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Destruction of liver haem by norethindrone

Conversion into green pigments

Ian N. H. WHITE
Toxicology Unit, M.R.C. Laboratories, Woodmansterne Road, Carshalton, Surrey, U.K.

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1. Factors affecting the norethindrone-mediated conversion of hepatic haem into green pigments have been studied in the rat. Concentrations of haem and green pigments were estimated spectrophotometrically after esterification and separation by silica gel high-pressure liquid chromatography (h.p.l.c.). 2. Accumulation of green pigments in the liver was dependent on the dose of steroid and the time after dosing, maximum values being reached after 4–8h. Phenobarbitone pretreatment of rats resulted in an 8-fold increase in the concentration of green pigments at these times. 3. In microsomal systems *in vitro*, the formation of green pigments in the presence of NADPH and norethindrone was also dependent on the concentration of steroid and incubation times. Reaction rates very rapidly became non-linear with time, consistent with the self-catalysed destruction of the form(s) of cytochrome *P*-450 responsible for the metabolic activation of norethindrone. Microsomal mixtures incubated for a short period of time (1 min) with norethindrone gave only one green-pigment peak after h.p.l.c. Longer incubation times gave four or five additional green pigments. Results suggested that multiple green pigments may arise by metabolic transformation of a single precursor. 4. When liver haem was prelabelled with ^{14}C by using 5-amino[4-^{14}C]laevulinic acid, subsequent dosing with norethindrone *in vivo* gave rise to three major ^{14}C-labelled-green-pigment peaks on h.p.l.c. None of these components had the same retention times as the green pigments produced by microsomal fractions *in vitro*. 5. When liver haem was prelabelled with ^{59}Fe by using $^{59}FeCl_3$, norethindrone administration resulted in the detection of ^{59}Fe-labelled green pigments if subsequent esterification was carried out under neutral conditions with trimethyloxonium tetrafluoroborate, but not when carried out under acidic conditions with methanol/H_2SO_4. These results suggested that green pigments normally contain chelated iron and that metal-free green pigments are not produced by the liver.

The contraceptive steroid norethindrone (17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one) causes the destruction of the haem moiety of hepatic cytochrome *P*-450 in rats (White & Muller-Eberhard, 1977). This is brought about by the metabolic activation of the 17α-ethynyl substituent of the steroid by NADPH-dependent microsomal mixed-function oxidases. Destruction of cytochrome *P*-450 in this way is not confined to norethindrone but occurs with a number of other ethynyl-substituted compounds (White, 1978, 1980; Ortiz de Montellano & Kunze, 1980*a*). Haem that is destroyed is converted into abnormal green pigments as a result of a 1:1 covalent interaction between the steroid and the porphyrin ring of haem (Ortiz de Montellano *et al.*, 1979). Field-desorption mass-spectrometric studies of green pigments extracted from the livers of rats dosed with ethynyl-substituted compounds have a molecular ion corresponding to the molecular weights of protoporphyrin IX plus the ethynyl substrate and oxygen (Ortiz de Montellano & Kunze, 1980*a*). The precise chemical structure of these green pigments is not known. However, De Matteis & Cantoni (1979) and De Matteis *et al.* (1980) showed them to have characteristics typical of *N*-alkylated porphyrins. Similar destruction of hepatic haem and the formation of green pigments occurs in rats after the administration of 2-allyl-2-isopropylacetamide, first reported by De Matteis

Abbreviation used: h.p.l.c., high-pressure liquid chromatography.

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(1971). Although much progress has been made on the chemical identity of green pigments, only in the pioneering studies of De Matteis (1971) and more recently by Bonkowsky *et al.* (1980), both using 2-allyl-2-isopropylacetamide, has any progress been made in the study of factors affecting the turnover of these compounds in the liver.

A new procedure is now described, based on h.p.l.c., which permits the simultaneous resolution and estimation of esterfied haem and green pigments in extracts of liver and in microsomal preparations *in vitro*. Norethindrone is used as a model ethynyl-substituted compound to study factors affecting the destruction of haem and the accumulation of green pigments in the liver. The results are reported in the present paper.

Experimental

Chemicals

[6,7-^{3}H]Norethindrone (>99% pure, sp. radioactivity 55 Ci/mmol) was from New England Nuclear, Winchester, Hants., U.K. 5-Amino[4-^{14}C]-laevulinic acid (sp. radioactivity 25 Ci/mol) and $^{59}FeCl_3$ (sp. radioactivity 7.4 mCi/mg of Fe) were from The Radiochemical Centre, Amersham, Bucks., U.K. [^{14}C]Haem (sp. radioactivity 0.36 Ci/mol, >98% radiochemical purity) was prepared from hen erythrocytes incubated with 5-amino-[4-^{14}C]laevulinic acid as described by Unseld (1976). Trimethyloxonium tetrafluoroborate was prepared by the method of Curphey (1971).

