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Biochemistry

Self-catalyzed destruction of cytochrome *P*-450: Covalent binding of ethynyl sterols to prosthetic heme

(biooxidation/porphyrins/green pigment/acetylene metabolism)

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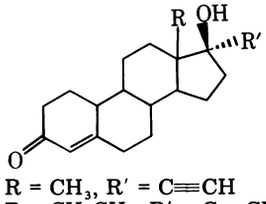
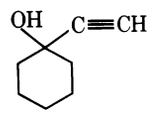
ABSTRACT **The hepatic pigment accumulated as a consequence of the self-catalyzed destruction of cytochrome *P*-450 by norethisterone (17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one), after acidic methylation and purification, consists of two virtually identical, probably isomeric, porphyrins. Radiolabeled norethisterone is incorporated into both porphyrin products. The major of the two porphyrins exhibits a mass spectrometric molecular ion exactly equivalent to the sum of norethisterone and dimethylprotoporphyrin IX, less two hydrogen atoms: unequivocably demonstrating covalent association of the sterol with this porphyrin in a 1:1 ratio. Cytochrome *P*-450 is therefore destroyed by self-catalyzed addition of norethisterone to its heme prosthetic group. Cytochrome *P*-450 is also destroyed by norgestrel (13-ethyl-17-hydroxyl-18,19-dinor-17α-pregn-4-en-20-yn-3-one) and by 1-ethynylcyclohexanol but not by 17-hydroxy-19-nor-17α-pregn-4,20-dien-3-one. The destructive potential is thus clearly a property of the propargylic alcohol function. A mechanism involving enzymatic oxidation of the triple bond is postulated.**

Norethisterone (17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one, **1**), which contains the 17β-hydroxyl-17α-ethynyl substitution pattern characteristic of virtually all orally active contraceptive agents (1), diminishes the drug-metabolizing activity of the liver (2, 3). One contribution to this perturbation was revealed by the recent discovery that norethisterone causes a time- and dose-dependent inactivation of cytochrome *P*-450 both *in vitro* and *in vivo* (4). Hemoprotein loss occurs only in the presence of NADPH and oxygen and is inhibited by carbon monoxide, characteristics that implicate catalytic turnover of cytochrome *P*-450 in its own destruction (4). A green-brown pigment, extracted with acidic methanol from the livers of norethisterone-treated rats, provides a firm link between this process and the analogous destruction of cytochrome *P*-450 by 2-isopropyl-4-pentenamide (allylisopropylacetamide), in which a similar pigment is formed (5–9). Our recent discovery that 2-isopropyl-4-pentenamide is covalently bound to cytochrome *P*-450 heme (10, 11) in a suicidal interaction led us to investigate the norethisterone-mediated process, the first results of which are presented here.



1 R = CH_3, R' = C≡CH
2 R = CH_2CH_3, R' = C≡CH
3 R = CH_3, R' = CH=CH_2



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MATERIALS AND METHODS

Pigment Isolation and Characterization. Sprague–Dawley male rats weighing approximately 250 g were treated with phenobarbital (80 mg/kg daily) for 4 days prior to intraperitoneal injection of norethisterone (25 mg/kg) in 0.25 ml of trioctanoin (mixture sonicated prior to injection). Four hours after injection of the sterol, the rats were decapitated and the livers were perfused with cold 0.9% saline. The excised livers were homogenized in 5% (vol/vol) H_2SO_4/methanol (4 ml/g of liver) and allowed to stand in the same mixture overnight in the dark at 4°C (4). The mixture was filtered and equal volumes of water and chloroform were added. The chloroform extract, after washing with water, drying (Na_2SO_4), and solvent removal, was chromatographed on an Analtech preparative thin-layer chromatography plate [chloroform/acetone, 2:1 (vol/vol)]. Unless isolated for individual study, the brown-green bands with intense red fluorescence ($R_F \approx 0.4$ and 0.6) were combined and demetalated in 1 ml of 5% H_2SO_4 in methanol (12). The organic layer obtained by partitioning the mixture between chloroform and water (12) was dried, concentrated, and analyzed by high-pressure liquid chromatography (HPLC) on a Whatman 10-PAC column. The eluent was monitored by a variable wavelength detector (417 nm). Eluent fractions were collected as needed for further study or for radioscintillation counting (Aquasol). Zinc complexes were prepared by addition of zinc acetate as described (10, 11).

