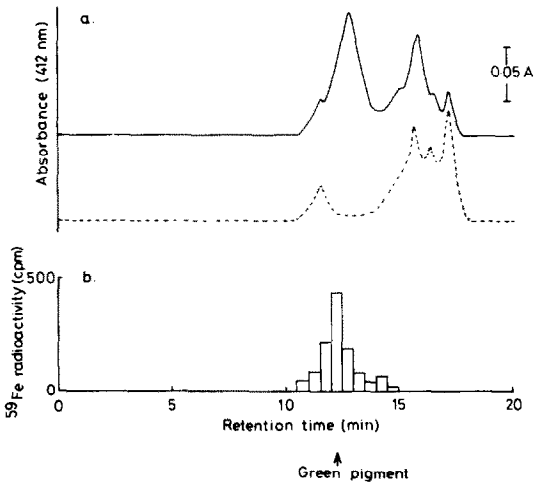

Fig. 7. Gel permeation chromatography of green pigments purified by TLC. Experimental conditions were the same as described in Fig. 6. Following TLC the 0.5 cm band, R_f 0.23, was scraped off the plate and eluted with $CHCl_3$–methanol (1:1). The concentrated extract was subjected to gel permeation chromatography using a 60 × 0.7 cm column and a $CHCl_3$–methanol (95:5) solvent system at a flow rate of 1 ml min^{-1}. (a) Absorbances measured at 412 nm. —— Norethindrone dosed, - - - controls. (b) ^{59}Fe radioactivity in 0.5 ml fractions collected from the detector output; norethindrone dosed rats. In extracts from control rats, radioactivity was not significantly greater than the background levels.

since it has previously been reported that haem cannot be detected in normal rat bile [14].

Faecal excretion of green pigments. The results presented in Table 2 show that administration of norethindrone to normal (non-cannulated) rats leads to the elimination of green pigments in the faeces, the majority within 48 hr of dosing. The retention times following HPLC and spectral properties of the faecal green pigments esterified with methanol–H_2SO_4 appeared to be the same as those isolated from the bile. Esterification of faecal extracts with trimethyloxonium tetrafluoroborate followed by TLC showed there to be a mixture of green pigments of low R_f (0.23) which did not fluoresce under u.v. light and of higher R_f (0.45 and 0.37) which fluoresced red. These may represent iron containing and iron free green pigments respectively. The relative proportions of the two species was not quantitated.

Green pigments in plasma. Extracts of plasma taken from norethindrone dosed rats when subjected to HPLC, showed the presence of green pigments. Their concentrations were very much lower than in bile or in liver (Fig. 8). The time course for the accumulation of these components in plasma was similar to that seen in liver [7] reaching a maximum 4–6 hr after dosing. Because of the high concentration of haemoglobin in erythrocytes, the green pigment concentration in whole blood could not be examined by the present procedures.

No green pigments could be detected in the urine of rats given norethindrone in samples examined by TLC and HPLC up to 72 hr after dosing.

Table 2. Faecal excretion of green pigments in norethindrone dosed rats

Time after dosing (hr)	Green pigments excreted* (nmoles)
0–24	100 ± 16
24–48	133 ± 33
48–72	1.8 ± 0.7

* Results represent the mean ± S.E.M. of four experiments.

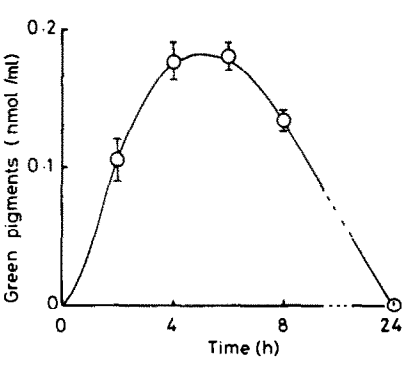

Fig. 8. Effects of time on the concentration of green pigments in plasma following the administration of norethindrone. At various times after dosing with norethindrone acetate (100 mg/kg, i.p.), blood was collected from the dorsal aorta and green pigment concentrations estimated in samples of plasma following esterification with methanol–H_2SO_4 and HPLC. Each time point represents the mean ± S.E.M. from 4 rats.