Animals and dosing

Male rats (130–150 g) were of the Fischer F.344 strain. Norethindrone was administered intraperitoneally dissolved in trioctanoylglycerol (trioctanoin) vehicle usually at a dose of 100 mg/kg body wt. Pretreatment of rats with phenobarbitone and the preparation of liver microsomal fractions were carried out as described previously (White, 1978). Aroclor 1254 (Monsanto Co., St. Louis, MO, U.S.A.) dissolved in trioctanoin was given as a intraperitoneal single dose of 400 mg/kg body wt. and the rats were killed 5 days after dosing.

To prelabel liver haem with ^{14}C, phenobarbitone-pretreated rats were given 10 μCi of 5-amino-[4-^{14}C]laevulinic acid intraperitoneally and killed 2 h after dosing. Liver haem labelled with ^{59}Fe was prepared by injecting phenobarbitone-pretreated rats with 25 μCi of $^{59}FeCl_3$ intraperitoneally, 18 h before use.

Extraction and esterification of liver haem and green pigment

Livers of rats killed by decapitation were thoroughly perfused *in situ* with 0.14 M-NaCl through the hepatic portal vein. All chromatographic procedures were carried out on esterified haem and green pigments. Formation of methyl esters was carried out under either (*a*) acidic (methanol/H_2SO_4) or (*b*) neutral (trimethyloxonium tetrafluoroborate) conditions.

(*a*) *Methanol/H_2SO_4* procedure. Portions of liver (1 g) were homogenized in 20 ml of ice-cold 5% (v/v) H_2SO_4 in methanol. After 18 h at 4°C in the dark, the esterified products were extracted into chloroform, concentrated and subjected either to t.l.c. as described previously (White & Muller-Eberhard, 1977) or to h.p.l.c. as described below.

(*b*) *Esterification with trimethyloxonium tetrafluoroborate.* A 10% (w/v) liver homogenate was prepared in ice-cold 1.15% (w/v) KCl. This was adjusted to pH 2.0 with 4 M-HCl and extracted three times with equal volumes of butanone at 0°C as described by Teale (1959). Esterification was carried out essentially as described by Dean *et al.* (1976). To the combined butanone phases was added $\frac{1}{3}$ vol. of ethanol followed by the dropwise addition of trimethyloxonium tetrafluoroborate (1 g) in acetonitrile. The pH of the reaction mixture was kept at a nominal value of 7.4 (glass electrode) with diisopropylethylamine. The mixture was washed three times with equal volumes of 1 M-NaCl, dried (over anhydrous Na_2SO_4) and rotary-evaporated to dryness at 30°C.

Formation of green pigments by microsomal systems in vitro

Reaction mixtures of 2 ml volume were as described by White (1978). Norethindrone (0.5 mM) was added in a solution of 20 μl of propylene glycol. Reactions were terminated after 1 min incubation at 37°C by the addition of 40 ml of ice-cold 5% (v/v) H_2SO_4 in methanol. The mixtures were allowed to stand for 18 h at 4°C in the dark, then centrifuged and the supernatants were extracted into chloroform as described by White & Muller-Eberhard (1977). In some instances, the concentration of norethindrone or the times of incubation were changed. Control incubation mixtures contained no steroid.

Gel-permeation column chromatography

Green pigments were separated from haem by chromatography on a column (45 cm × 1.5 cm) of Biobeads SX-2 (Bio-Rad Laboratories, Watford, Herts., U.K.) equilibrated with dichloromethane/methanol (9:1, v/v). The flow rate was 1 ml/min. Fractions (1 ml) were collected and assayed for radioactivity or A_{417}.

H.p.l.c.

An isocratic solvent system of cyclohexane/chloroform/methanol (3:2:1, by vol.) containing 0.2% (v/v) acetic acid was used. Solvent flow

(Waters 6000A pump) was 1.5 ml/min. Absorbance was measured at 417 nm. Peak areas were computed with a Pye–Unicam DP88 integrator. The column (25 cm × 0.4 cm) was packed with 5 μm silica gel (Machery Nagel)/Nucleosil, 10:1, Camlab, Cambridge, U.K.). Retention times of haem and green pigments were found to be very dependent on the brand of silica gel employed. Sample size was generally 20 μl. Riboflavin (retention time 13.8 min) was used as an internal standard.

Purification of green pigments

Green pigments, esterified with methanol/H_2SO_4, were purified by Biobeads gel-permeation chromatography followed by h.p.l.c. The individual peaks were collected, concentrated under N_2 at 30°C and demetallated as described by Ortiz de Montellano & Kunze (1980*a*). The components were rechromatographed by h.p.l.c. to ensure their homogeneity and their absorption spectra and radioactivity were determined. Zinc chelates of the green pigments were prepared by using the procedure of Ortiz de Montellano & Kunze (1980*a*).

