Factors responsible for the formation of different *N*-alkylated porphyrins in rat liver microsomal systems exposed to norethindrone

The role of 3 α -hydroxysteroid dehydrogenase

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1. Incubation of rat liver microsomes with norethindrone and a NADPH-generating system leads to the formation of one *N*-alkylated porphyrin (green pigment, GP1). Administration of this steroid to male rats *in vivo* results in the formation of three more-polar green pigments (GP2, 3 and 4). 2. A cytosolic protein (green-pigment converting protein) has been purified from rat liver that, when added to liver microsomal mixtures containing norethindrone (0.03 mM) and a NADPH-generating system, results in the formation of all four green pigments (GP1, 2, 3 and 4). Field-desorption mass spectrometry of the purified green pigments gave protonated molecules, $[M+H]^+$, at m/z 905 for GP1, m/z 909 for GP2, m/z 925 for GP3 and 4. 3. The M_r of the purified cytosolic protein on SDS/polyacrylamide-gel electrophoresis or gel filtration was 37000. Polyacrylamide-gel isoelectric focusing gave a pI value of 5.9. Antibodies raised in rabbits against this protein, after preincubation with rat liver cytosol, subsequently prevented the formation of the more-polar norethindrone-induced green pigments (GP2, 3 and 4). 4. The purified protein in the presence of either NADH or NADPH catalysed the reduction of Δ^4 -ring-reduced norethindrone, 5 α -oestrane-17 α -ethynyl-17 β -ol-3-one and, with the appropriate cofactor, the oxidation and reduction of steroids lacking the ethynyl function, e.g. androsterone or dihydrotestosterone. Indomethacin inhibited the reduction of dihydrotestosterone by this protein with an I_{50} (concn. causing 50% inhibition) value of 4.9 μ M. From its physical and enzymic properties it is concluded that green-pigment converting protein is the same as 3 α -hydroxysteroid dehydrogenase (EC 1.1.1.50).

INTRODUCTION

Metabolic activation of the ethynyl function of certain contraceptive steroids such as norethindrone (17 α -hydroxy-19-nor-17 α -pregn-4-en-20-yn-3-one, leads to the destruction of hepatic cytochrome *P*-450 and the formation of *N*-alkylated porphyrins ('green pigments', GPs) (White & Muller-Eberhard, 1977; Ortiz de Montellano *et al.*, 1979; for review, see White, 1984).

The pattern of *N*-alkylated porphyrins extracted from the liver after the administration of norethindrone and separated by h.p.l.c. or t.l.c. differs between species and even between male and female rats (White *et al.*, 1984). By contrast, the present study showed that hepatic microsomal preparations incubated with norethindrone and a NADPH-generating system for short time periods produced the same single GP component. Experiments using male-rat hepatocyte cultures suggested further metabolism of the steroid or the steroid-protoporphyrin IX adduct results in the formation of the more-polar GPs of the type extracted from the livers of rats given norethindrone *in vivo* (Blakey & White, 1986). High concentrations of this compound, certain naturally

occurring steroids without an ethynyl substituent or mixed-function-oxidase inhibitors such as SKF 525A, were able to preferentially inhibit the transformation to the more-polar GP forms (Blakey & White, 1986). Microsomal systems alone incubated with norethindrone and NADPH were not able to produce these types of polar *N*-alkylated porphyrins (White, 1978, 1980). Preliminary results suggested that, in such systems *in vitro*, an additional cytosolic factor was required (White *et al.*, 1984), although the role of this factor, called here 'GP converting protein' was not defined.

Evidence is now presented for the identity of this protein and of the different GP forms produced in the livers of rats given norethindrone.

EXPERIMENTAL

Chemicals

Norethindrone, androsterone, epiandrosterone, dihydrotestosterone, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, 3 α -hydroxysteroid dehydrogenase (*Pseudomonas testosteroni*), NAD⁺ and NADP⁺ were

Abbreviations used: GP, green pigment; f.d., field desorption.

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from the Sigma Chemical Co. Ltd., Poole, Dorset, U.K. 5α -Oestrane- 17α -ethynyl- 17β -ol-3-one was from Steraloids Ltd., Croydon, Surrey, U.K. [$1,2$ - ^3H]Dihydrotestosterone ($> 98\%$ pure, sp. radioactivity 51.6 Ci/mmol) was from New England Nuclear, Stevenage, Herts., U.K.

