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The Allylisopropylacetamide and Novonal Prosthetic Heme Adducts

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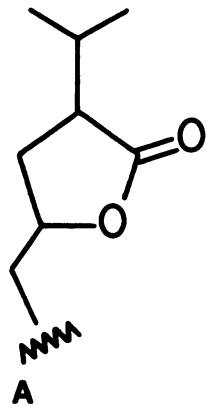
SUMMARY

Administration of 2-isopropyl-4-pentenamide (AIA) and 2,2-diethyl-4-pentenamide (novonal) to phenobarbital-pretreated rats gives rise to abnormal porphyrins derived from the prosthetic heme group of inactivated cytochrome P-450. The abnormal porphyrins, identified by NMR and other spectroscopic methods, are *N*-alkylated protoporphyrin IX derivatives in which the *N*-alkyl moiety is derived from the parent drug by addition of a hydroxyl group to the internal carbon and of a porphyrin nitrogen to the terminal carbon of the π-bond. A secondary reaction of the hydroxyl with the amide group converts the *N*-alkyl moiety into a lactone. The indicated alkylation-lactonization sequence is supported by the fact that the AIA adduct formed under an atmosphere of $^{18}O_2$ incorporates one labeled oxygen atom. The regiochemistry of heme alkylation is consistent with a previously postulated active site topology [*J. Biol. Chem.* **258**:4202–4207 (1983)].

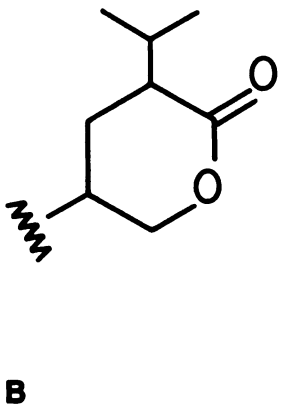
INTRODUCTION

AIA[1] has been used for more than three decades as an experimental probe of the heme biosynthetic pathway (1–3). Its utility stems from its ability to stimulate heme biosynthesis greatly and to engender in animals a biochemical state that in many respects resembles that associated with the genetically determined human porphyrias (3). Administration of AIA to rodents causes a major increase in the activity of 5-aminolevulinic acid synthetase (the rate-limiting step in heme biosynthesis) (1–3), the appearance of an abnormal hepatic green pigment (4), and a decrease in the concentration and activity of hepatic cytochrome P-450 (5–7). These three phenomena are causally linked to alkylation of the prosthetic heme group of cytochrome P-450 by AIA, a suicide substrate for the enzyme (8–10). Heme alkylation, which inactivates the enzyme, converts the prosthetic group into the green hepatic pigment. The link between these events and induction of heme biosynthesis is provided by the finding that replacement of damaged prosthetic groups by exogenous heme resuscitates the inactivated enzyme (11, 12). Given that the activity of 5-aminolevulinic acid synthetase is believed to be regulated by the size of an uncommitted heme pool (13), the exceptional induction of this critical enzyme by AIA is readily rationalized if the regulatory heme reservoir is drained by cyclical inactivation and reconstitution of cytochrome P-450 (12, 14).

The green pigment formed during the suicidal inactivation of cytochrome P-450 by AIA, partially characterized by radiolabeling and mass spectrometric studies, is a porphyrin with a molecular weight equal to the sum of protoporphyrin IX (dimethyl ester) + AIA + an oxygen atom − ammonia (9, 10). The electronic absorption spectra of the pigment (10) in the free-base and zinc-complexed forms are those of an *N*-alkylated protoporphyrin IX structure (15, 16). These results, in view of the structures established for adducts with simple olefins (17, 18), suggest that in the AIA adduct protoporphyrin IX bears substructure *A* or *B* as the *N*-alkyl group (19).



A



B

Novonal, sold in Germany as an over-the-counter drug until 1978, is currently available as a prescription drug. This close congener of AIA has been reported to decrease

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[1] The abbreviations used are: AIA, 2-isopropyl-4-pentenamide (frequently referred to as allylisopropylacetamide); novonal, 2,2-diethyl-4-pentenamide; heme, iron protoporphyrin IX regardless of iron oxidation state; DETAPAC, diethylenetriaminepentaacetic acid; NOE, nuclear Overhauser effect.

