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SUICIDAL INACTIVATION OF CYTOCHROME P-450. FORMATION OF A HEME-SUBSTRATE COVALENT ADDUCT

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SUMMARY: The green pigment accumulated in the livers of phenobarbital pretreated rats after administration of 2-(^{14}C)-2-isopropyl-4-pentenamide (allylisopropylacetamide, AIA) is radiolabeled. The single primary green prophyrin component isolated by HPLC (λ_{max} ($CHCl_3$) 417, 512, 545, 594, 652 nm) is cleanly converted to a zinc complex (λ_{max} ($CHCl_3$) 431, 547, 591, 634, 669 nm). The radiolabel quantitatively shifts with the chromophore on TLC and HPLC upon formation of the zinc complex. Correlation of chromophore absorbance with radiolabel content suggests the formation of a 1:1 porphyrin-AIA adduct. Cytochrome P-450 is therefore destroyed by self-catalyzed addition of AIA to its heme prosthetic group.

A number of xenobiotics incorporating a gamma unsaturated amide substructure, exemplified by secobarbital and 2-isopropyl-4-pentenamide (allylisopropylacetamide, AIA), are potent inducers of porphyrin biosynthesis (1,2). These agents are differentiated from other inducers by their potency, exploited in models of clinical porphyrias (2), and by the accumulation in treated animals of a long known but uncharacterized green pigment (3). More recently it has been established that AIA specifically destroys the cytochrome P-450 isoenzyme(s) induced by phenobarbital without, in so far as can be determined, damaging any other enzyme (4-6). Catalytic turnover of the victimized cytochrome P-450 is required for its inactivation (4,5). The green pigment is furthermore derived from the prosthetic heme of destroyed cytochrome P-450, since there is a quantitative relationship between spectroscopic loss of cytochrome P-450, loss of labeled cytochrome P-450 heme, and formation of green pigment (4,5). Efforts to characterize the pigment since original

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work in 1955 (3) have been unsuccessful, although the pigment has recently been fractionated into two major TLC separable components whose ultraviolet spectra are diagnostic for the presence of an intact porphyrin ring (7,8). We have discovered, however, that one of the bands observed on TLC is an artifact due to complexation by metals on the plate, so that there is in fact only one major green pigment (P. Ortiz de Montellano, B. A. Mico, and G. S. Yost, unpublished). We now present unequivocal evidence that this major green pigment is not an abnormal catabolite of heme but is instead produced by covalent attachment of AIA to the prosthetic group of cytochrome P-450.

Materials and Methods

Sprague-Dawley rats weighing 240 ± 10g were injected intraperitoneally once a day for four days with a solution of sodium phenobarbital (75 mg/Kg). Twenty-four hours after the final phenobarbital injection, 2-isopropyl-4-pentenamide (AIA) was injected subcutaneously at a dose of either 160 or 200 mg/Kg, the lower dose being used in experiments with 2-(^{14}C)-2-isopropyl-4-pentenamide (2-(^{14}C)-AIA, specific activity 1.43 μCi/mg). The rats were sacrificed by decapitation 3 hr later, their livers were perfused, excised and homogenized, and the green pigment was extracted and methylated exactly as described by McDonagh (7). The crude methylated pigment was isolated and purified by high pressure liquid chromatography (HPLC), using a Whatman Partisil 10-PAC column and 5:5:1 tetrahydrofuran (THF)-hexane-MeOH as the eluting solvent. The column effluent was monitored with a variable wavelength detector set at 417 nm and, in experiments with labeled substances, by collection and liquid scintillation counting of effluent fractions. Freshly purified pigment exhibited the spectrum shown in Figure 1a and ran as a single spot on TLC (Eastman silica gel plates, 25% acetone-$CHCl_3$), although metal complex formation occasionally resulted in observation of a second spot (λ_{max} ($CHCl_3$) 430, 545, 592, 635 nm).

The zinc complex of the HPLC isolated green pigment was obtained by addition of an 0.5% solution of zinc acetate in 5% methanol-chloroform to a chloroform solution of the pigment (10). The resulting complex, apparently formed in quantitative yield, was purified by HPLC on the same column used to obtain the green pigment itself, except that a more polar solvent (4:1 MeOH-THF) was required. The zinc complex appears as a single spot on TLC, although its position relative to the green pigment itself depends on the type of TLC plate used. On Eastman silica gel plates (25% acetone-$CHCl_3$), the green pigment had an R_f of 0.54 and the zinc complex of 0.32.

The known metabolites of AIA, 4,5-dihydroxy-2-isopropylpentanamide and 2-isopropyl-4-hydroxymethyl-gamma-butyrolactone, were synthesized by modifications of the procedures reported by Doedens (9).

Results and Discussion

Administration of 2-isopropyl-4-pentenamide (AIA) to phenobarbital pretreated rats resulted in the formation of a green pigment which, after

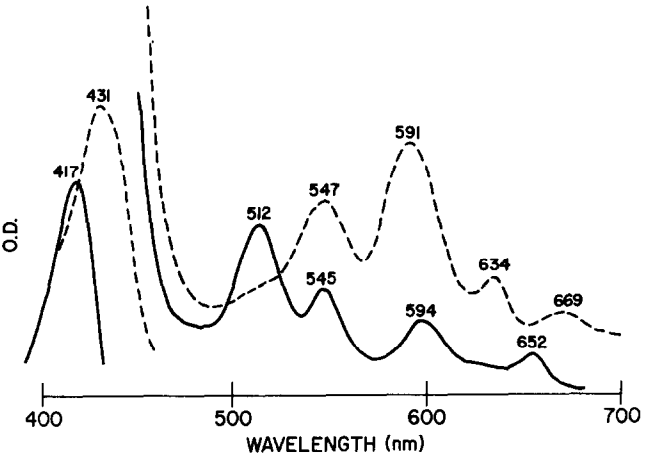


Figure 1. Spectra in $CHCl_3$ after HPLC purification of (a) the methylated green porphyrin[3](solid line) and (b) the corresponding zinc complex (dashed line).

methylation and isolation by HPLC, exhibited the previously reported ultraviolet spectrum (Figure 1a) (7,8). The purified pigment obtained by this procedure appears to be a single substance, as judged by TLC and HPLC, although the possibility exists that it is made up of closely related isomers which are not resolved. This possibility is to be clearly distinguished from the frequent observation of a well resolved artifactual metal complex (λ_{max} ($CHCl_3$) 430, 545, 592, 635 nm) on thin layer chromatography of the pigment.