Results

Separation of haem and green pigments by h.p.l.c.

H.p.l.c. of chloroform extracts of control rat liver showed dimethylhaem as a major component, retention time 4.2 min (Fig. 1*a*). In rats dosed wtih norethindrone, three additional components, retention times 7.8, 10.4 and 11.6 min, were observed (Fig. 1*b*). The green pigments separated by h.p.l.c. corresponded on rechromatography to the three major green bands seen after silica gel t.l.c. of such extracts (R_F values 0.45, 0.39 and 0.37 respectively). These green pigments were present in extracts from both control and phenobarbitone-pretreated rats given norethindrone; in the former two additional minor green components, retention times 7.4 and 12.4 min, were also observed. When haem of rat liver was prelabelled with ^{14}C by giving 5-amino[4-^{14}C]laevulinic acid before the administration of norethindrone, radioactive label appeared associated with all the green-pigment peaks (Fig. 1*c*). These radioactive peaks were not seen in liver extracts from control rats not given norethindrone. Recoveries of radioactivity from the h.p.l.c. column were 92.8 ± 1.1% (mean ± S.E.M., for four determinations). Recoveries of authentic [^{14}C]haem or purified ^{14}C-labelled esterified green pigments added to unlabelled rat liver before homogenization in methanol/H_2SO_4 were 87.3 ± 0.9% and 93.5 ± 1.2% (mean ± S.E.M. for four determinations) respectively.

Assuming covalent binding of norethindrone to the porphyrin ring of haem in a 1:1 molar ratio (Ortiz de Montellano *et al.*, 1979) and the absence of isotopic effects, administration of [^3H]norethindrone of known specific radioactivity to rats followed by extraction and purification of the esterified green pigments gave a product with an absorption coefficient in chloroform of 106 000 litre · mol^{-1} · cm^{-1} at 417 nm. This value

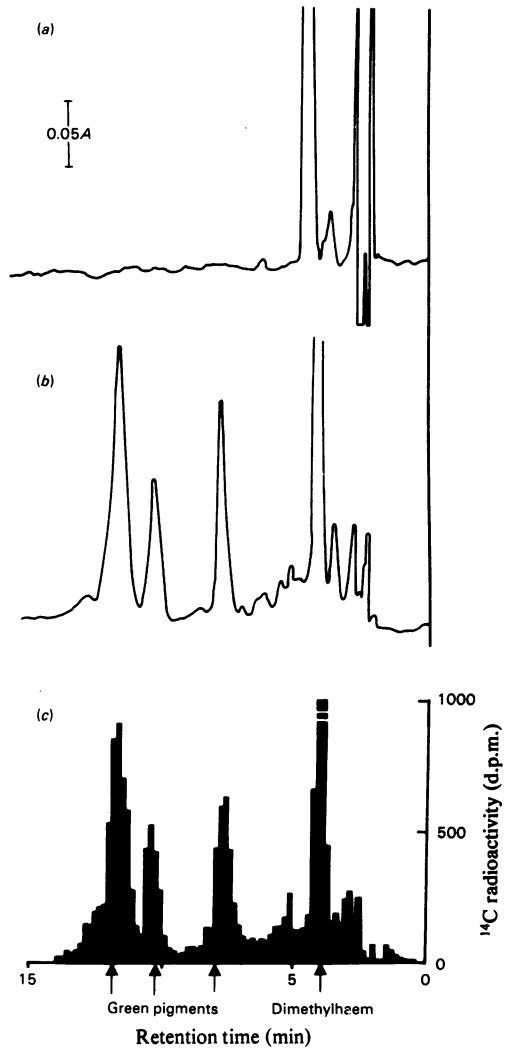

Fig. 1. *Separation of esterified haem and green pigments by h.p.l.c.*

Phenobarbitone-pretreated rats, with liver haem prelabelled with ^{14}C as described in the Experimental section, were dosed either with trioctanoin vehicle (*a*) or 100 mg of norethindrone acetate/kg body wt. (*b*) and killed 8 h later. Livers were homogenized in methanol/H_2SO_4 and the concentrated chloroform extracts subjected to h.p.l.c. The ordinates in (*a*) and (*b*) represent relative aborbance at 417 nm. (*c*) represents ^{14}C radioactivity in 0.3 ml fractions collected at the detector output from (*b*).

was used to calculate the concentration of green pigments formed by the liver. There appeared to be no difference between the absorption coefficients of the three major green pigments.