The isolation of pigment from 1-ethynylcyclohexanol-treated rats (50 mg/kg in 0.25 ml of ethanol) was performed by an identical procedure.

Mass spectrometric data were obtained on an MS-9 instrument adapted to a field-desorption ionization mode at the Berkeley Biomedical Mass Spectrometry Resource. All absorption spectra were recorded in chloroform on a Varian-Cary 118 spectrophotometer.

Cytochrome *P*-450 Destruction Assay. The loss of spectroscopically observable cytochrome *P*-450 in liver microsomes from phenobarbital-pretreated rats as a function of the incubation time with test substrates was measured essentially as described (6, 11). Each incubation mixture contained the following: substrate (1 mM), microsomal protein (1 mg/ml), NADPH (1 mM), KCl (150 mM), and EDTA (1.5 mM), all in 0.033 M phosphate buffer (pH 7.4). An Aminco DW-2 spectrophotometer was used. Protein concentrations were determined by the Lowry procedure (13).

Substrates. Specifically labeled [9,11-^{3}H]norethisterone [200 mCi (1 Ci = 3.7×10^{10} becquerels)/mmol] was a gift from Syntex. This substance was purified by silica thin-layer chromatography (chloroform/acetone, 2:1) and was diluted with unlabeled sterol to a specific activity of 0.22 mCi/mmol. Norgestrel (13-ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-

Abbreviation: HPLC, high-pressure liquid chromatography.

20-yn-3-one, **2**) was obtained from Wyeth Laboratories (Philadelphia, PA). Vinyl norethisterone analogue **3** (17-hydroxy-19-nor-17α-pregn-4,20-dien-3-one) was prepared by a literature procedure (14). Aldrich was the commercial source of 1-ethynylcyclohexanol (**4**), which was distilled prior to use.

RESULTS

The crude methylated pigment extracted with acidic methanol from the livers of norethisterone-treated rats or from hepatic microsomes incubated with the sterol was fractionated by thin-layer chromatography to remove an intractable contaminant that interfered with HPLC analysis. Thin-layer chromatography, however, resulted in the formation of artifactual metal complexes and, consequently, in the isolation of two or more green-brown bands exhibiting characteristic red fluorescence under ultraviolet light. The artifactual metal complexes were distinguished by their electronic absorption spectra with a Soret band at 432 nm and a trio of smaller peaks at 551, 592, and 632 nm in a pattern similar to that of the zinc complex (Fig. 1). The relationship of the artifactual complexes with the uncomplexed substance was established by the fact that removal of the metal with acidic methanol (12) converted the former to the latter, in striking analogy to our observations with the pigments obtained from rats treated with 2-isopropyl-4-pentenamide (10, 11). In practice, therefore, all the green-brown bands exhibiting intense red fluorescence were removed from the thin-layer plate, combined, and demetalated (12) prior to more detailed HPLC analysis.

Analysis of the demetalated sample by HPLC revealed the presence of two clearly resolved substances (see Fig. 2*A*) whose electronic absorption spectra, after isolation, were superimposable (Fig. 1). Addition of zinc acetate to a solution of either of the two isolated substances resulted in rapid formation of the corresponding zinc complex, both complexes again possessing identical electronic absorption spectra and, in this instance, identical HPLC retention times (see Fig. 2 *B* and *C*). Each parent substance, uncontaminated by any trace of the other, was regenerated from the corresponding zinc complex by demetalation with acidic methanol. The spectroscopic, complexing, and chromatographic properties of the two pigment components clearly characterize them as closely interrelated, probably isomeric, porphyrins.