DISCUSSION

The results demonstrate that bile is a major route for the excretion of green pigments produced in the liver following treatment of rats with norethindrone. Only very low levels were detected in plasma and none at all in extracts of urine.

Chromatographic properties of green pigments. From their behaviour on the TLC and HPLC and from their absorption spectra, the green pigments excreted into the bile appear to be the same as two of the major components found in the liver after the administration of norethindrone. Although green pigments are formed as a result of alkylation of the protoporphyrin IX ring of haem [4], the chemical identity of the different chromatographic forms is not known. It has been suggested that initially one green pigment may be formed and this subsequently undergoes metabolic transformation into the three major components separable by HPLC [7]. Alternatively it has been suggested these components represent different isomeric forms [3, 6, 15].

Concentration of green pigments in the bile. Assuming that the biliary green pigments are the same as those in the liver and that they have the same extinction coefficient, then, somewhat unexpectedly, their total concentration in the bile excreted in the 24 hr following the administration of norethindrone (277 nmoles/100 g body wt) was more than the concentration of cytochrome P-450 in the liver (202 nmoles/liver/100 g body wt). In addition, previous studies both *in vivo* and *in vitro* indicated that only 30–40% of the total hepatic cytochrome P-450 was susceptible to destruction by the active metabolites of norethindrone [1–3]. As the liver is the only site of formation of green pigments, at least detectable using the present analytical techniques (I. N. H. White, unpublished observations), the results suggest that either synthesis of new cytochrome P-450 occurs following norethindrone dosing or that non-cytochrome haem such as that from the hepatic 'free haem pool' can combine with apocytochrome P-450 to give a functionally active holoenzyme. Experiments with the protein synthesis inhibitor cycloheximide suggest the first of these alternatives may be the more important mechanism.

The dose of cycloheximide used (1.5 mg/kg) inhibits 93% of hepatic protein synthesis [16]. Although reports differ on the overall inhibitory effect that cycloheximide has on bile flow [16, 17], in the present experiments when this compound was administered 15 min prior to dosing with norethindrone, it did not depress bile flow or bilirubin concentrations beyond that caused by norethindrone alone [13] (Figs. 1 and 2). The cycloheximide was sufficient however to cause a 43% reduction in the total concentration of green pigments excreted into the bile over 8 hr (Fig. 3). Cycloheximide did not appear to affect the total metabolic activation of norethindrone since both the concentration of green pigments excreted into the bile in the first 2 hr and the destruction of cytochrome P-450 measured 8 hr after dosing were similar in the norethindrone and the cycloheximide + norethindrone dosed rats (Table 1).

The role of iron in biliary green pigments. Green pigments formed in the liver following the administration of norethindrone, like haem, contain iron. Unlike haem which is broken down to bilirubin and loses its iron before being excreted into the bile, the evidence suggests that the majority of the green pigments are excreted unchanged into the bile still retaining their iron. During the passage through the gut, some of the iron is lost but the green pigments themselves appear remarkably resistant to degradation by enzymes from the intestine or gut flora.

Green pigments in the plasma. It has been suggested that the major portion of the normal hepatic haem breakdown product bilirubin effluxes into the plasma before its excretion into the bile [18]. Green pigments were certainly present in the plasma of rats given norethindrone although it is not known if this is part of the biliary excretion pathway. Bilirubin is largely conjugated before excretion into the bile [19, 20]. The present evidence from field desorption mass spectrometry gives no indication for the present in the liver of any green pigment conjugates following dosing of rats with a wide variety of ethynyl-substituted compounds [3]. The very low concentrations of green pigments in plasma suggest that very much more sensitive assay techniques will be required before concentrations can be monitored in women taking these types of contraceptive steroids.

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