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the microsomal concentration of cytochrome P-450 (19, 20), but little is known about the mechanism underlying its effect on the monooxygenase system. In view of the continued use of novonal as a therapeutic agent, and of the fact that much of our understanding of heme biosynthesis rests on observations that stem from inactivation of cytochrome P-450 by AIA, we have undertaken to establish whether novonal also alkylates the prosthetic heme group and to determine the precise structure of the resulting heme adducts.

EXPERIMENTAL PROCEDURES

Materials. AIA was provided by Hoffmann-La Roche (Nutley, N. J.) and novonal by Hoechst AG (Frankfurt, Germany). Glucose-6-phosphate dehydrogenase, glucose-6-phosphate, and NADP were purchased from Sigma Chemical Company (St. Louis, Mo.). High-purity $^{18}O_2$ was obtained from the Isomet Corporation. The glass-distilled solvents used for chromatographic purifications were from Burdick and Jackson Laboratories Inc. (Muskegon, Mich.). Other reagents were of the highest commercially available grade.

Isolation of novonal pigment. Thirty male Sprague-Dawley rats (250–280 g) injected i.p. with an 80 mg/kg dose of phenobarbital at 24-hr intervals for 4 days, were administered a 200 mg/kg dose of novonal in dimethyl sulfoxide (60 mg/0.3 ml of dimethyl sulfoxide) on the 5th day. Four hours later the rats were decapitated and their livers were perfused *in situ* with ice-cold 0.9% saline solution. The livers, homogenized in a Waring blender for 3 min, were then allowed to stand for 36 hr in the dark at 0° in 5 liters of 5% (v/v) H_2SO_4/methanol. The filtrate obtained by vacuum filtration was combined with an equal volume of CH_2Cl_2, and the mixture was washed twice with equal volumes of water and once with brine. After drying over anhydrous sodium sulfate, the solvent was removed at a rotary evaporator. The brown viscous residue was dissolved in 30 ml of CH_2Cl_2 and was applied to six preparative silica gel G plates. A single red-fluorescing band (R_F 0.46) was observed under long wavelength UV light after the plates were developed with 5% methanol in CH_2Cl_2. The red-fluorescing fraction was recovered with the same solvent, and a few drops of saturated methanolic zinc acetate solution were added to convert the porphyrin to its zinc complex. Excess zinc was removed by washing with water and brine. The zinc-complexed porphyrin was chromatographed on six 500-μm silica gel G plates developed with 3:1 $CHCl_3$/acetone. The pigment (R_F 0.85), removed from the plates with 5% methanol in CH_2Cl_2, was demetalated by stirring in a 1:1 mixture of CH_2Cl_2 and 5% H_2SO_4/methanol for 10 min. The organic phase was washed twice with water, once with saturated sodium bicarbonate, and once with brine before it was dried over anhydrous sodium sulfate. The residue obtained on solvent removal was chromatographed on three 500-μm silica gel G plates with 5% methanol/CH_2Cl_2. The red-fluorescing band was recovered with the same solvent from the silica and was converted (for NMR work) to its zinc complex as described above.

Isolation of the AIA pigment. The AIA pigment was isolated as before (10) by a procedure that differs from that used with novonal inasmuch as high-pressure liquid chromatography on a PAC 10 column (25 cm) with a linear gradient of methanol into 1:1 tetrahydrofuran/hexane was used after the first thin-layer plate to isolate the zinc complex. The metal-free pigment was fractionated into the two isomers by chromatography on a Whatman Partisil PX5 silica gel column (25 cm). The column, prewashed extensively with 1:7 tetrahydrofuran/hexane, was eluted isocratically with this same solvent after introduction of the sample. The partially resolved isomer peaks were separated, and each peak was reinjected into the column for a second separation. The individual isomer fractions, converted to the zinc complexes with a saturated solution of zinc acetate in methanol, were chromatographed on the PAC 10 column with the gradient of methanol into tetrahydrofuran/hexane described above. For the NMR studies, solutions of each of the zinc-complexed isomers in CH_2Cl_2 were shaken with saturated NaCl solution and dried over sodium sulfate before the solvent was removed under vacuum. The samples were held under vacuum overnight to remove all traces of solvent.

