Biochemical Pharmacology, Vol. 33, No. 3, pp. 459–464, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

SPECIES DIFFERENCES IN THE HEPATIC FORMATION OF GREEN PIGMENTS FOLLOWING THE ADMINISTRATION OF NORETHINDRONE

IAN N. H. WHITE
MRC Toxicology Unit, Woodmansterne Road, Carshalton, Surrey SM5 4EF, U.K.

ALAN R. BOOBIS and DONALD S. DAVIES
Dept of Clinical Pharmacology, Royal Postgraduate Medical School, Ducane Road, London W12 0NS, U.K.

(*Received* 19 *July* 1983; *accepted* 15 *September* 1983)

Abstract—Metabolic activation of the ethynyl substituent of the contraceptive steroid norethindrone to cause the loss of hepatic cytochrome P-450 and the formation of green pigments has been compared *in vivo* and *in vitro* in rat, hamster, guinea pig, rabbit, mouse and hen and with marmoset and human liver microsomal preparations *in vitro*.

In vivo green pigment accumulation in the liver 4 hr after the administration of norethindrone (100 mg/kg, i.p.) varied 60-fold between species. Male rat was the most active in this respect, the hen was the least active. The accumulation of green pigments in female rats was 27% that of male animals. This sex-dependent difference was not seen in male and female mice. Cytochrome P-450 destruction *in vivo* was also greatest in the male rat given norethindrone, whereas no loss was detected in the hen. In other species, however, the correlation between green pigment accumulation and cytochrome P-450 destruction was not particularly good.

When liver microsomes were incubated with norethindrone and an NADPH generating system *in vitro*, the ranking order between species with respect to the initial rates of green pigment formation was similar to that based on the hepatic accumulation of these compounds found *in vivo*.

Human liver microsomes showed initial rates of green pigment formation which were only 2% of that seen in the male rat. No destruction of human microsomal cytochrome P-450 caused by norethindrone could be detected.

The HPLC elution profile of the green pigments produced in the liver following the administration of norethindrone differed between species. Hepatic microsomal preparations in contrast, at least with short incubation times, formed only one green pigment. Results suggest that further metabolism of either norethindrone or the green pigment, involving a cytosolic factor(s), results in the varied HPLC patterns seen *in vivo*.

Metabolic activation of the 17α-ethynyl substituent of certain contraceptive steroids such as norethindrone (17-hydroxy-19nor-17α-pregn-4-en-20yn-3-one), results in the formation of reactive intermediate(s) which cause the destruction of hepatic cytochrome P-450 and the formation of abnormal green pigments [1, 2]. Such green pigments represent a covalent adduct between the activated steroid and the protoporphyrin IX ring of haem in a 1:1 molar ratio [3]; the site of alkylation being one of the nitrogen atoms of the porphyrin tetrapyrrole ring [4].

Inactivation of cytochrome P-450 by acetylenes is not confined to ethynyl-steroids but occurs with many terminally substituted lipophilic ethynyl-compounds [5, 6]. The mechanism of oxidation of the triple bond which leads to the formation of the reactive species has not been defined, though the evidence so far suggests the initial formation of an unstable oxirene intermediate [7, 8]. Activation of the ethynyl substituent of norethindrone to an intermediate responsible for green porphyrin formation is carried out by the phenobarbital-inducible NADPH-dependent mixed function oxidases [1]. Factors affecting the activities of this enzyme system have been examined in the rat both *in vivo* and *in vitro* [1,2,6]. However, as far as is known, no studies have been carried out on this aspect of norethindrone activation in other species or in man.

Results in this paper compare the norethindrone mediated destruction of hepatic cytochrome P-450 and the formation of green pigments in various laboratory species *in vivo* and *in vitro* with data obtained using human liver microsomal preparations *in vitro*.

MATERIALS AND METHODS

Norethindrone, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were from the Sigma Chemical Co. (Poole, Dorset).