Formation of green pigments in vivo

Fig. 2 shows that in phenobarbitone-pretreated rats, norethindrone (100mg/kg body wt. injected intraperitoneally) caused a rapid marked decrease in the concentration of haem in the liver with time and the appearance of green pigments, the latter reaching a maximum concentration 4–8h after dosing. Similar though less marked effects were seen in control rats dosed with norethindrone. In neither group was there a significant change in the liver weight expressed as a percentage of body weight in the 24h after dosing. The peak concentration of green pigments was about 7-fold greater in phenobarbitone-pretreated rats than in control animals. The accumulation of green pigments was also dependent on the dose of norethindrone administered (Fig. 3). The ratio of the concentrations of the different green-pigment components did not remain constant and was dependent on both the dose of norethindrone and the time animals were killed after dosing. However, the component, retention time 11.6min, was always present in the highest concentration. Green pigments were also found in the duodenal contents of rats dosed with norethindrone, suggesting biliary excretion may be important in the elimination of these compounds from the liver (I. N. H. White, unpublished work).

The role of iron in green pigments: esterification under neutral conditions

Green pigments could also be separated from haem by Biobeads gel-permeation chromatography. The green pigments were eluted first as a single component followed by haem. Similar elution patterns were obtained, whether esterification of liver

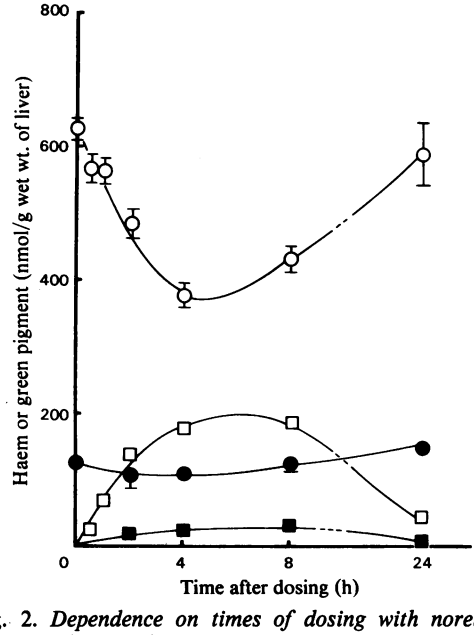

Fig. 2. *Dependence on times of dosing with norethindrone of hepatic haem degradation and green-pigment formation in vivo*

Either control rats (closed symbols) or phenobarbitone-pretreated animals (open symbols) were given norethindrone acetate (100mg/kg body wt.) intraperitoneally. At various times after dosing, animals were killed and livers homogenized in methanol/H_2SO_4. Concentrations of haem and green pigments were estimated by h.p.l.c. as described in the Experimental section. ○, and ●, liver haem; □, and ■, green pigments. Results are means ± S.E.M. for four determinations.

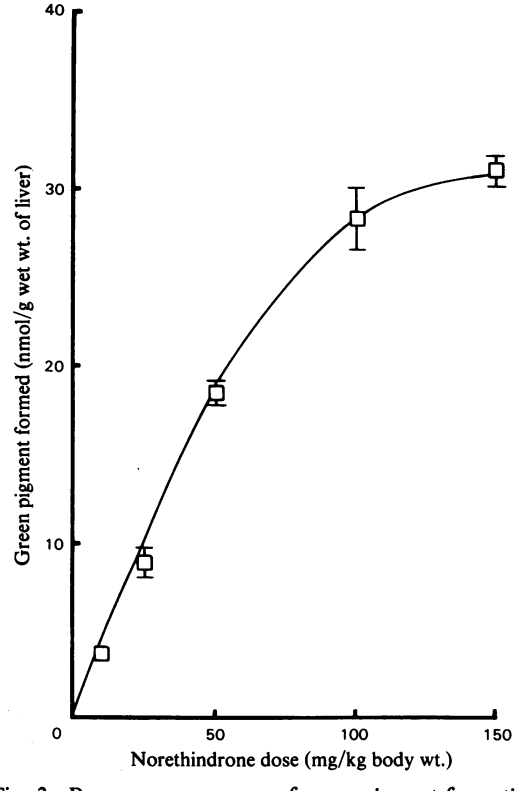

Fig. 3. *Dose–response curve of green-pigment formation in rat liver caused by the administration of norethindrone*

Rats were given various doses of norethindrone acetate intraperitoneally and killed 8h later. Portions of liver were homogenized in methanol/H_2SO_4 and the concentrations of green pigments were estimated by h.p.l.c. Results are means ± S.E.M. for four determinations.