Radiolabeled pigment was isolated from the livers of three rats treated with [9,11-^{3}H]norethisterone by the usual extraction/methylation, thin-layer chromatography, and demetalation sequence. The use of this specifically labeled substrate is important because, in contrast to the commercially available randomly labeled material, there is little exchange of label into the medium (15, 16). HPLC analysis of the demetalated, red-fluorescing, porphyrins (Fig. 2*A*) revealed that, in addition to radioactivity contributed by the nonchromophoric (at 417 nm) norethisterone, label was associated with both of the pigment porphyrin components. Label also was found in the zinc complex of the porphyrins formed in the HPLC precolumn, a reaction almost impossible to suppress due to traces of zinc left on the column by previous analyses. Each of the two free porphyrin peaks was collected and its electronic absorption spectrum was recorded to confirm its identity (Fig. 1). HPLC analysis of these collected fractions (Fig. 2 *B* and *C*) demonstrated that each was uncontaminated with label from the other. Addition of zinc acetate to each of the two fractions resulted in formation of the corresponding zinc complex, as judged by appearance of the correct absorption spectrum (Fig. 1). Most importantly, covalent association of the porphyrin chromophore with the sterol radioactivity was unambiguously demonstrated by the coincident HPLC shift in both chromophore and label accompanying the porphyrin-specific complexation step (Fig. 2 *B* and *C*). As expected, control experiments showed that the retention time of norethisterone is not affected by the presence of zinc acetate. In addition, correlation of associated radioactivity with chromophore absorbance for each of the two zinc complexes shows that the same amount of label *per* absorbance unit was present in both cases. More quantitative measurements, assuming a 1:1 sterol-to-porphyrin adduct (see below), suggest that the molar absorbance values (at 432 nm) for each of the complexes is approximately 123,000–125,000.

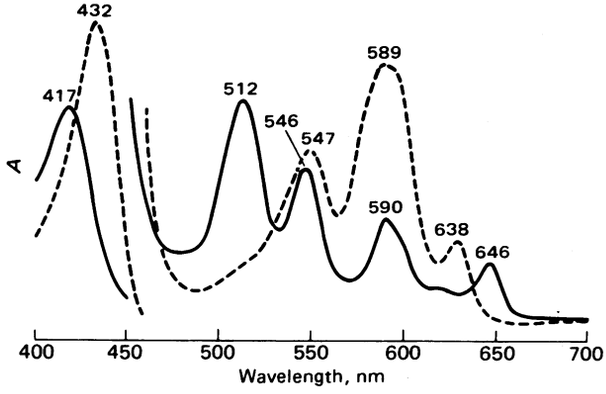


FIG. 1. Electronic absorption spectra in $CHCl_3$ of the sterol–porphyrin adducts isolated by HPLC (solid line) and the corresponding zinc complexes (broken line). The spectra of the two adducts separable by HPLC are superimposable in each instance.

A more detailed characterization of the pigment porphyrins has been achieved by mass spectrometric analysis, a nontrivial task given their high molecular weight, low vapor pressure, and instability as well as the microscopic amounts of available material. Nevertheless, a molecular ion is obtained for the major isomer at m/e 886 (after subtraction of 23 mass units due to complexed sodium) by field desorption mass spectrometry. This value, which is clearly different from the parent ion m/e 730 obtained for the pigment–porphyrin produced by 2-isopropyl-4-pentenamide (unpublished data), corresponds precisely to the sum of dimethylprotoporphyrin IX, the porphyrin expected from the prosthetic heme of cytochrome *P*-450, plus norethisterone minus two hydrogen atoms. The adducts are therefore 1:1 adducts of protoporphyrin IX with norethisterone, clearly pointing to a destructive mechanism in which the substrate is bound to the cytochrome *P*-450 prosthetic heme.

Intervention of the acetylenic group in the destructive process has been conclusively established by studies with substrate analogues, the results of which are summarized in Table 1. In contrast to the efficient destruction of cytochrome *P*-450 caused by *in vitro* incubation of norethisterone (**1**) as norgestrel (**2**) with liver microsomes, no destruction was caused by **3**, in which the

Table 1. *In vitro* destruction of hepatic microsomal cytochrome *P*-450

Compound (at 1 mM)	% destruction at given incubation time*		
	10 min	20 min	30 min
Norethindrone (**1**)	20 ± 5	25 ± 2	27 ± 3
Norgestrel (**2**)	18	34	34
Vinyl analogue **3**	0	0	3 ± 2
1-Ethynylcyclohexanol (**4**)	18 ± 6	22 ± 6	26 ± 6

* The values given are averages of three independent experiments, except for norgestrel, which was only examined once. SD is given where applicable.