Animals and dosing. Male and female Fischer F.344/N rats (140–160 g), C57 BL/10 ScSn mice (20–25 g) and 2 female marmosets (275 and 320 g) were bred on site. Male Syrian golden hamsters (100–120 g) and male albino Hartley strain guinea pigs were from OLAC (1976) Ltd (Bicester, Oxfordshire). Male Dutch rabbits (2.0–2.3 kg) were from Hylene Rabbits Ltd (Northwich, Cheshire) and female chickens (1.2–1.8 kg) were obtained from Southern Biological Supplies (Carshalton, Surrey).

Human liver samples were obtained from renal transplant donors who had met traumatic death and were maintained on life support systems until the kidneys were removed. Donors were not receiving any chronic drug treatment but were given such drugs as necessary while on life support systems.

Norethindrone acetate was dissolved in trioctanoin (usually 50 mg/ml) and administered intraperitoneally at a dose of 100 mg/kg. Animals were killed 4 hr after dosing unless indicated otherwise. Controls received trioctanoin only. In some instances animals were pretreated with phenobarbitone in their drinking water (0.1% w/v) for 7 days prior to dosing with norethindrone.

Estimation of cytochrome P-450. Livers were perfused with ice-cold 0.9% w/v saline, rinsed, blotted dry and weighed. Since green pigments are excreted into the bile [9], in those species with gall bladders, care was taken to remove this organ. Liver homogenates 10% (w/v) were prepared in ice-cold 0.25 M sucrose. In order to reduce interference from contaminating haemoglobin, cytochrome P-450 was estimated in liver homogenates by the CO-reduced vs. the CO-oxidised difference spectrum as described by McLean and Day [10].

Cytosolic and washed microsomal fractions were prepared from untreated animal [11] or human [12] liver homogenates by differential centrifugation. Microsomal pellets were suspended in 0.25 M phosphate buffer pH 7.4 containing 30% (v/v) glycerol. Protein concentrations were estimated by the method of Lowry *et al.* [13] using a bovine serum albumin standard.

Estimation of cytochrome P-450 *destruction* in vitro. Incubation mixtures of 3 ml volume and containing norethindrone (0.3 mM) a NADPH generating system and liver microsomes (4–5 mg protein) were as described previously [6]. Following incubation for 10 min at 37°, flasks were placed on an ice–salt mixture and cytochrome P-450 estimated from the CO-reduced vs. the reduced difference spectrum [14]. Results were expressed relative to controls incubated for the same time but with norethindrone omitted from the reaction mixture. In some instances where indicated, the reaction time, or the norethindrone concentration was changed or cytosol (0.5 ml, 5–6 mg protein) was included in the incubation mixtures.

Estimation of green pigment formation: (a) in vitro. The composition of the reaction mixtures were the same as those described above. Following incubation for various times up to 10 min, the reactions were stopped by taking 2 ml of the mixture and adding it to 40 ml ice-cold 5% (v/v) concentrated H_2SO_4 in methanol. Tubes were left for 18 hr at 4° in the dark. The esterified products were extracted into chloroform as described previously [6].

Estimation of green pigment formation: (b) in vivo. Tissues (1 g) were homogenised in 20 ml 5% H_2SO_4 in methanol using an Ultraturrax homogeniser. The tubes were processed as described above.

Separation of haem from green-pigments. This was carried out by high performance liquid chromatography. Silica gel columns (25 × 0.47 cm, Machery Nagel, Nucleosil 50:5, Camlab, Cambridge) were used with a cyclohexane/chloroform/methanol (4:2:1 v/v) solvent system containing 0.2% (v/v) acetic acid. The flow rate was 1.5 ml min^{-1}. Detector wavelength was 417 nm. Peak areas were determined using a Pye-Unicam DP88 integrator. Green pigments were located initially in HPLC eluents by fluorescence detection using a Kratos FS-970 fluorimeter coupled to the outlet of the absorbance detector. Excitation wavelength was 417 nm, emission filter was >550 nm.

TLC. TLC was carried out using silica gel 60 plates layer thickness 0.25 mm (E. Merck, Darmstadt, F.R.G.) with a solvent of chloroform/methanol (85:15 v/v). In some instances the green pigments were scraped off and eluted with chloroform/methanol (1:1 v/v).