Animals and dosing

Male and female rats (150–180 g body wt.) were of the Fischer F344/N strain. Norethindrone was administered intraperitoneally dissolved in triolein/glycerol vehicle at a dose of 100 mg/kg body weight. Washed liver microsomal and cytosolic fractions were prepared as described previously (White, 1978).

Formation and assay of GPs by microsomal systems

Reaction mixtures of 2 ml volume were as described previously (White, 1978). The norethindrone concentration was 0.03 mM. In some instances, serial dilutions of liver cytosol or purified protein was included in the mixtures. Reactions were usually terminated after 15 min incubation at 37 °C. Esterification, extraction and resolution by h.p.l.c. of the GPs were carried out as described by White (1981).

Purification of GPs: m.s.

Non-esterified GPs were extracted with 1.7% (v/v) HCl in acetone and partially purified by Sephadex LH-20 column chromatography (White *et al.*, 1983). After esterification the individual metal-free GPs were further purified by t.l.c. and h.p.l.c. (White, 1981).

M.s. of the purified GPs was performed in the f.d. mode on a Finnigan MAT 731 double-focusing spectrometer equipped with a combined electron-impact/f.d. ion source. High-temperature activation of the f.d. emitters, sample transfer and desorption by indirect heating applying an emitter heating current were as previously described (Schulten, 1979). All f.d. spectra were produced at ion-source potentials of 8 kV for the emitter and –3 kV for the slotted cathode plate. Chloroform was used as solvent. Small added amounts of methanol and acetone had a strong influence on the spectra, promoting protonation reactions.

Assay for 3α -hydroxysteroid dehydrogenase (EC 1.1.1.50)

Reaction volumes were 3 ml. For both oxidation and reduction, initial velocities were determined at 340 nm on a recording spectrophotometer fitted with a thermostatically controlled cell compartment at 37 °C. Reactions were started with steroid (20 μM) dissolved in acetonitrile (10 μl). Oxidations were carried out in 0.03 M-sodium pyrophosphate buffer, pH 8.9 and either NAD^+ or NADP^+ (2 mM). Reductive reactions were carried out in 0.1 M-phosphate buffer, pH 7.4, containing either NADH or NADPH (0.18 mM). In some instances, indomethacin (1–30 μM) dissolved in dimethyl sulphoxide (10 μl) was added before the steroid substrate. Where [$1,2$ - ^3H]dihydrotestosterone (30 μCi) was used, this was diluted with unlabelled material to give a final concentration of 20 μM . After 5 min incubation with NADPH, the reaction was stopped with ice-cold 0.1 M-NaOH (3 ml). Reaction products were extracted (thrice) into ethyl acetate (3 ml). The combined organic phases were washed with water, dried (over anhydrous Na_2SO_4) and concentrated to dryness under N_2 at 37 °C. The residue was dissolved in methanol (100 μl) and a portion was subjected to h.p.l.c. or to t.l.c. A similar procedure

was used with 5α -estrane- 17α -ethynyl- 17β -ol-3-one as substrate.

H.p.l.c. of steroids was carried out with a C_{18} column (15 cm $\times$ 0.47 cm, 5 μm -particle-size Magnusphere) and a solvent system of methanol/water (7:3, v/v) at a flow rate of 1.2 ml/min. Fractions (0.3 ml) were collected from the detector outlet and, after the addition of scintillant, assayed for radioactivity.

T.l.c.

Silica-gel plates (silica-gel 60, layer thickness 0.25 mm; E Merck, Darmstadt, Germany) were used. Steroids were chromatographed in a chloroform/acetone (4:1, v/v) solvent system. Steroids were detected with a conc. H_2SO_4 spray or 0.4% (w/v) dinitrophenylhydrazine in 0.3% (v/v) HCl/methanol spray. GPs were separated with a chloroform/methanol (9:1, v/v) solvent system.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with a bovine serum albumin standard.

Glutathione transferase assay (EC 2.5.1.18)

Glutathione transferase was assayed spectrophotometrically by using 1,2-dichloro-4-nitrobenzene (1 mM) as substrate as described by Habig & Jakoby (1981).

Purification of liver cytosolic GP converting protein

Enzyme purifications were carried out at 4 °C, and all buffer solutions used for chromatography and dialysis after the DEAE-cellulose stage contained 20% (v/v) glycerol, 1.0 mM-EDTA and 0.5 mM-dithiothreitol.