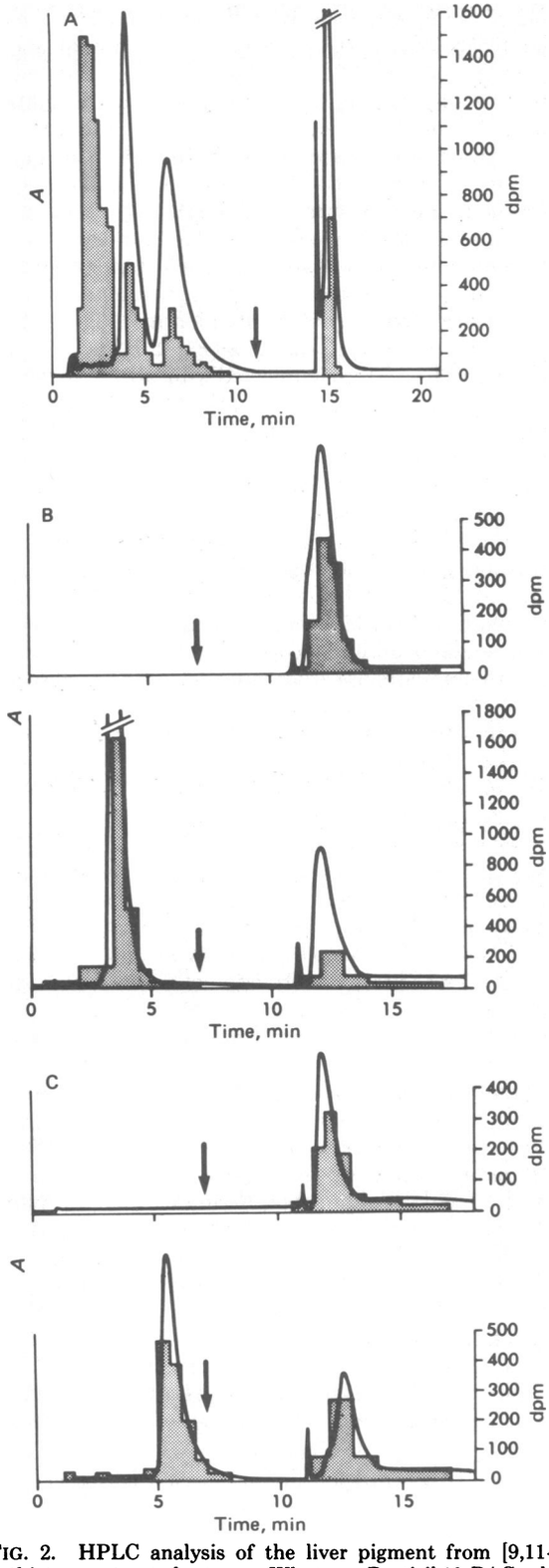


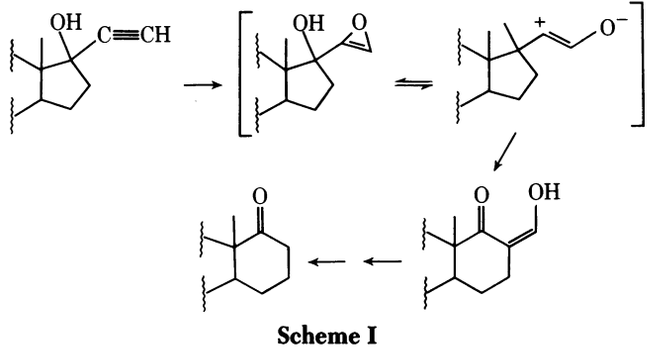
FIG. 2. HPLC analysis of the liver pigment from [9,11-^{3}H]-norethisterone-treated rats on a Whatman Partisil 10-PAC column, eluted first with hexane/THF/MeOH, 10:3:1 (vol/vol), and then (arrow) with MeOH/THF, 4:1. The curve is the absorbance at 417 nm; the bars indicate the eluted radioactivity in dpm. (*A*) Crude methylated pigment after thin-layer chromatographic prepurification and demetalation. (*B*) Reanalysis of first chromophoric peak (from *A*) after isolation, before (*Lower*) and after (*Upper*) addition of zinc acetate. (*C*) Reanalysis of second chromophoric peak (from *A*) after isolation, before (*Lower*) and after (*Upper*) chromophoric peak (from *A*) after isolation, before (*Lower*) and after (*Upper*) addition of zinc acetate.