^{18}O Incorporation into the AIA pigment. Microsomes were prepared from the livers of eight phenobarbital-pretreated (see above) Sprague-Dawley male rats as previously reported (21) except that the final buffer contained 1.5 mM DETAPAC rather than the equivalent amount of EDTA. A total of 2180 nmoles of cytochrome P-450 were estimated to be present by spectroscopic analysis of an aliquot. The microsomes were diluted to a total volume of 200 ml (10.8 nmoles of cytochrome P-450/ml, 4.4 mg of protein/ml) in 0.1 N Na/K phosphate buffer (pH 7.4) containing 150 mM KCl, 1.5 mM DETAPAC, and 10 mM AIA. The mixture was placed in a 500-ml vacuum flask with valves leading to a water aspirator, a nitrogen inlet, and two 100-ml break-seal ampules containing 99.8% ^{18}O-oxygen gas. A balloon on the same valve as the break-seal flasks provided a reservoir for the gas. The system was cycled 10 times through a 3-min evacuation with the water aspirator followed by introduction of nitrogen gas. After the last evacuation, the pressure was brought up by breaking the seal to the labeled oxygen ampules. The metabolic reaction was then initiated by adding an NADPH-regenerating system that also had been purged of oxygen by repetitive evacuation-nitrogen cycles. The regenerating system consisted of 300 mg of glucose-6-phosphate, 100 units of glucose-6-phosphate dehydrogenase, 80 mg of NADP, and 80 mg of $MgCl_2 \cdot 6H_2O$ in 5 ml of the same buffer mixture used for the microsomal preparation. After incubation for 1 hr at 37° in a reciprocating water bath, the microsomal suspension was poured into 3 liters of 5% H_2SO_4 in methanol. The mixture was allowed to stand for 15 hr at 4° in the dark before the mixture was worked up and the pigment was purified as described above for *in vivo* AIA experiments. Approximately 50 μg of the pure abnormal porphyrin were obtained.

Spectroscopic studies. Electronic absorption spectra were recorded in CH_2Cl_2 on a Hewlett-Packard 8450A spectrophotometer. Mass spectra were obtained by field desorption on a Kratos/AEI MS-902 instrument at the Berkeley Biomedical Mass Spectrometry Resource under conditions that have been reported (10). NMR spectra were recorded on a 360-MHz Varian instrument in deuterated chloroform at the NMR Facility of the University of California, Davis, or on a 240 MHz custom-built instrument in San Francisco. The preparation of samples for NMR work and the general instrumental parameters have been described (17).

RESULTS

Administration of novonal to phenobarbital-pretreated rats resulted in the accumulation of an abnormal hepatic pigment analogous to that obtained from rats injected with AIA. The pigment migrated as a single band on thin-layer chromatography even though NMR studies subsequently demonstrated the presence of two unresolved isomeric structures. Separation of the pigment by high-pressure liquid chromatography into a major peak with a slower-moving shoulder confirmed the presence of isomeric structures even though the peaks were too poorly resolved to be separated (data not shown). The electronic absorption spectra of the pigment as the free base and the zinc complex were virtually identical with those found for the AIA adduct (Table 1) (9). The Soret band in the spectrum of the zinc complex did not exhibit the long-wavelength shoulder associated with *N*-alkylation of the vinyl-substituted protoporphyrin IX pyrrole rings (22). The field desorption mass spectrum of the dimethyl-esterified porphyrin exhibited the monoprotonated molecular ion at *m/e* 745 (data not shown) expected if, by analogy with the AIA adduct (10), the structure derived from the sum novonal (M_r = 155) + an oxygen atom (atomic weight = 16) + the dimethyl

TABLE 1

Electronic absorption maxima of the novonal and AIA adducts

Adduct	Maxima (relative intensity)[a]
	nm
Novonal	
Free base	416 (1.00), 512 (0.12), 546 (0.08), 594 (0.06), 652 (0.04)
Zinc complex	430 (1.00), 546 (0.11), 590 (0.14), 632 (0.08)
Diprotonated	420 (1.00), 568 (0.05), 612 (0.02)
AIA	
Free base	416 (1.00), 512 (0.14), 544 (0.09), 594 (0.07), 654 (0.06)
Zinc complex	432 (1.00), 546 (0.09), 590 (0.12), 632 (0.06)
Diprotonated	418 (1.00), 566 (0.08), 610 (0.05)

[a] Relative peak absorbance with respect to the Soret band.

ester of protoporphyrin IX (M_r = 590) − a molecule of ammonia (M_r = 17). The novonal and AIA adducts thus arise by similar mechanisms and give rise to ostensibly similar heme adducts.