1. $(\text{NH}_4)_2\text{SO}_4$ fractionation. The cytosolic fraction prepared from the livers of six male rats (45 g) was treated with $(\text{NH}_4)_2\text{SO}_4$ ('reduced in metals'; Sigma Chemical Co.). The precipitate obtained from 40–75% (w/v)-satd. $(\text{NH}_4)_2\text{SO}_4$ was dialysed against three changes of 2 litres of 0.05 M-Tris/HCl buffer, pH 8.0, over 20 h.

2. DEAE-cellulose column chromatography. The dialysed $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to a column (30 cm $\times$ 2.5 cm) of DEAE-cellulose (DE-52; Whatman, Maidstone, Kent, U.K.) equilibrated with 0.05 M-Tris/HCl buffer, pH 8.0. A linear gradient of 0–0.5 M-NaCl in the same buffer was applied at a flow rate of 15 ml/h. Fractions (5 ml) were collected. The dark-red GP converting protein fractions (0.21–0.32 M-NaCl) were pooled and dialysed against 0.01 M-phosphate buffer, pH 7.4, for 20 h.

3. Blue Sepharose chromatography. The non-diffusable material was loaded on a Blue Sepharose column (20 cm $\times$ 1.6 cm) equilibrated with the dialysis buffer. A linear gradient of 0–1 M-KCl in the equilibration buffer at a flow rate of 30 ml/h was used. Fractions (5 ml) were collected. The colourless eluate (0.63–0.8 M-KCl) containing GP-converting activity was pooled and concentrated by ultrafiltration to about 3 ml by using a Amicon Diaflo PM 10 membrane.

4. Gel filtration. The concentrated protein was subjected to gel filtration on an Ultrogel AcA 44 (LKB, Croydon, Surrey, U.K.) column (90 cm $\times$ 2.5 cm) equilibrated with 0.025 M-imidazole/HCl buffer, pH 7.4. The

column was eluted with this buffer at a flow rate of 15 ml/h and 5 ml fractions were collected. The fractions eluted at total eluant volume 355–395 ml were pooled. At the end of the purification procedure, the column was calibrated with the following proteins of known M_r : phosphorylase *b*, 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; trypsin inhibitor, 20000; and α -lactalbumin 14400. The GP converting protein was re-run on this column to obtain an estimate of M_r .

5. Chromatofocusing. The pooled fractions from gel filtration were pumped directly (30 ml/h) on to a column (20 cm $\times$ 1.6 cm) of PBE 94 Polybuffer exchange resin (Pharmacia, Hounslow, Middx., U.K.) equilibrated with 0.025 M-imidazole buffer, pH 7.4. After application of the sample, which was retained on the column, the eluting buffer was changed to Polybuffer 7–4/HCl, pH 4.0, at a flow rate of 30 ml/h. Fractions (3 ml) were collected. The pH value of each fraction was determined and the active ones eluted between pH 6.0 and 5.8 were pooled, then concentrated with an Amicon membrane to about 5 ml. The concentrated material was dialysed against 0.01 M-phosphate buffer, pH 7.4, for 20 h.

6. Hydroxyapatite chromatography. The non-diffusable material was finally subjected to hydroxyapatite-gel chromatography (Ultrogel HA; LKB) on a column (20 cm $\times$ 1.6 cm) equilibrated with the dialysis buffer. The flow rate was 20 ml/h. A linear gradient of 0.01–0.5 M-phosphate buffer, pH 7.4, was used. Fractions (3 ml) eluted between 0.13 and 0.18 M-phosphate were pooled and concentrated with an Amicon membrane.

SDS/polyacrylamide-gel electrophoresis

This was performed on a 3% (w/v) stacking/12.5% running gel prepared in 0.37 M-Tris/HCl buffer, pH 8.8, containing 0.1% SDS as described by Laemmli (1970). Protein was stained with 0.01% Kenacid Blue R.

Isoelectric focusing

Preformed polyacrylamide gels (24.5 cm $\times$ 11 cm) of 1 mm thickness (pH range 4–6.5; Ampholine PAG-Plates; LKB) were used. Gels were run for 1.6 h at 15 W (1500 V). Proteins were stained as described above. The pH gradient was assessed in unstained gels after electrophoresis, 0.5 cm strips of the gel being first suspended in deionized water for 18 h at 4 °C.