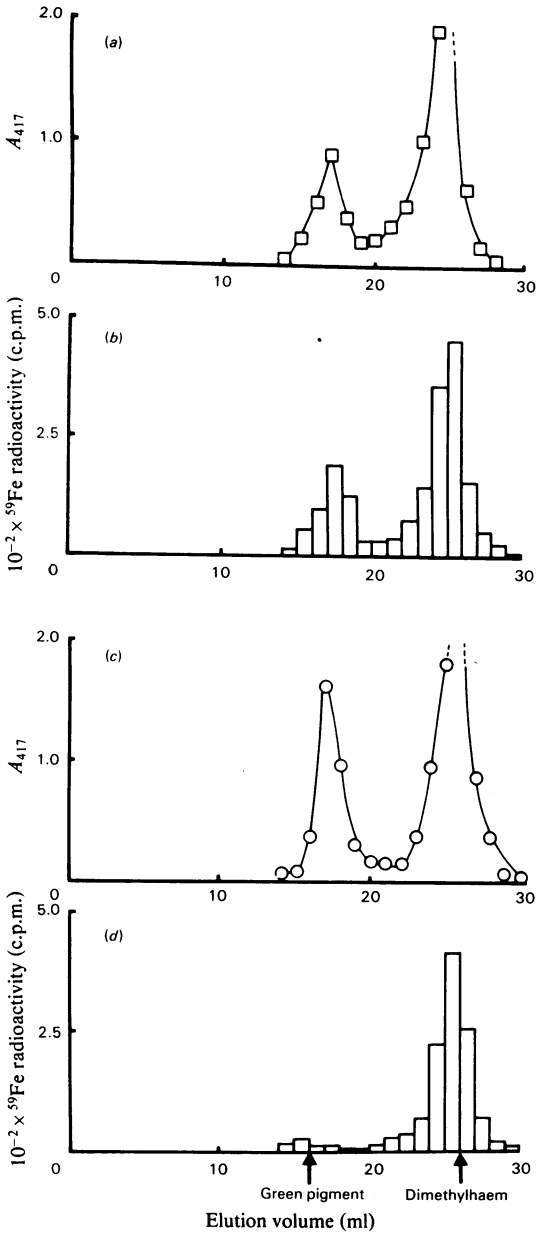

Fig. 4. *Separation of green pigments and haem by Biobeads gel-permeation chromatography*

The haem of rat liver was prelabelled with ^{59}Fe, then animals were dosed with 100mg of norethindrone/kg body wt. At 8h after dosing, rats were killed and portions of liver were extracted with butanone; the extracts were esterified with trimethyloxonium tetrafluoroborate before chromatography (*a* and *b*). Alternatively livers were homogenized in methanol/H_2SO_4 and extracted into chloroform before chromatography (*c* and *d*) as described in the Experimental section. Fractions (1ml) were collected and assayed for A_{417} or for ^{59}Fe radioactivity.

extracts was carried out under neutral conditions with trimethyloxonium tetrafluoroborate (Fig. 4*a*) or in acidic conditions with methanol/H_2SO_4 (Fig. 4*c*). When haem iron in rat liver was prelabelled by the administration of ^{59}Fe, radioactivity remained associated with the green-pigment peak after esterification under neutral conditions and chromatography (Fig. 4*b*), but only traces of radioactivity remained when esterification took place under acidic conditions (Fig. 4*d*). The iron-containing green pigments (fractions 16–20, Fig. 4*b*) migrated as two poorly defined components on silica gel t.l.c. (R_F 0.23 and 0.18). Unlike the metal-free green components, they did not fluoresce red under u.v. light. Because of their apparent instability on silica gel, attempts to separate these compounds by h.p.l.c. have not been successful. The iron-containing green pigments separated by Biobeads chromatography had an absorption maximum at 412nm (Fig. 5) but showed no sharp absorption bands in the region 500–650nm. Iron could readily be removed from these components under acidic conditions using the demetallation procedure described by Ortiz de Montellano & Kunze (1980*a*) to give products that behaved on t.l.c. and h.p.l.c. in a similar manner to the green pigments prepared from liver extracts by

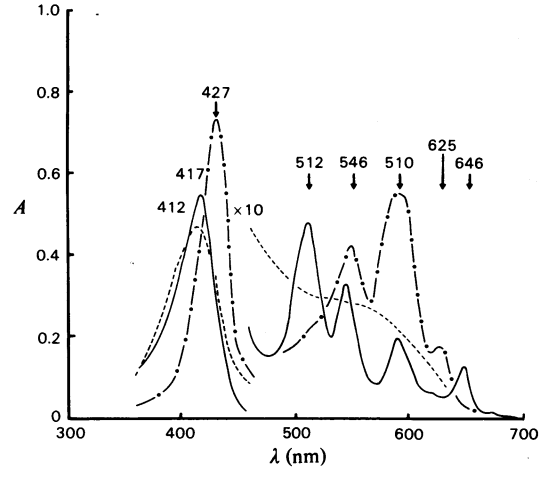

Fig. 5. *Absorption spectra of purified green pigments*

Absorption spectra in chloroform of green pigments purified by gel-permeation chromatography and h.p.l.c. were recorded on a Varian DMS 90 spectrophotometer calibrated with a didymium filter. ——, Metal-free green pigment produced by microsomal fraction incubated for 1min with norethindrone *in vitro*; –·–·, zinc chelate of the same pigment; ––––, green pigments produced by rat liver *in vivo* after norethindrone administration, esterified with trimethyloxonium tetrafluoroborate and separated from haem by gel-permeation chromatography.

esterification with methanol/H_2SO_4. As judged by t.l.c. of liver extracts esterified with trimethyloxonium tetrafluoroborate, green pigments produced *in vivo* after treatment of rats with norethindrone normally contained iron. There was no evidence of the presence of metal-free green pigments in the time period up to 24h after dosing with norethindrone.