The structures of the AIA and novonal adducts, partially defined by the electronic absorption and mass spectrometric data, have been firmly defined by NMR studies. Although the AIA adduct has been under study much longer than the novonal adduct, the absence of the chiral carbon in novonal that is present in AIA reduces the number of potential isomers and simplifies analysis of the NMR spectrum. NMR characterization of the novonal adduct thus is discussed first and is subsequently used as a point of reference for analysis of the NMR spectrum of the AIA adduct.

Novonal NMR. The NMR spectrum of the isolated novonal adduct clearly shows that two unresolved isomers are present in the sample because more than four *meso* proton signals are found in the 10.0–10.5 ppm region and more than six methyl signals in the 3.4–3.7 ppm region (Fig. 1). The number of peaks expected from one dimethyl-esterified protoporphyrin IX derivative is thus exceeded. The initial analysis also confirms that the *N*-alkyl group in the porphyrin is on one of the propionic-acid substituted rings, as suggested by the absence of a shoulder on the Soret band of the zinc complex, because the internal vinyl proton signals at approximately 8.2 ppm are not clearly separated whereas the propionic acid methylene group signals are (data not shown). The methylene protons adjacent to the porphyrin appear at 4.2 and 4.35 ppm and the methylene protons next to the carboxyl groups at approximately 3.2, 2.9, and 2.8 ppm. This pattern of vinyl and methylene signals is characteristic of protoporphyrin IX derivatives *N*-alkylated on pyrrole rings C or D (23).

The *N*-alkyl group in the novonal adduct is clearly identified by the NMR spectrum as the lactone reproduced in the *inset* of Fig. 2. The methylene protons (H_a) on the carbon bound to the pyrrole nitrogen, due to the ring current effect of the porphyrin, appear at very high field (−4.9 to −5.0 ppm). The lactone ring proton *trans* to the porphyrin (H_d) is found at approximately 0.05 ppm whereas the proton *cis* to the porphyrin (H_c) is found at −1.25 ppm. The signal of H_b, the tertiary proton vicinal to H_a, H_d, and H_c, is almost completely buried in the signal of an impurity at 1.5 ppm. Its presence and identity, however, are confirmed by the fact that irradiation at 1.5 ppm results in decoupling of the signals attributed to the four vicinal protons (Fig. 2). The barely visible multiplet due to H_b on the upfield side of the signal at 1.5 ppm furthermore sharpens and becomes more visible on irradiation of H_a, H_d, or H_c (data not shown). The methyl protons (H_g) *cis* to H_c and the

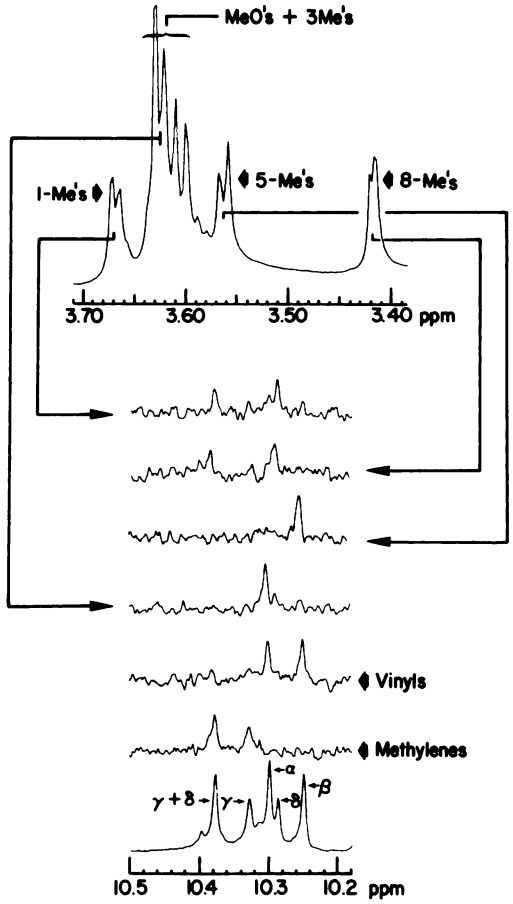


FIG. 1. *Meso proton NOEs observed when the methyl protons of the novonal adduct are irradiated*

The meso proton NMR signals (360 MHz) of the zinc-complexed novonal adduct are reproduced at the bottom and the porphyrin methyl group signals at the top. The meso proton NOE observed on irradiating a given set of methyl protons is identified by the connecting *arrow*. NOEs observed on irradiating the internal vinyl or the internal propionate methylene protons are so labeled. The meso and methyl proton signal assignments are given.