RESULTS AND DISCUSSION

Separation of green pigments formed in vivo *by HPLC.* Liver extracts from male rats given norethindrone and subjected to HPLC gave a complex pattern of 3 major green pigments (retention times 7.2, 10.1, 11.2 min, Fig. 1(a)). Unexpectedly, female rats given norethindrone in addition to accumulating less green pigment than males, also showed a different pattern following HPLC. Only one major green pigment was isolated (retention time 7.2 min, Fig. 1(b)). Female rats killed at various times (1–8 hr) after dosing with norethindrone formed only this component. However, pretreating female rats with the mixed function oxidase inducer phenobarbitone, prior to the administration of norethindrone resulted in an elution pattern of green pigments similar to that seen in male rats (Fig. 1(a)). This result suggests that in the rat, the later eluting, more polar, green pigments may be the result of further mixed function oxidase dependent metabolism of the steroid either prior to or after alkylation of haem. Treatment of rats or mice with ethylene gas or diethylnitrosamine results in the protoporphyrin IX ring of hepatic haem being *N*-hydroxyethylated [4, 15] while treatment with the compound 3,5-diethoxycarbonyl-4-ethyl-1,3-dihydro-2,6-dimethyl pyridine results in *N*-ethylation [16]. In each of the above examples, only one component was resolved by HPLC [15]. If metabolic modification of the protoporphyrin IX ring of the green pigment could occur, multiple components might have been expected. It is therefore suggested that with norethindrone, metabolic modification of the steroid moiety rather than the protoporphyrin IX ring is responsible for the multiple green pigment components reported in the present experiments. Attempts to identify further the individual norethindrone green pigments have not so far been successful. Previous studies have shown that those produced by the male rat all have similar absorption spectra and extinction coefficients [2].

Other species showed HPLC elution profiles which differed from that found in the rat. One major component Green Pigment 1 (retention time 6.2 min) appeared to be common to these other species but additional green pigments differed between species. Fig. 1(c)–(e) shows the results for mouse, hamster and rabbit. The HPLC trace for hen liver extracts was similar to that of rabbits, guinea pig was similar,

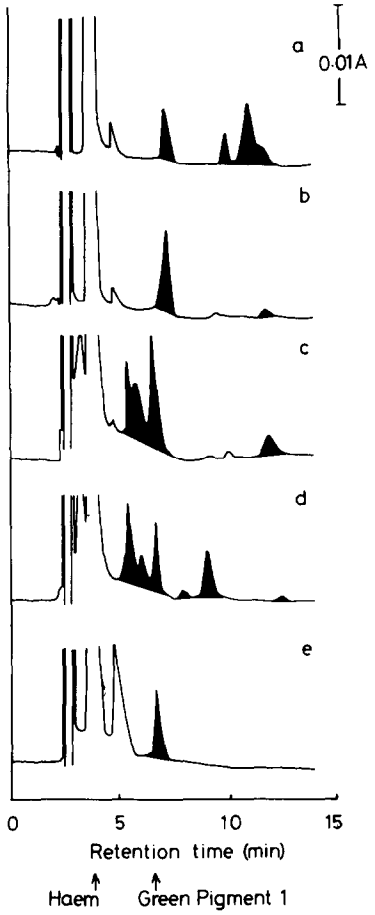

Fig. 1. HPLC elution profiles of green pigments isolated from the livers of various species given norethindrone (100 mg/kg) intraperitoneally and killed 4 hr after dosing. (a) Male rat; (b) female rat; (c) mouse; (d) hamster; (e) rabbit. Ordinates represent relative absorption at 417 nm. Green pigment peaks are shown shaded black.

though not identical, to mouse (not shown). Pretreatment of mice with phenobarbitone, although increasing the amount of the green pigment formed, did not change the HPLC elution profile to one similar to that seen in rats.

The major green pigments isolated and purified by TLC from the various species all showed a similar neutral aetio-type absorption spectrum in chloroform λ_{max} 417, 513, 546, 592 and 647 nm. Concentrations of green pigments were estimated from the peak areas following HPLC based on an extinction coefficient in chloroform of 106,000 l mol^{-1}cm^{-1} at 417 nm [2].