Antibody production

Antibodies were raised in male Sandy Lop rabbits. Pure antigen (0.5 mg in 1 ml) was mixed 1:1 (v/v) with Freund's complete adjuvant and administered intramuscularly. Blood was withdrawn after 1 month from the marginal ear vein. After clotting (30 min at room temperature, then for 18 h at 4 °C) and centrifugation (10000 *g* for 15 min at 4 °C) the serum was tested for activity by Ouchterlony double-immunodiffusion analysis. Booster injections of the same dose were given at monthly intervals. The IgG fraction from reactive serum was partially purified by DEAE Affi-Gel Blue (Bio-Rad Laboratories, Watford, Herts., U.K.) column chromatography as described by Hudson & Hay (1976) and stored at –70 °C until required.

For antibody–antigen reactions serial dilutions of antibody were reacted with cytosol or purified protein at 4 °C for 18 h in 0.1 M-phosphate buffer, pH 7.4.

RESULTS AND DISCUSSION

Effects of cytosol on the h.p.l.c. pattern of GPs formed in the presence of norethindrone in male-rat liver microsomes

Fig. 1(a) shows the pattern of GPs separated by h.p.l.c. from an extract of a rat liver microsomal mixture incubated for 3 min with 0.03 mM-norethindrone and an NADPH-generating system. Only one major *N*-alkylated porphyrin (GP1) was formed. As previously reported (White, 1981), longer incubation times (15 min) resulted in additional GP adducts of greater and lesser retention times. None of these co-chromatographed with the three major GPs (GP2, 3 and 4) extracted from the livers of male rats given norethindrone *in vivo* (Fig. 1b). However, if either male- or female-rat liver cytosol (3 mg of protein)

Fig. 1. H.p.l.c. of GPs

Microsomal reaction mixtures were esterified with methanol/ H_2SO_4 and the concentrated chloroform extracts subjected to silica-gel h.p.l.c. GPs detected by absorbance and fluorescence (excitation 417, emission > 550 nm) peaks are shown shaded. (a) Liver microsomal extract after 3 min incubation with norethindrone (0.03 mM) and an NADPH-generating system. (b) Liver extract from a male rat dosed 3 h earlier with norethindrone (100 mg/kg body wt.). (c) Liver microsomal extract after 15 min incubation with rat liver cytosolic fraction (3 mg of protein), norethindrone (0.03 mM) and an NADPH-generating system.

Fig. 2. F.d. mass spectra of purified GP methyl esters

(a) GP1; (b) GP2; (c) GP3; (d) GP4. Only the sections of the quasimolecular ions and relevant fragments are shown. Fractionated desorption (Schulten *et al.*, 1983) was performed and fifteen f.d. mass spectra were recorded and summed between 20 and 25 mA emitter heating current [best anode temperature; for a detailed description of the procedure, see Schulten (1979) and references cited therein].

was added to the microsomal incubation mixtures, GPs could be extracted that had the same retention times (Fig. 1c) and co-chromatographed with the three major adducts *in vivo*.

Similar results were obtained using t.l.c. The microsomal product GP1 (R_F 0.56) did not co-chromatograph with GP2, 3 or 4 (R_F 0.46, 0.41 and 0.36 respectively). After the addition of cytosol, four GPs were extracted (R_F 0.56, 0.46, 0.41 and 0.36) which fluoresced red under u.v. light following t.l.c. All had similar actio-type absorption spectra in chloroform at 417 nm (Soret) and 512, 546, 610 and 646 nm.

M.s.

The purified GP dimethyl esters were subjected to soft-ionization m.s. (Schulten, 1985). The f.d. mass spectra of GP 1, 2, 3 and 4 are shown schematically in Figs. 2(a)–2(d) and given intense quasimolecular ions due to proton and metal-cation attachment. From these signals the M_r values are easily derived. In addition, some structurally characteristic fragmentation is observed, although in part with low relative abundances. Cleavage and hydrogen transfer at C-17 of the steroid moiety gives the ions at m/z 272 and 273 for GP1, m/z 276 and 277 for GP2 as well as m/z 292 and 293 for GP3 and 4. Furthermore, fission between the bridging methylene group and the porphyrin moiety leads to an ion at m/z 590 (protoporphyrin IX dimethyl ester) for all GPs.