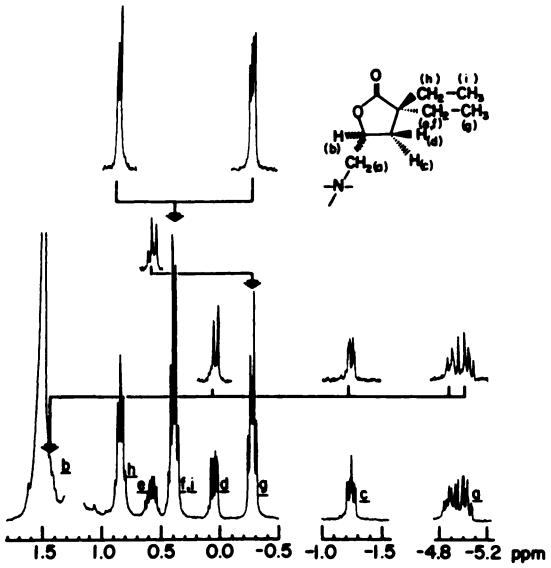


FIG. 2. *N-Alkyl group region of the 360-MHz NMR spectrum of the zinc-complexed novonal adduct*

The porphyrin proton signals in the region below 1.5 ppm are not shown. Irradiation of the protons indicated by the *arrowheads* causes the signal changes given in the associated *inset*. The NMR peaks are labeled to identify the protons in the *N*-alkyl moiety (*inset*) responsible for them. An impurity peak at 1.24 ppm has been deleted from the spectrum.

porphyrin are located at −0.28 ppm, whereas the methyl protons (H_i) of the *trans* ethyl moiety are found at 0.4 ppm. The methylene protons (H_e and H_f) *cis* to the porphyrin are clearly resolved and appear at 0.6 and 0.4 ppm, but the methylene protons (H_h) of the other ethyl group are equivalent and appear together at 0.85 ppm. The number of protons associated with each signal and the relationships between them, confirmed in all cases by decoupling experiments (Fig. 2), clearly exclude a six-membered lactone structure. For example, a six-membered lactone (structure B) would only give rise to a one-proton signal at −5.0 ppm.

The multiplicity of the lactone proton signals in the NMR spectrum can be explained only if they represent the superposition of signals from protons of two similar but nonidentical structures. The protons designated as H_a, for example, are not equivalent and should give rise to two distinct doublets of doublets due to coupling with each other and with H_b. However, the multiplet at approximately −5.0 ppm is closer to two octets than two quartets. Only when H_b is decoupled by irradiation (Fig. 2) does one observe the expected pattern. A similar doubling of the expected multiplicity characterizes the signals of most of the *N*-alkyl group protons. To pick a second example, irradiation of H_b results in collapse of H_c not to a doublet, as expected, but to a pair of doublets (Fig. 2). Two types of closely related isomeric structures are possible for the novonal adduct. The lactone group could be located on the nitrogens of two different pyrrole rings (those of pyrrole rings C and D), or the structures could be diastereomeric due to a difference in the absolute stereochemistry of the chiral carbon to which H_b is attached. The protons of the lactone moiety would, in both instances, bear the same relationship to each other but would be in slightly different environments. A detailed analysis of the porphyrin proton signals (below) suggests that both isomers are alkylated on pyrrole ring D, and thus that they differ in the chirality of the asymmetrical carbon created during catalytic introduction of the oxygen atom.

The meso and methyl proton signals, their identities, and the NOEs between them, are given in Fig. 1. NOEs decrease sharply as the distance between two protons increases and are only observed when the protons in question are very close to each other (24). We, following an earlier lead (25), have demonstrated that the meso protons of *N*-alkyl porphyrins give NOEs when the adjacent methyl(s) or methylenes are irradiated (23). The γ and δ meso proton signals are thus easily identified because the former exhibits an NOE on irradiation of the propionic acid side chain methylene groups whereas the latter exhibits an NOE on irradiation of *two* different methyl groups. On this basis it has been found that the peak at 10.38 ppm is due to superimposed γ and δ meso protons, the signal at 10.33 ppm to a γ meso proton, and that at 10.29 ppm to a δ meso proton. The signals at 10.30 and 10.25 ppm, which also show an NOE when the internal vinyl protons are irradiated, have been assigned, respectively, to the α and β meso protons after identification by spin-decoupling experiments of the methyls which elicit the indicated NOE (see below).