Formation of green pigments in vitro

Esterification of haem and green pigments *in vitro* was obtained when methanol/H_2SO_4 was added in a 20-fold excess to aqueous microsomal reaction mixtures. Recoveries of [^{14}C]haem from such mixtures was 90.4 ± 0.9% (mean ± S.E.M. for four determinations). Fig. 6(*a*) shows the h.p.l.c. chromatogram of extracts of mixture incubated in the absence (Fig. 6*a*) or presence (Fig. 6*b*) of norethindrone. After incubation with norethindrone for short periods of time (approx. 1 min) in addition to dimethylhaem (retention time 4.2 min) a single green-pigment peak was produced (retention time 7.4 min; Fig. 6*b*). The zinc chelate of this pigment had a retention time of 3.1 min. When the haem of the microsomal fraction had been prelabelled with ^{14}C, as described in the Experimental section, radioactivity appeared associated wtih the green-pigment peak (Fig. 6*c*). Longer incubation times resulted in the appearance of four or five additional green-coloured peaks (Fig. 6*d*) that fluoresced red under u.v. light. None of these components, including the primary peak obtained after 1 min incubation, had the same retention times as the major green pigments produced by the liver of rats dosed with norethindrone *in vivo*. The microsomal green pigment produced *in vitro* did not appear to be an artefact of the extraction procedure since: (*a*) a similar product was obtained when the microsomal pellet was treated with methanol/H_2SO_4 after centrifugation (105000 *g* for 40 min at 0°C) of the incubation mixtures; (*b*) the usual three major green pigments were obtained when liver microsomal fraction from norethindrone-dosed rats was added to incubation mixtures *in vitro*. Purification of the primary microsomal green pigment showed it to have similar spectroscopic properties (Fig. 5) and the same absorption coefficient as the green pigments produced *in vivo* (Ortiz de Montellano *et al.*, 1979).

Kinetics of norethindrone-mediated production of green pigments in vitro

Fig. 7 shows the time course for green-pigment formation *in vitro*. Reaction rates rapidly became non-linear with time, consistent with the self-catalysed destruction of the cytochrome *P*-450 forms responsible for the metabolic activation of norethindrone. Initial reaction rates showed microsomal preparations from phenobarbitone-pretreated

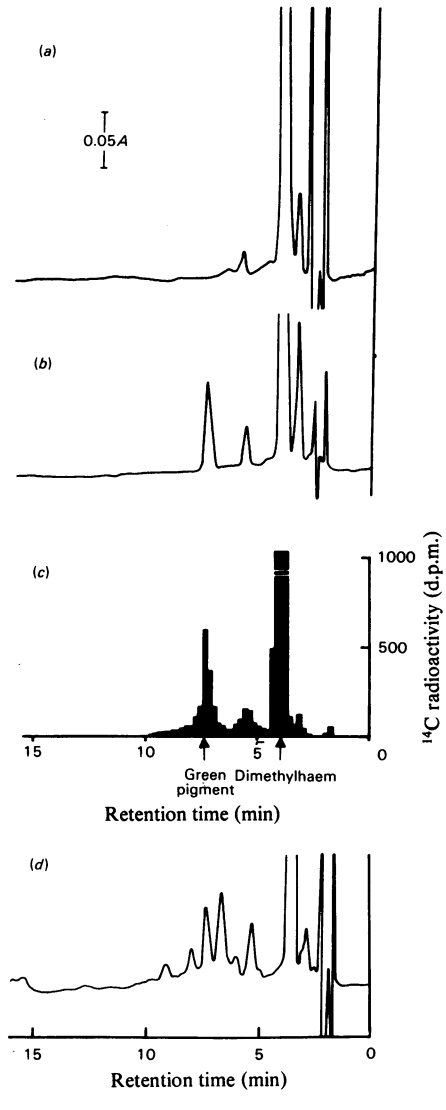

Fig. 6. *Separation by h.p.l.c. of haem and green pigments produced by microsomal fraction in vitro*