Fig. 3. Effects of cytosol on GP formation

(a) Accumulation of GPs with time after incubation of norethindrone with male-rat liver microsomes + cytosol. Rat liver microsomal fractions (2–3 mg of protein) were incubated with cytosol (3 mg of protein), norethindrone (0.03 mM) and an NADPH-generating system for various lengths of time as indicated. Reactions were stopped with methanol/ H_2SO_4 and the concentrated chloroform extracts subjected to silica-gel h.p.l.c. Concentrations of green pigments were calculated from their relative absorbances at 417 nm. $\square$, GP1; $\triangle$, GP2; ∇ , GP3; $\circ$, GP4; $\bullet$, total GPs, including minor components. Results represent the means $\pm$ S.E.M. for four experiments. (b) Effects of cytosolic protein concentration on the accumulation of GP2, 3 and 4 after incubation with norethindrone and rat liver microsomes. Conditions were the same as in the legend to Fig. 2(a), except that the incubation time was 15 min and the concentration of cytosolic protein was varied as indicated. The concentration of GP2, 3 and 4 formed is expressed as a percentage of the total GPs extracted and represents the means $\pm$ S.E.M. for four experiments.

Such studies show these adducts are not structural isomers as has been described for certain *N*-ethylated porphyrins (De Matteis *et al.*, 1983); they suggest

Table 1. Purification of GP converting protein from male-rat liver cytosol

3 α -Hydroxysteroid dehydrogenase activities with dihydrotestosterone (20 μ M) as the substrate and NADH as cofactor at pH 7.4 and the titration of GP converting activities were determined as described in the Experimental section.

Purification stage	GP conversion				3 α -Hydroxysteroid dehydrogenase			
	Volume (ml)	Total protein (mg)	End-point titration (mg of protein)	Purification factor	Total activity (μ mol/min)	Specific activity (μ mol/min per mg of protein)	Purification factor	Yield (%)
Cytosol	150	2700	0.2	1	26.4	0.009	1	100
(NH ₄) ₂ SO ₄	28	885	0.12	2	20.9	0.24	3	79
DEAE-cellulose	65	195	0.04	6	17.9	0.091	10	68
Blue Sepharose	80	30	0.013	19	8.3	0.27	30	31
Gel filtration	70	6	0.006	45	4.1	0.67	75	15
Chromatofocusing	30	4	0.005	52	3.3	0.83	82	10
Hydroxyapatite	35	2	0.004	63	2.2	1.1	120	8

norethindrone GPs arise as a result of metabolic modification of the steroid before activation of the ethynyl function.

Effects of concentration of cytosolic protein on GP2, 3 and 4 formation

In incubation mixtures containing liver cytosol, microsomes and a NADPH-generating system, concentrations of norethindrone greater than 0.03 mM progressively inhibited the formation of GP3 and 4, but not GP1 or 2. Fig. 3(a) shows that, in the presence of 0.03 mM-norethindrone, GP1 was formed first, reaching a maximum level after 2.5–5 min, declining in concentration thereafter. GP2 could not be detected until 2.5 min, whereas the more polar adducts, GP3 and 4, did not start to accumulate until 7.5–10 min. In order to obtain data on the ability of cytosolic fractions to promote the formation of GP2, 3 and 4, an incubation time of 15 min was chosen. This represents the time of maximal accumulation of total GPs in such incubation mixtures.

For the purpose of assessing GP converting activity during protein purification, the concentration of GP2, 3 and 4 formed after 15 min incubation was expressed as a percentage of the total GPs extracted (Fig. 3b). The titration end point was taken as the protein concentration just insufficient to permit the formation of any of the more polar adducts (0.25 mg in Fig. 3b).

Purification and identity of GP converting activity

Liver cytosolic glutathione transferases bind steroids and porphyrins (Smith *et al.*, 1985). It was originally considered that the GP converting protein might be the same as this enzyme. This proved not to be the case. No glutathione transferase activity could be detected in the purified product.

Table 1 outlines the recoveries and yields from the protein purification procedure used. Converting activity became very labile as the protein became more pure. Gel filtration suggested this protein had an M_r of 37000 (results not shown). SDS/polyacrylamide-gel electrophoresis also showed a single component (Fig. 4) with an M_r of 37000. A component corresponding in electrophoretic mobility to the purified protein appeared to be present in the liver cytosolic fraction from both male and female rats. Isoelectric focusing gave a pI value of 5.9.

Fig. 4. SDS/polyacrylamide-gel electrophoresis of liver cytosolic fractions and purified GP converting protein

SDS/polyacrylamide gels were prepared and run as described in the Experimental section. Proteins were stained with Kenacid Blue R. Lanes 1 and 5, M_r markers; from top to bottom these were: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α -lactalbumin; lane 2, female-rat liver cytosol; lane 3, male-rat liver cytosol; lane 4, GP converting protein.