As already noted, more methyl group signals are present than are expected for a single isomer (Fig. 2). A correlation of the methyl group signals with the meso proton NOE which they elicit indicates that the peaks at 3.665 and 3.67 are those of the 1-methyl protons, and those at 3.42 and 3.435 ppm those of the 8-methyl protons, in two distinct isomers. The 1- and 8-methyl groups were distinguished by the fact that irradiation of the 1-methyl protons resulted in long-distance decoupling and consequent sharpening of the internal vinyl proton signals, whereas no such effect was noted when the 8-methyl protons were irradiated (data not shown). The remaining signals at 3.615, 3.610, 3.60. 3.57, and 3.56 are those of the methoxy groups and the 3- and 5-methyls of the two isomers. Two conclusions follow from the data on the meso and methyl protons. The first is that pyrrole ring D must be alkylated in both isomers because the 8-methyl protons are found at highest field in both structures. We have previously shown that the methyl substituent of the alkylated pyrrole ring is shifted upfield because it is less influenced by the ring current of the aromatic system. It is also striking that the methyl group pattern is the same in both isomers (8-methyl at highest field and 1-methyl at lowest), a result inconsistent with *N*-alkylation of two different rings. The fact that the α and β, but not γ and δ, meso protons have similar chemical shifts in the two isomers supports the argument that their differences are localized on ring D because it is flanked by the γ and δ meso protons. If, as appears likely, the same pyrrole nitrogen is alkylated in both isomers, the two structures must differ in the *absolute* stereochemistry of either the carbon bearing H_b or the alkylated nitrogen. The former alternative appears more

probable because the available evidence suggests that the prosthetic heme of cytochrome P-450 is only alkylated from one side (18, 26). The isomers of the novonal adduct therefore are probably **1a** and **1b** (Fig. 3).

NMR of the isomeric AIA adducts. We have already reported that the AIA adduct is probably *N*-alkylprotoporphyrin IX with substructure A or B as the *N*-alkyl group (19). Our NMR studies therefore focused on differentiation of these two alternatives and on the question of regioisomers (due to alkylation of different nitrogens) and stereoisomers (due to chiral centers in the *N*-alkyl lactone moiety). Indeed, high-pressure liquid chromatographic and NMR analyses rapidly established that two isomeric porphyrins were formed in variable amounts. The most rapidly eluted of the two isomers (I), generally formed in highest yield, is only poorly separated by high-pressure liquid chromatography from the trailing isomer (II) (Fig. 4). It has therefore been possible to obtain isomer I reasonably free of isomer II, but not the reverse. A more detailed structural analysis has therefore been possible of isomer I than isomer II.

The *N*-alkyl moiety in the AIA adduct is unambiguously shown by the NMR data to be γ-lactone *A* rather than δ-lactone *B*. Immediate support for this conclusion is provided by the fact that two protons appear at very high field, one at −5.1 and one at −4.9 ppm, each of

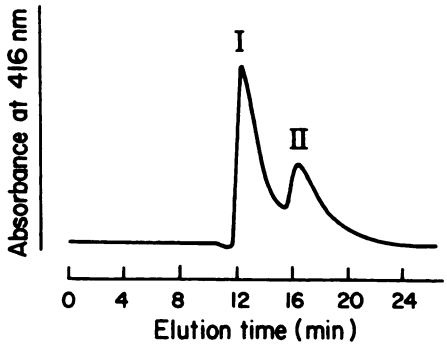


FIG. 4. *Separation of the two isomers of the AIA adduct by high-pressure liquid chromatography*

Experimental conditions for the separation are given under Experimental Procedures. The metal-free isomers are labeled in order of their elution from the column.

which gives rise to a doublet of doublets (Fig. 5). This extraordinary upfield chemical shift places the protons (H_a, $H_{a'}$) on the carbon attached to the porphyrin because only there is the ring current sufficiently strong to cause the observed shift. The two protons are non-equivalent and are coupled to a vicinal proton (H_b) with the following coupling constants: $J_{aa'} = 14.8$, $J_{ab} = 8.2$, and $J_{a'b} = 3.7$ Hz. The lactone ring proton (H_b) to which the N-