Liver microsomal fraction containing haem prelabelled with ^{14}C was incubated in the absence (*a*) or presence (*b*) of norethindrone. Washed liver microsomal fractions were prepared and suspended in 0.25 M-sucrose so that 1 ml of microsomal suspension was equivalent to 0.5 g wet wt. of liver. Incubation mixtures were of 2 ml volume. Reactions were started with the addition of 0.2 ml of microsomes (2.1 mg of microsomal protein). After incubation for 1 min, reactions at 37°C were stopped with methanol/H_2SO_4 and the methylated products were extracted with chloroform as described in the Experimental section. The ordinates show relative absorbance at 417 nm, apart from (*c*), which shows the radioactivity in 0.3 ml fractions collected at the detector outlet in (*b*). (*d*) is the h.p.l.c. chromatogram obtained after 40 min incubation of microsomal fraction with norethindrone.

rats to be about eight times more effective than controls in catalysing the formation of green pigments. There was excellent linearity between microsomal protein concentration and green-pigment formation over the range 0.2–4mg of protein/reaction mixture (results not shown). Green-pigment formation was also dependent on the concentration of norethindrone in the reaction mixture (Fig. 8). No conversion of haem into green pigments was observed in the absence of NADPH or if NADH was substituted for NADPH. Exogenous [^{14}C]haem (10–50nmol) pre-incubated with the microsomal preparation (5min at 37°C) had no effect on the subsequent norethindrone-mediated formation of green pigments and no incorporation of radioactivity could be detected in the green pigment peak.

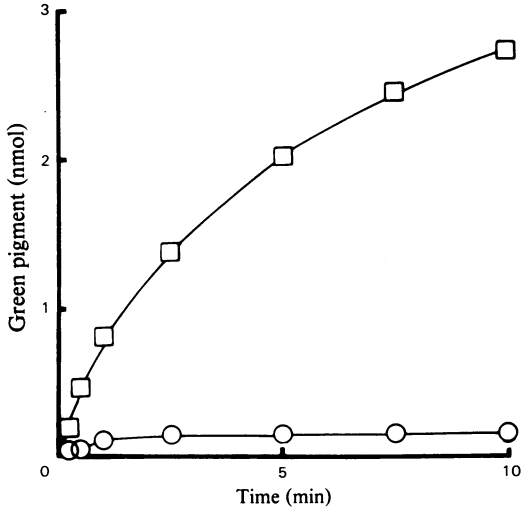

Fig. 7. *Time course for green-pigment formation in vitro*
Liver microsomal fraction from control (○) or phenobarbitone-pretreated (□) rats were incubated for various lengths of time with norethindrone and NADPH as described in the Experimental section. Incubation mixtures were of 2ml volume and contained 2.0 and 3.9mg of microsomal protein for control and phenobarbitone-pretreated rats respectively. Reactions were stopped with methanol/H_2SO_4 and the concentrations of green pigments were estimated by h.p.l.c. Results are the means for two experiments.

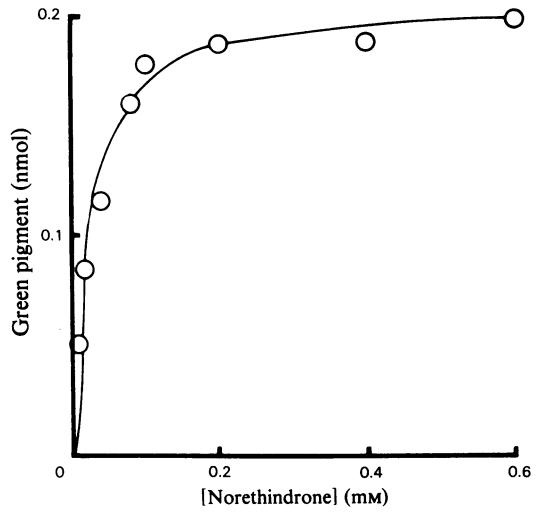

Fig. 8. *Green-pigment formation in vitro: dependence on norethindrone concentration*
Reaction mixtures of 2ml volume were incubated with liver microsomal fractions (2.0mg of microsomal protein) for 1min at 37°C with various concentrations of norethindrone as indicated. Reactions were stopped with methanol/H_2SO_4 and the concentrations of green pigments were estimated by h.p.l.c. Results are means for two experiments.

Table 1. *Effects of pretreating rats with mixed-function oxidase inducers on norethindrone-mediated conversion of liver microsomal haem into green pigments in vitro*
Male Fischer rats were pretreated with the microsomal enzyme inducers as described in the Experimental section. Washed liver microsomal fractions were prepared and suspended in 0.25M-sucrose so that 1ml of microsomal suspension was equivalent to 0.5g wet wt. of liver. Cytochrome *P*-450 concentrations were estimated as described by Omura & Sato (1964). Incubation mixtures were of 2ml volume. Reactions were started by the addition of 0.2ml of the microsomal suspension. Green-pigment formation was determined by h.p.l.c. after 1min incubation at 37°C in the presence of 0.5mM-norethindrone and an NADPH-generating system. Results are means ± S.E.M. for four determinations.