Time course for green pigment formation. Figure 2 shows the time course for the accumulation of green pigments in the livers of male mice given norethindrone. Concentrations rose rapidly in the liver to reach maximum values 2–4 hr after dosing, then declining to low levels by 24 hr. A similar time course has been reported in the rat [2]. In order to compare the accumulation of green pigments in the liver between species the concentrations 4 hr after dosing were used. Norethindrone treatment did not significantly affect the liver weight expressed as a percentage of the body weight at this time.

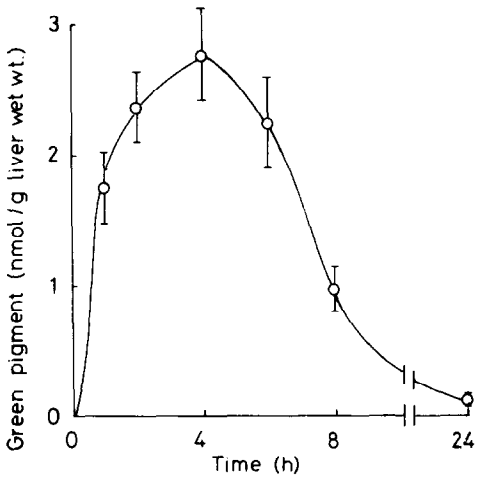

Fig. 2. Accumulation of green pigments in the livers of male mice at various times following the administration of norethindrone (100 mg/kg) intraperitoneally. Results represent the mean ± S.E. of four animals/group.

Species variations in hepatic green pigment concentrations following norethindrone treatment: relationship to cytochrome P-450 destruction. Table 1 shows that the concentrations of green pigments accumulated in the liver 4 hr after dosing with norethindrone varied over a 60-fold range of values between the species examined. Male rats were the most active in this respect; hens were the least active. While female rats accumulated less green pigment than males, this sex-dependent difference was not observed in mice. Lower rates of metabolism of xenobiotics have been observed generally in female rats, relative to males, but this sex-dependent difference appears to be mostly absent in other species, including man [17]. No green pigment formation could be detected in organs apart from the liver even though in some species, e.g. rabbit, relatively high levels of mixed function oxidases are found in other organs such as the lung [18, 19].

The correlation between the concentrations of green pigments accumulated in the liver and the amount of hepatic cytochrome P-450 destroyed following dosing with norethindrone was not particularly good (Table 1). Although the male rat, most active in the accumulation of green pigments, also showed the greatest loss of cytochrome P-450, in other species, e.g. the mouse, much greater cytochrome P-450 destruction occurred than would have been predicted from the amount of green pigment formed. Previous studies in the rat showed there to be generally good correlation between these parameters [2]. In other species, factors such as lipid peroxidation may contribute to cytochrome P-450 loss [20]. The present results indicate green pigment formation to be a more sensitive and more specific index of metabolic activation of the ethynyl substituent of norethindrone than cytochrome P-450 destruction.

Formation of green pigments by microsomal systems in vitro. Figure 3 shows that following incubation of microsomes from untreated animals with norethindrone and a NADPH generating system,

Table 1. Formation of green pigments and loss of cytochrome P-450 in the livers of various species after the administration of norethindrone *in vivo**

Species		Green pigments (nmol/g liver wet-wt)	Initial homogenate cytochrome P-450 (nmol/g liver wet-wt)	Cytochrome P-450 remaining (%)†
Rat	♂	31.3 ± 4.0	48.4 ± 1.6	63.6 ± 3.6
Rat	♀	8.5 ± 0.9	56.4 ± 1.8	87.6 ± 4.7
Hamster	♂	9.8 ± 0.4	63.4 ± 5.2	87.7 ± 8.0
Guinea pig	♂	3.7 ± 1.0	47.8 ± 2.4	98.1 ± 9.3
Rabbit	♂	3.3 ± 1.2	57.3 ± 5.2	91.4 ± 8.1
Mouse	♂	3.0 ± 0.6	56.2 ± 2.0	70.7 ± 6.4
Mouse	♀	2.9 ± 0.8	56.9 ± 2.0	70.0 ± 5.8
Hen	♀	0.5 ± 0.2	18.2 ± 2.8	99.0 ± 8.1

* Results represent the mean ± S.E. from 4 animals. Norethindrone was administered intraperitoneally at a dose of 100 mg/kg and the animals were killed 4 hr after dosing.