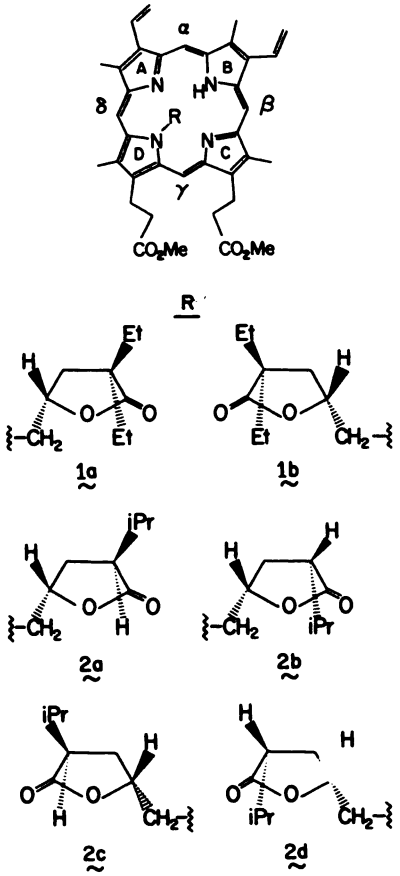


FIG. 3. *Possible isomeric structures for the porphyrins isolated from rats treated with novonal (1) and AIA (2)*

The pyrrole rings and meso positions of the porphyrin ring are labeled. Pyrrole ring C may be alkylated rather than pyrrole ring D in the AIA-derived structures.

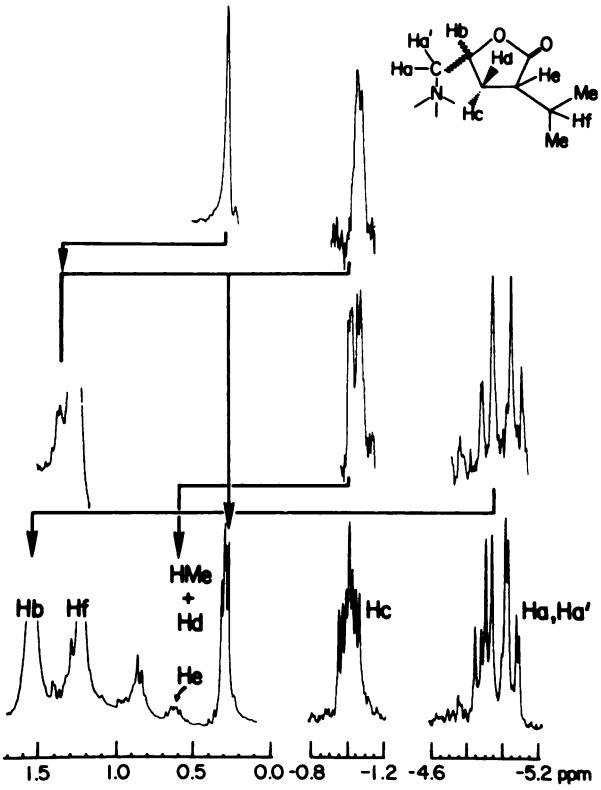


FIG. 5. *N-Alkyl group region of the 240-MHz NMR spectrum of zinc-complexed isomer I of the AIA adduct*

Irradiation of the region indicated by the *arrowheads* causes the signal changes given in the associated *insets*. The NMR peaks are labeled to identify the protons in the *N*-alkyl moiety (*inset*) responsible for them. The signals of H_b and H_f are obscured by large peaks due to impurities. The peak at 0.85 ppm is also due to water or some other contaminant.