Addition of zinc acetate to the methylated pigment in chloroform results in essentially quantitative formation of a zinc complex exhibiting an ultraviolet spectrum (Figure 1b) characteristic of a metal-porphyrin complex (13). The complex, prepared from preisolated or crude green pigment, can be purified by HPLC although a much more polar solvent is required to elute the complex than is required for isolation of the uncomplexed pigment. The selective complexing ability of the green porphyrin, coupled with the accompanying radical change in HPLC (and TLC) retention time, provides a specific and unique method for purification and characterization which we

have utilized in subsequent work with radiolabeled material.

Crude methylated green pigment obtained by the usual procedure using 2-(^{14}C)-AIA as the causative agent was analyzed by high pressure liquid chromatography (Figure 2a). Approximately one sixth of the radioactivity present in the sample was associated with the green pigment (the major spectroscopically observable peak), the rest of the label being contributed by a trace of contaminating labeled AIA. The green pigment peak, collected and shown to have the expected ultraviolet spectrum (Figure 1a), retained its radioactivity despite attempts to chromatographically dissociate the chromophore from the label. Covalent attachment of label to the porphyrin, suggested by these results, was unambiguously confirmed by observation that the radioactivity present in a purified sample of the methylated porphyrin was quantitatively shifted with the chromophore on addition of zinc acetate (Figure 2b). The ability of the green porphyrin to scavenge metals is in fact impressively demonstrated by the formation of a small amount of the complex on the column itself (Figure 2a). In contrast, we have specifically shown that the retention time of AIA and its two known metabolites (9,12) is not affected by addition of zinc acetate. Finally, TLC analysis of the HPLC purified green pigment before and after zinc complexation established that, here also, the label remained firmly associated with the porphyrin.

A rough estimate of the AIA to porphyrin ratio in the green adduct can be made by correlating the radiolabel content with the Soret band (417 nm) absorbance, given the known specific activity of AIA and making the reasonable assumption that the molar extiction coefficient of the adduct is in the range common to porphyrins (13). The calculated ratio thus extends from approximately 1.0 to 2.0, a value of 1.6 being obtained if the molar absorbance of protoporphyrin IX (13) is used. These values are therefore consistent with a 1:1 or (less probably) 2:1 AIA-porphyrin adduct, but they are much too high for incorporation of label into heme via initial degradation of AIA to a heme precursor since administration of even a direct

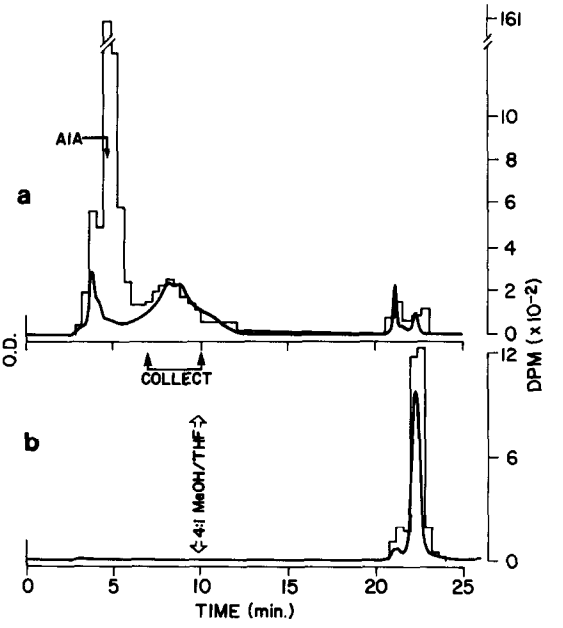
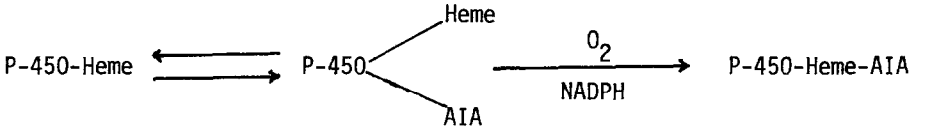


Figure 2. HPLC analysis of green pigment produced by 2-^{14}C-AIA on a Whatman Partisil 10-PAC column using 5:5:1 THF-hexane-MeOH followed by 4:1 MeOH-THF: curve is 417 nm detector response, bars indicate radioactivity in dpm. (a) Crude methylated pigment. Collected fraction is indicated by a bracket and retention time of AIA is shown by an arrow. (b) Analysis of collected fraction after addition of zinc acetate.

precursor of heme under analogous conditions results in dilution of the incorporated label by a factor of several hundred (4).

Our results establish that the green pigment, quantitatively derived from the prosthetic heme of destroyed cytochrome P-450 (4,5), is an AIA-porphyrin covalent adduct. Self-catalyzed inactivation of cytochrome P-450 is therefore mediated by the following unprecedented sequence of events:



Studies in progress of the precise structure of the adduct, and of the mechanism resulting in its formation, will be reported in due course.

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