† Cytochrome P-450 remaining is expressed relative to controls given trioactonoin vehicle only and killed at the same time.

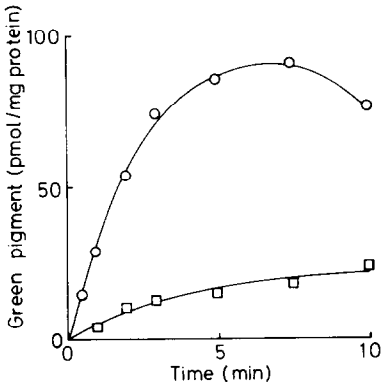

Fig. 3. Rate of formation of green pigments in liver microsomal preparations *in vitro*. Incubation mixtures of 3 ml volume contained 4–5 mg microsomal protein from untreated animals, norethindrone (0.3 mM) and an NADPH generating system. Reactions were stopped with 5% concentrated H_2SO_4 in methanol and green pigment concentrations subsequently estimated using HPLC. Results represent the mean of 2 experiments. Male rat liver microsomes (○——○). Female marmoset microsomes (□——□).

there was a time dependent increase in the formation of green pigment. Although the shape of the progress curve differed slightly between species, reaction rates always rapidly became non-linear with time. This was not due to substrate depletion since saturating concentrations were employed [2]. Such non-linearity has been attributed to the self-catalysed destruction of the forms of cytochrome P-450 responsible for the metabolic activation of norethindrone [8]. In order to make comparisons between species the 2 min time point from the linear part of the progress curve was generally used.

Table 2 shows initial rates of green pigment formation to span a 100-fold range of values. Highest activities were in hepatic microsomes from male rats, the lowest activities were in the hen. The ranking order between species in this respect (with the exception of the female rat) was the same both *in vivo* and *in vitro*. Female rat liver microsomes showed much lower rates of formation of green pigment *in vitro* than would have been predicted from the *in vivo* results. The reason for this disparity is not known.

Table 2. Formation of green pigment and loss of cytochrome P-450 caused by norethindrone in liver microsomal preparations *in vitro**

Species		Green pigments (pmol/min/mg protein)	Initial microsomal cytochrome P-450 (nmol/mg protein)	Cytochrome P-450 remaining (%)†
Rat	♂	40.8 ± 4.5	0.98 ± 0.06	82.6 ± 2.4
Rat	♀	3.2 ± 0.3	0.79 ± 0.04	100 ± 1.5
Hamster	♂	19.5 ± 6.0	1.24 ± 0.19	90.4 ± 2.0
Guinea pig	♂	15.5 ± 3.8	1.45 ± 0.03	88.6 ± 3.5
Rabbit	♂	10.6 ± 2.3	2.07 ± 0.22	101 ± 5.4
Mouse	♂	8.3 ± 1.3	1.30 ± 0.03	87.0 ± 1.5
Mouse	♀	8.5 ± 0.8	1.13 ± 0.05	87.5 ± 5.3
Marmoset	♀	1.8	0.39	100
Human‡	♂	0.8 ± 0.3†	0.07 ± 0.04	100 ± 3.6
Hen‡	♀	0.4 ± 0.2†	0.34 ± 0.03	91.3 ± 3.4

* Results represent the mean ± S.E. from 4 animals except for the marmoset where two animals were used.

† Loss of cytochrome P-450 caused by incubating hepatic microsomes with norethindrone (0.3 mM) and NADPH generating system for 10 min at 37° is expressed relative to controls incubated for the same time but with norethindrone omitted from the mixture.

‡ Green pigment formation determined following 10 min incubation. In all other instances, a 2 min incubation time was used.