Pretreatment	Microsomal cytochrome *P*-450 (nmol/g wet wt. of liver)	Green-pigment formation (nmol/min per g wet wt. of liver)
Controls	23.3 ± 0.6	0.11 ± 0.01
Phenobarbitone	86.3 ± 1.1	0.80 ± 0.06
Aroclor 1254	88.7 ± 2.3	0.41 ± 0.02
3-Methylcholanthrene	45.3 ± 1.5	0.10 ± 0.01

Effect of microsomal enzyme inducers on green-pigment formation in vitro

The results shown in Table 1 indicate that pretreatment of rats with phenobarbitone was the most effective in inducing the formation of green pigments *in vitro*. The chlorinated biphenyl Aroclor 1254 caused a similar induction of cytochrome *P*-450 concentration as phenobarbitone, but the yield of green pigments was relatively lower. The rate of formation of green pigments from 3-methylcholanthrene-pretreated animals was little different from that of controls.

Discussion

Haem degradation to green pigments

Liver microsomal haem, prelabelled with ^{14}C was degraded *in vivo* and *in vitro* in the presence of norethindrone to give ^{14}C-labelled green pigments (Figs. 1 and 6). There were no major ^{14}C-labelled components formed that were not associated with the green peaks. Previous studies showed that it was the haem of cytochrome *P*-450 that was destroyed by norethindrone. Haem from other sources in the endoplasmic reticulum, e.g. cytochrome b_5, was not degraded (White & Muller-Eberhard, 1977). At least *in vitro*, exogenous [^{14}C]haem added to microsomal incubation mixtures did not enhance or result in the detectable incorporation of ^{14}C label into green pigments. Such incorporation, however, has been reported after the administration of [^{14}C]haem *in vivo* (Correia *et al.*, 1979).

The norethindrone green pigments had many of the characteristics of the green pigments produced in rats given allyl-, vinyl- or allenic-substituted compounds (De Matteis *et al.*, 1980; Ziman *et al.*, 1980; Ortiz de Montellano & Kunze, 1980*b*). However, the norethindrone green pigments were rapidly lost from the liver. This was unlike the prolonged elevation of green-pigment concentrations seen in the livers of rats given allylisopropylacetamide (Bonkowsky *et al.*, 1980). It is not known whether metabolism and excretion of the parent drug or the green pigments themselves is the most important factor in determining their accumulation in the liver.

Green pigments as metabolic products

Silica gel h.p.l.c. permitted the separation of three major green pigments from rats given norethindrone *in vivo* (Fig. 1). Previous procedures utilizing reverse-phase h.p.l.c. have resolved two metal-free components (Ortiz de Montellano *et al.*, 1979). Green pigments are formed as a result of alkylating one of the nitrogen atoms of the porphyrin ring of haem (De Matteis & Cantoni, 1979; De Matteis *et al.*, 1980). De Matteis and his co-workers and Ortiz de Montellano & Kunze (1980*a*) have suggested that the two green pigments previously resolved represented different isomeric adducts. The data in the present paper suggest additional factors may be involved since: (*a*) three major metal-free green pigments were separated on h.p.l.c. (Fig. 1) and t.l.c. (White & Muller-Eberhard, 1977); (*b*) the ratio of the concentrations of the individual green pigments did not remain constant with the dose of norethindrone or time after dosing; (*c*) *in vitro*, only one green pigment was formed initially (Fig. 6) and with longer incubation times it was transformed into other green-coloured red-fluorescing products. It is suggested that *in vivo*, a similar metabolic transformation of a single green-pigment precursor could account for the multiple forms seen in liver extracts, although the chemical nature of the more polar products has not yet been deduced.

The data in the present paper are difficult to reconcile with the findings of Ortiz de Montellano & Kunze (1980*a*), who reported that green pigments produced by microsomes *in vitro* had identical chromatographic properties and molecular weights with the ones produced *in vivo* by norethindrone. Mass spectrometry of green pigments produced *in vivo* by other acetylenic-substituted compounds, apart from norethindrone, indicated the presence of an additional oxygen atom (Ortiz de Montellano & Kunze, 1980*a*). This result would be consistent with the formation of a hydroxylated porphyrin–drug adduct.

The role of iron in green pigments

When methylation of liver extracts from norethindrone-dosed rats was carried out under mild conditions at neutral pH values, iron remained associated both with the esterified haem and green pigment peaks (Fig. 4). The spectroscopic properties of the iron-chelated green pigment was quite different from the metal-free components (Fig. 5). These results are in agreement with the findings of Unseld (1976) and De Matteis & Unseld (1976) that, in the liver, non-esterified green pigments produced by allylisopropylacetamide contained iron. The presence of iron in these components may be of importance in assessing their physiological actions, recently investigated by De Matteis & Gibbs (1980), as the present results suggest that after norethindrone treatment of rats, metal-free green pigments are not present within the hepatic parenchymal cells.

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