After the addition of this single purified protein to a male rat liver microsomal system containing norethindrone (0.03 mM) and an NADPH-generating system, all four GPs (GP1, 2, 3 and 4) could be extracted and separated by h.p.l.c. or t.l.c. after 15 min incubation. Under identical conditions with microsomes from female rats, only GP1 and 2 were seen. This suggested differences in microsomal, rather than cytosolic, properties were responsible for the formation of GP3 and 4.

The physical characteristics of the purified protein matched those of 3 α -hydroxysteroid dehydrogenase (Penning *et al.*, 1984). After DEAE-cellulose chromatography, fractions were tested for this activity. With androsterone as the substrate with NAD⁺ as cofactor at

pH 8.9, two peaks of activity were seen (Fig. 5). However, with dihydrotestosterone as substrate and NADH as cofactor at pH 7.4, only one major active component was detected. This was coincident with GP converting activity.

Fig. 5. DEAE-cellulose column chromatography of proteins from male-rat liver cytosolic fraction

The dialysed 40–70% -satd. $(\text{NH}_4)_2\text{SO}_4$ fraction of a liver cytosolic preparation was subjected to DEAE-cellulose column chromatography at pH 8.0, an NaCl gradient (----) being used as the eluent. Fractions (5 ml) were collected and assayed for A_{280} (○) or GP converting activity (□). 3 α -Hydroxysteroid dehydrogenase activity was assayed using either androsterone and NAD⁺ at pH 8.9 (△) or dihydrotestosterone with NADH at pH 7.4 (●) as described in the experimental section.

In subsequent purification steps, the pigment converting protein retained 3 α -hydroxysteroid dehydrogenase activity. Table 2 shows that the purified protein was able to oxidize and reduce a number of steroids with either NAD(H) or NADP(H) as cofactors. In both oxidation and reduction reactions, the use of NADP(H) consistently resulted in higher V_{max} values than with NAD(H). The presence of a 17 α -ethynyl function in 5 α -oestrane-17 α -ethynyl-17 β -ol-3-one did not affect the ability of this enzyme to reduce the 3-oxo group. This enzyme was not able to oxidize the 3 β -hydroxysteroid epiandrosterone or reduce Δ^4 -3-oxo steroids such as norethindrone or testosterone. In order to confirm that utilization of cofactors was accompanied by modification of the steroid structure, in one experiment [1,2-³H]dihydrotestosterone was used as a substrate and NADPH as the cofactor. The purified converting protein catalysed the formation of a single reaction product [after separation by h.p.l.c. (retention time 11.7 min)]. This had the same retention time as authentic 5 α -androstane-3 α ,17 β -diol (Fig. 6). In an analogous manner, 5 α -oestrane-17 α -ethynyl-17 β -ol-3-one was incubated with the protein in the presence of NADH or NADPH. T.l.c. of ethyl acetate extracts of the reaction mixture after 15 min incubation with NADPH showed this steroid (R_F 0.53) was converted into a single more polar adduct (R_F 0.38) that gave no reaction after spraying with dinitrophenylhydrazine, consistent with the product being 5 α -oestrane-17 α -ethynyl-3 α ,17 β -diol. Such extracts when incubated with male-rat liver microsomes and a NADPH-generating system for 10 min in the absence of liver cytosol formed three GPs, as judged by h.p.l.c. These had retention times the same as, and co-chromatographed with, GP 2, 3 and 4. Similar results were obtained when 5 α -oestrane-17 α -ethynyl-17 β -ol-3-one was preincubated with bacterial 3 α -hydroxysteroid dehydrogenase.

5 α -Oestrane-17 α -ethynyl-17 β -ol-3-one (0.03 mM) when incubated with rat liver microsomes and a NADPH-generating system in the absence of cytosol formed a

Table 2. Kinetic data for purified 3 α -hydroxysteroid dehydrogenase

Steroid reductions were carried out in 0.03 M-sodium pyrophosphate buffer, pH 8.9, with either NADH or NADPH (0.18 mM) and steroid (1–20 μM) dissolved in acetonitrile (10 μl). Oxidations were in 0.1 M-phosphate buffer, pH 7.4, containing either NAD⁺ or NADP⁺ (2.3 mM) and steroid (1–20 μM). Reactions were monitored by the change in absorbance at 340 nm in a thermostatically controlled spectrophotometer cuvette at 37 °C. Values for V_{max} and K_m were calculated by the method of Wilkinson (1961).