Cytochrome P-450 destruction again proved to be a less sensitive and less specific guide to the metabolic activation of norethindrone. Longer incubation times (10 min) had to be used in order to obtain a measurable loss of this cytochrome while in some species, e.g. hen or marmoset, no cytochrome P-450 destruction could be detected over this time.

Separation of green pigments formed in vitro *by HPLC.* The elution profile of the green pigment produced by liver microsomes in the presence of an NADPH generating system and norethindrone was the same in all species examined, at least when short (2 min) incubation times were used. Figure 4(a)–(d) shows absorbance and fluorescence HPLC traces for rat and human microsomal extracts. Only one green pigment is formed. This had a retention time of 6.2 min and co-chromatographed both on HPLC and TLC with Green pigment 1 produced by the majority of species *in vivo*. The aetio-type absorption spectrum of the purified microsomal green pigment was similar to that of green pigment 1.

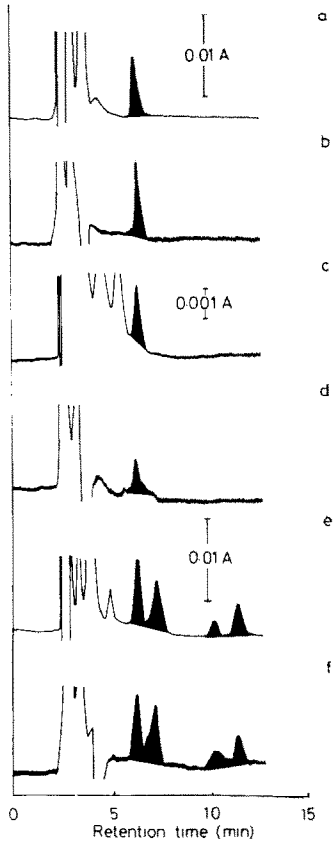

Fig. 4. HPLC elution profile of green pigment produced by microsomal suspensions *in vitro* when incubated with norethindrone and an NADPH generating system. (a), (b): Male rat liver. Incubation time 2 min. Norethindrone concentration 0.3 mM. (c), (d): Human liver microsomes incubated for 10 min with 0.3 mM norethindrone. (e), (f): Microsomes from a phenobarbitone pretreated male rat were incubated for 20 min with 0.05 mM norethindrone and rat liver cytosol (6–7 mg protein). Ordinates a, c and e represent relative absorbance at 417 nm, b, d and f represent relative fluorescence. Excitation wavelength 417 nm, emission >550 nm. Green pigment peaks are shown shaded black.

Male rat liver microsomes incubated with norethindrone and a NADPH generating system for longer periods of time (20 min) do not give an *in vivo* pattern of green pigments on HPLC [2]. However, if cytosol (5–6 mg cytosolic protein) is added to the incubation mixture a pattern of green pigments approaching that seen *in vivo*, but with the addition of green pigment 1, is produced (Fig. 4(e) and (f)). Similar *in vivo* type chromatograms are obtained in other species using microsomes + cytosol, e.g. mouse liver microsomes + mouse liver cytosol. The identity of the cytosolic factor and its interaction with the microsomal mixed function oxidases is being further investigated (D. C. Blakey and I. N. H. White, unpublished work).

Relationship between pigment porphyrins produced in vivo *and* in vitro. It is proposed that green pigment 1 is produced initially *in vivo* and *in vitro* in the livers of all the animal species examined here following exposure to norethindrone and this product represents the 1:1 covalent adduct between the activated steroid and the protoporphyrin IX ring of haem. *In vivo* in some species, e.g. the rabbit (Fig. 1(e)), no further metabolism occurs and only this component accumulates in the liver. In other species, e.g. the mouse and hamster (Fig. 1(c) and (d)) green pigment 1 accumulates in the liver but additional metabolism occurs to form components with both shorter and longer retention times following HPLC. In the case of the rat *in vivo*, even at the earliest times after dosing no Green Pigment 1 can be detected. As this component is formed in rat liver microsomal incubation mixtures *in vitro*, it is suggested that its formation *in vivo* is transitory, being rapidly lost due to further metabolism. More detailed characterisation of the individual green pigments remains to be achieved.