norethisterone triple bond has been reduced to a double bond. More impressive, however, is the discovery that 1-ethynylcyclohexanol (**4**), which is naked of all functionality except for the propargylic alcohol and is free of the rigid sterol structure, nevertheless was equally potent as a destructive agent. In order to rule out a difference in the destructive mechanism, we injected 1-ethynylcyclohexanol into phenobarbital-pretreated rats and found in preliminary experiments, that a virtually identical pigment porphyrin (λ max 418, 511, 545, 590, 648 nm) is obtained and, in turn, can be transformed into a zinc complex (λ max 430, 546, 589, 629 nm).

DISCUSSION

Previous workers have established that the destruction due to norethisterone is specific for the phenobarbital-inducible isozymes of cytochrome *P*-450 (**4**). This specificity, the demonstration that enzymatic turnover is required for destruction, and the failure of glutathione to protect the hemoprotein all suggest that destruction is mediated by a short-lived norethisterone metabolite formed in the affected active site (**4**). In this respect, it is instructive that the nonspecific binding of norethisterone to proteins, caused by enzymatic production of the 4,5-epoxy derivative, is in fact attenuated by glutathione (16, 17). Our present demonstration that norethisterone is covalently attached to heme in a 1:1 ratio during the destructive process, however, provides firm evidence for such a mutually destructive interaction. Although cytochrome *P*-450 has not been specifically shown to provide the protoporphyrin IX incorporated into the norethisterone adduct, the close analogy between this example and inactivation caused by 2-isopropyl-4-pentenamide, where the prosthetic heme-to-adduct porphyrin connection has been clearly made (5–11), leaves little doubt that the adduct porphyrin also derives here from cytochrome *P*-450 heme.

The nature of the transient species that undergoes reaction with the prosthetic group, however, can not be precisely defined with the data in hand. The fact that norethisterone (**1**), norgestrel (**2**), ethynylestradiol (**4**), and particularly 1-ethynylcyclohexanol inactive cytochrome *P*-450, whereas norethisterone analogues with a vinyl (**3**) or an ethyl (**4**) substituent in place of the acetylenic group do not, convincingly localizes the destructive potential in the acetylenic moiety. Destructive mechanisms invoking direct electron transfer between the acetylenic group and heme, based on the postulated transfer of electrons from unsaturated bonds to metalloporphyrins (18), are ruled out by the essential requirement for oxygen (**4**). Clues to a probable mechanism, however, are provided by the products formed on oxidative metabolism of the ethynyl group in norethisterone (19), noregestrel (20, 21), and 17-ethynylestradiol (22, 23). All of these clinically used ethynyl sterols have been reported to undergo oxidative expansion of the D ring, with eventual loss of one carbon from the acetylenic group, yielding D-homo ketone or alcohol products (**Scheme 1**) (22). Transfer of oxygen from cytochrome *P*-450 to the acetylenic



Scheme I

moiety, the critical step in the metabolic scheme, formally results in an oxirene intermediate, an antiaromatic species of such instability that it has never been isolated in the laboratory (24, 25). It is therefore most reasonable to postulate that the cytochrome *P*-450 prosthetic group is alkylated by this highly energetic species or by a less-reactive intermediate generated during the precipitous rearrangement of this species to a structure of lower energy. Although nothing more definite can yet be said about the alkylation reaction, the possibilities are constrained by our observation that the adduct, after an acidic isolation which may alter the original structure, corresponds in molecular weight to the sum of the parent porphyrin and sterol, less two hydrogen atoms.