Substrate	Parameter value			
	Cofactor . . . NAD ⁺		NADP ⁺	
	K_m (μM)	V_{max} ($\mu\text{mol/min}$ per mg of protein)	K_m (μM)	V_{max} ($\mu\text{mol/min}$ per mg of protein)
Oxidation				
Androsterone	14.6	3.5	6.0	10.8
Androstane-3 α ,17 β -diol	2.8	4.1	0.8	5.8
Reduction	Cofactor . . . NADH		NADPH	
	K_m	V_{max}	K_m	V_{max}
5 α -Androstane-3,17-dione	1.4	1.2	0.56	1.5
5 α -Androstan-17 β -ol-3-one	5.5	1.0	0.89	1.3
5 α -Oestrane-17 α -ethynyl-17 β -ol-3-one	2.5	1.7	0.77	1.9

Fig. 6. H.p.l.c. of reaction products formed by incubating [1,2- ^3H]dihydrotestosterone with purified GP converting protein and NADPH

Incubations were performed at pH 7.4 and 37 °C for 15 min. The concentrated ethyl acetate extracts of the reaction mixture were subjected to reverse-phase h.p.l.c. with a methanol/water (7:3, v/v) solvent system. Fractions (0.3 ml) were collected from the detector outlet and assayed for radioactivity. The retention times for authentic dihydrotestosterone and 5 α -androstane-3 α ,17 β -diol are as indicated.

major GP component (retention time 3.4 min). This was eluted before GP1. For this reason it is thought that this steroid does not accumulate in microsome+cytosol incubation mixtures but as acted on directly by the cytoplasmic 3 α -hydroxysteroid dehydrogenase.

Using dihydrotestosterone as the substrate, with NADPH as cofactor at pH 7.4, indomethacin inhibited the purified enzyme with an I_{50} value of $4.9 \pm 0.7 \mu\text{M}$ (mean $\pm$ S.E.M., four experiments).

Although it cannot be excluded that some minor protein co-purifies with the purified protein, its M_r , isoelectric point, enzymic activity towards steroids such as androsterone or dihydrotestosterone (Table 2) and the inhibition of enzymic activity towards the latter substrate by indomethacin (Smithgall & Penning, 1985), strongly suggest that the GP converting protein is the same as 3 α -hydroxysteroid dehydrogenase.

Reaction of GP converting protein with antibodies

Antibodies raised in rabbits against the purified protein gave a single Ouchterlony immunoprecipitation line against cytosol from either male or female rats (Fig. 7a). They showed no cross-reactivity with the proteins that were eluted in the void volume after DEAE-cellulose chromatography and which were able to oxidize androsterone (Fig. 5). The antibody progressively inhibited 3 α -hydroxysteroid dehydrogenase activity of cytosol (Fig. 7b). The ability of cytosol to promote the formation of GP 2, 3 and 4 was similarly inhibited after

Fig. 7. Properties of rabbit antibodies to rat liver GP converting protein

(a) Ouchterlony double-immunodiffusion plates of immune rabbit serum versus rat liver cytosol and purified converting protein. Ouchterlony plates were prepared from 1% (w/v) agarose as described in the Experimental section. The centre well contains: DEAE mAffi-Gel-Blue-purified rabbit serum. Outer wells contain: 1, male rat liver cytosol; 2, female rat liver cytosol; 3, void volume proteins from DEAE-cellulose column chromatography of male rat liver cytosolic fraction; 4, purified GP converting protein. (b) Effects of pre-incubation of rat liver cytosol with rabbit anti-(GP converting protein) serum on 3 α -hydroxysteroid dehydrogenase activities or the accumulation of GP 2, 3 and 4 from microsomal incubation mixtures exposed to norethindrone. Dialysed male-rat liver cytosol (2 mg of protein) was incubated with various amounts (as indicated) of DEAE Affi-Gel-Blue-purified rabbit serum IgG fraction (35 mg/ml of protein) at 4 °C for 18 h. Portions were then assayed either for 3 α -hydroxysteroid dehydrogenase activities using dihydrotestosterone as substrate and NADPH as cofactor at pH 7.4 (○) or for GP converting activity. This was calculated as GP 2, 3 and 4, expressed as a percentage of total GP formed after 15 min incubation with rat liver microsomes, norethindrone and an NADPH-generating system (□). Results represent the means for two experiments.

preincubation with this antibody. DEAE Affi-Gel-Blue-purified pre-immune rabbit serum had little effect on either reaction.