Metabolic activation of norethindrone by human liver microsomal preparations. Table 2 shows that rate of green pigment formation by human liver microsomes incubated with norethindrone to be only about 2% that of male rat liver microsomes. Rates were also low compared with most other mammalian species tested. No loss of cytochrome P-450 caused by norethindrone could be detected in human liver microsomal preparations during 10 min incubation.

As reported by Walker [17] mixed function oxidases of human liver microsomes (expressed on a per mg of protein basis) are generally of lower activity in man than in rodent. However, Benford *et al.* [21] have reported that towards certain xenobiotics e.g. 3′ and 4′-biphenyl hydroxylation, human liver microsomes do show similar levels of activity to those of rats. Chronic administration of norethindrone to rats results in an increased incidence of liver tumours [20]. The slightly increased incidence of liver tumours in women taking oral contraceptives may be related to the use of these steroids [23, 24]. It is not known the role, if any, that metabolic activation of the ethynyl substituent of these steroids plays in such tumorigenic side effects. The present results do show however that in humans, hepatic microsomal activation of the ethynyl substituent of the contraceptive steroids, as judged by green pigment formation, to be very low relative to rodent species examined here.

Acknowledgement—The expert technical assistance of Janet Cox is gratefully acknowledged.

REFERENCES

1. I. N. H. White and U. Muller-Eberhard, *Biochem. J.* **166**, 59 (1977).
2. I. N. H. White, *Biochem. J.* **196**, 575 (1981).
3. P. R. Ortiz de Montellano, K. L. Kunze, G. S. Yost and B. A. Mico, *Proc. natn. Acad. Sci. U.S.A.* **76**, 746 (1979).
4. F. De Matteis and A. H. Gibbs, *Biochem. J.* **187**, 285 (1980).
5. I. N. H. White, *Biochem. Pharmac.* **29**, 3253 (1980).
6. I. N. H. White, *Biochem. J.* **174**, 835 (1978).
7. A. Wade, A. M. Symons, L. Martin and D. V. Parke, *Biochem. J.* **184**, 509 (1979).
8. P. R. Ortiz de Montellano and K. L. Kunze, *J. biol. Chem.* **255**, 5578 (1980).
9. I. N. H. White, *Biochem. Pharmac.* **31**, 1337 (1982).
10. A. E. M. McLean and P. A. Day, *Biochem. Pharmac.* **23**, 1174 (1974).
11. I. N. H. White, *Chem. Biol. Interact.* **29**, 103 (1980).
12. A. R. Boobis, M. J. Brodie, G. C. Kahn, D. R. Fletcher, J. H. Saunders and D. S. Davies, *Br. J. clin. Pharmac.* **9**, 11 (1980).
13. O. H. Lowry, N. J. Rosebrough, A. L. Fair and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
14. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
15. I. N. H. White, A. G. Smith and P. B. Farmer, *Biochem. J.* **212**, 599 (1983).
16. F. De Matteis, A. H. Gibbs, P. B. Farmer and J. H. Lamb, *FEBS Lett.* **129**, 328 (1981).
17. C. H. Walker, *Drug Metab. Rev.* **7**, 295 (1980).
18. R. Kato, *Drug Metab. Rev.* **3**, 1 (1974).
19. T. E. Gram, *Drug Metab. Rev.* **2**, 1 (1973).
20. S. G. Levine, *Life Sci.* **31**, 779 (1982).
21. D. J. Benford, J. W. Bridges, A. R. Boobis, G. C. Kahn, M. J. Brodie and D. S. Davies, *Biochem. Pharmac.* **30**, 1702 (1982).
22. *Carcinogenicity Tests of Oral Contraceptives Committee on the Safety of Medicines.* Her Majesty's Stationery Office, London (1972).
23. K. G. Ishak, in *Recent Results in Cancer Research* (Ed. C. H. Lingeman) Vol. 66, pp. 73–128. Springer Verlag, Berlin (1979).
24. S. Sherlock, in *Primary Liver Tumours* (Eds. H. Remmer, H. M. Bolt, P. Bannash and H. Popper), Falk Symposium No. 25, pp. 201–216. M.T.P. Press Ltd., Lancaster (1978).