We acknowledge the able assistance of Ms. Virginia Pascucci, of Dr. Hedaeki Kumbara who helped us obtain the mass spectrometric data, and of Prof. A. Burlingame who facilitated our use of the Berkeley Biomedical and Environmental Mass Spectrometry Resource (National Institutes of Health Grant RR 00719). We are also indebted to Syntex and Wyeth Research Laboratories for generous gifts of sterol substrates. This research has been supported by Grants GM-25515 and P 50 AM-18520 from the National Institutes of Health and by the award of an Alfred P. Sloan Fellowship to P.O.M.

1. Onken, D. & Heublein, D. (1970) *Pharmazie* **25,** 3–9.
2. Einarsson, K., Ericsson, J. L. E., Gustafsson, J. A., Sjövall, J. & Zietz, E. (1974) *Biochim. Biophys. Acta* **369,** 278–293.
3. Freudenthal, R. I., Amerson, E., Martin, J. & Wall, M. E. (1974) *Pharmacol. Res. Commun.* **6,** 457–468.
4. White, I. N. H. & Muller-Eberhard, U. (1977) *Biochem. J.* **166,** 57–64.
5. DeMatteis, F. (1973) *Drug. Metab. Dispos.* **1,** 267–274.
6. Levin, W., Jacobson, M., Sernatinger, E. & Kuntzman, R. (1973) *Drug Metab. Dispos.* **1,** 275–285.
7. McDonagh, A. F., Pospisil, R. & Meyer, U. S. (1976) *Biochem. Soc. Trans.* **4,** 297–298.
8. DeMatteis, F. & Unseld, A. (1976) *Biochem. Soc. Trans.* **4,** 205–209.
9. DeMatteis, F. (1978) in *Heme and Hemoproteins, Handbook of Experimental Pharmacology,* eds. DeMatteis, F. & Aldridge, W. N. (Springer, New York), Vol. **44,** pp. 95–127.
10. Ortiz de Montellano, P. R., Mico, B. A. & Yost, G. S. (1978) *Biochem. Biophys. Res. Commun.* **83,** 132–137.
11. Ortiz de Montellano, P.R., Mico, B. A., Yost, G. S. & Correia, M. A. (1978) in *Enzyme Activated Irreversible Inhibitors,* eds. Seiler, N., Jung, M. J. & Koch-Weser, J. (Elsevier, Amsterdam), pp. 337–352.
12. With, T. K. (1976) in *Porphyrins in Human Diseases,* ed. Doss, M. (Karger, Basel, Switzerland), pp. 481–485.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. C. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
14. Sandoval, A., Thomas, G. H., Djerassi, C., Rosenkranz, G. & Sondheimer, F. (1955) *J. Am. Chem. Soc.* **77,** 148–151.
15. Hanasono, G. K. & Fischer, L. J. (1974) *Drug. Metab. Dispos.* **2,** 159–168.
16. Kappus, H. & Bolt, H. M. (1976) *Steroids* **27,** 29–45.
17. Kappus, H. & Remmer, H. (1975) *Drug Metab. Dispos.* **3,** 338–344.
18. Tsutsui, M., Velapoldi, R. A., Hoffman, L., Suzuki, K. & Ferrarri, A. (1969) *J. Am. Chem. Soc.* **91,** 3337–3341.
19. Palmer, K. H., Feierabend, J. F., Baggett, B. & Wall, M. E. (1969) *J. Pharmacol. Exp. Ther.* **167,** 217–222.
20. Sisenwine, S. F., Kimmel, H. B., Liu, A. L. & Ruelius, H. W. (1974) *Drug. Metab. Dispos.* **2,** 65–70.
21. Sisenwine, S. F., Kimmel, H. B., Liu, A. L. & Ruelius, H. W. (1973) *Acta Endocrinol.* **73,** 91–104.
22. Abdel-Aziz, M. T. & Williams, I. H. (1969) *Steroids* **13,** 809–820.
23. Williams, J. G. & Williams, K. I. H. (1975) *Steroids* **26,** 707–720.
24. Concannon, P. W. & Ciabattoni, J. (1973) *J. Am. Chem. Soc.* **95,** 3284–3288.
25. Zeller, K. P. (1977) *Angew. Chem. Int. Ed. Engl.* **16,** 781–782.