Scheme 1. Proposed scheme of metabolism of norethindrone and structures of GPs

Metabolic modification of norethindrone leading to the formation of GP1, 2, 3 and 4

Scheme 1 shows the proposed scheme for the metabolism of norethindrone. Each green pigment arises as a result of the cytochrome *P*-450-dependent oxidative activation of the 17 α -ethynyl substituent of the steroid (White & Muller-Eberhard, 1977; Ortiz de Montellano *et al.*, 1979), presumably via an oxirene intermediate (Kunze *et al.*, 1983; Ortiz de Montellano & Kunze, 1980). From the time of appearance of the GPs in microsome + cytosol experiments (Fig. 3*a*) or in hepatocyte systems *in vitro* (Blakey & White, 1986) and from m.s. evidence (Fig. 2), it is suggested that GP1 formed first represents the 1:1 covalent adduct between the activated norethindrone and protoporphyrin IX as proposed by Ortiz de Montellano & Kunze (1980) (GP1, Scheme 1). Little or none of this component accumulates in the liver of rats dosed with norethindrone *in vivo* (Fig. 1*b*; White, 1981).

Our results suggest that first, microsomal Δ^4 -3-oxo-steroid 5 α -reductase acts on norethindrone to yield 5 α -oestrane-17 α -ethynyl-17 β -ol-3-one (Scheme 1, II). This is followed in concert by the cytoplasmic converting protein, 3 α -hydroxysteroid dehydrogenase, yielding 5 α -oestrane-17 α -ethynyl-3 α ,17 β -diol (Scheme 1, III). Oxidative activation of the ethynyl substituent of this metabolite results in the formation of GP2. This is consistent with m.s. data (Fig. 2*b*) and the finding that this steroid, formed enzymically when added to microsome systems *in vitro*, results in the formation of GP2,

3 or 4. Such a reductive pathway is known to be a major route of disposition of norethindrone in rats *in vivo* (Ranney, 1977).

M.s. (Figs. 2*c*, and 2*d*) suggests GP 3 and 4 arise as a result of further hydroxylation of this 5 α -oestrane-17 α -ethynyl-3 α ,17 β -diol intermediate (Scheme 1, IV). Although the site of hydroxylation of the steroid ring is not known, such a cytochrome *P*-450-dependent reaction would explain why the formation of GP3 and 4 is preferentially inhibited by high concentrations of steroids or certain mixed-function-oxidase inhibitors such as compound SKF 525A (Blakey & White, 1985).

Role of cytochrome *P*-450

Female-rat liver microsomes incubated with norethindrone and a NADPH-generating system form GP at a much lower rate than do microsomes from male rats. In addition, female rats given norethindrone *in vivo* form only GP1 and 2 in their livers (White *et al.*, 1984). Female-rat cytosol contains 3 α -hydroxysteroid dehydrogenase protein (Fig. 4) (Lax *et al.*, 1983). The present results suggest they also contain sufficient microsomal norethindrone Δ^4 -3-oxosteroid reductase required for the formation of 5 α -oestrane-17 α -ethynyl-3 α ,17 β -diol (Scheme 1, III), but lack, or have very low levels of, the cytochrome *P*-450-dependent enzymes needed to carry out ring hydroxylation of this intermediate necessary for the subsequent production of GP 3 and 4.

The evidence suggests that the different patterns of GPs after the administration of 17 α -ethynyl-substituted

steroids apart from norethindrone in the rat (White, 1981) or norethindrone in species other than the rat (White *et al.*, 1984) reflect the different routes of metabolism of these compounds. As a consequence of the high affinity for the steroids by enzymes involved in their metabolism (Table 2; White, 1981), only low concentrations of metabolite need to accumulate before either further metabolism occurs at a site apart from the ethynyl function, or activation of the ethynyl substituent occurs, and this can result in the formation of a new *N*-alkylated porphyrin. Alkylating agents which arise as a result of metabolic activation of chemically less complex structures, e.g. ethylene or diethylnitrosamine, form only one GP, as judged by h.p.l.c. (White *et al.*, 1983). In such cases the possibility of further metabolism of the parent compound while retaining the ability for subsequent metabolic activation is limited. Among non-steroidal compounds giving rise to *N*-alkylated porphyrins (e.g. De Matteis *et al.*, 1980; Ortiz de Montellano & Mathews, 1981; Coffman *et al.*, 1982), it is to be expected that further examples of the formation of multiple GPs arising by routes analogous to those described here will be found.

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