CH_2- protons are coupled is hidden under the signal at 1.6 ppm, as shown by the fact that irradiation at this frequency reduces the signals at −5.1 and −4.9 ppm to a doublet each (Fig. 5). A single high-field proton with a more complex coupling pattern would be expected for the six-membered lactone. H_b is also coupled to H_c (J_{bc} = 4.9 Hz) and H_d, the lactone ring protons located at −1.04 and 0.28 ppm, respectively. H_c is, as required, coupled to H_d (J_{cd} = 13.7 Hz). The chemical shift values for H_a, $H_{a'}$, H_b, and H_d in the NMR spectrum of the AIA adduct are similar to those of the corresponding protons of the novonal adduct. The lactone ring proton (H_e) not present in the novonal structure is located at 0.6 ppm and is coupled to H_c (J_{ce} = 9.5 Hz), H_d, and H_f. H_f, the tertiary isopropyl proton, is at 1.34 ppm and is, as expected, coupled to the two isopropyl methyl group protons at 0.28. The signal at 0.28 thus consists of the isopropyl methyl protons superimposed on H_d. Each of these coupling assignments has been confirmed by decoupling experiments (Fig. 5). For example, irradiation of H_f at 1.34 ppm reduces the isopropyl methyl doublet at 0.28 ppm to a singlet. It is to be noticed that, in contrast with the NMR of the novonal adduct, each signal has the multiplicity expected for the protons of a single isomer.

The NMR signals of the porphyrin protons confirm that protoporphyrin IX serves as the framework of the adduct. The two internal vinyl protons appear as distinct but overlapping triplets at 8.24 and 8.18 ppm and the external vinyl protons as a complex multiplet centered at approximately 6.25 ppm (data not shown). The internal methylene protons of the propionate side chains give rise to resolved multiplets at 4.2 and 4.4 ppm and the external methylene protons to multiplets at 3.6 and 2.8 ppm. The poor separation of the internal vinyl proton signals and the non-equivalence of the analogous protons on the two propionate side chains points, as with novonal, to *N*-alkylation of either pyrrole ring C or D in protoporphyrin IX (23). NMR thus places the *N*-alkyl group on the same pyrrole rings as the absence of a shoulder on the Soret band of the zinc complex (9, 22). Except for minor signals due to a trace of isomer II, only the four singlets expected of a single isomer are found in the meso proton region. Their chemical shift values are 10.40, 10.39, 10.35, and 10.30 ppm (data not shown). However, because the signals have not been assigned to individual meso protons by NOE experiments, we cannot differentiate between alkylation of pyrrole rings C and D. Alkylation of ring D is favored by analogy with the adducts obtained with novonal and all other olefins so far investigated (17, 18).

The NMR spectrum of isomer II of the AIA adduct is somewhat obscured by the presence of peaks due to isomer I. Despite this complication, NMR decoupling experiments have allowed us to identify the protons of the *N*-alkyl moiety. The positions of these protons, designated by the same letters as in isomer I (Fig. 5), are the following: H_a, −4.8; $H_{a'}$, − 5; H_b, 1.6: H_c, −1.3; H_d, 0.20; H_e, 0.28; H_f, 1.25; and H_{Me}, 0.31 and −0.13 ppm. These assignments confirm that the *N*-alkyl moiety in isomer II is also a γ-lactone. Three notable differences exist between the signals of the *N*-alkyl moieties in isomers I and II, however. H_c, the lactone ring proton *cis* to the porphyrin, is at −1.3 rather than at −1.04 ppm as in isomer I. H_e, the tertiary isopropyl proton, is upfield (0.28 ppm) of its position in the NMR of isomer I (0.6 ppm). Finally, and most significantly, the two isopropyl methyl groups are non-equivalent and have chemical shift values that differ by almost 0.5 ppm. Their identity is clearly established by the fact that irradiation of H_f at 1.25 ppm causes both methyl signals to collapse to singlets (Fig. 6). These differences suggest that isomers I and II differ in the orientation of the isopropyl substituent. Indeed, the fact that the isopropyl methyls are clearly differentiated in one isomer of the AIA adduct but much less so in the other parallels the observation that the methylene protons are only non-equivalent for the ethyl group *cis* to the porphyrin in the novonal adduct (Fig. 2). The isopropyl group in isomer II may thus be *cis* and that in isomer I *trans* to the porphyrin.

The vinyl group protons of isomer II, as with isomer I, have only slightly different NMR chemical shifts whereas the methylene protons of the two propionate side chains are clearly differentiated (data not shown). The now-familiar NMR pattern and the absence of a shoulder on the Soret band of the zinc complex establishes that isomer II, like isomer I, is alkylated on pyrrole ring C or D. The clear non-equivalence of the isopropyl methyls in isomer II but not isomer I, however, cannot be explained if the *N*-alkyl groups are otherwise stereochemically identical by alkylation of ring C in one isomer

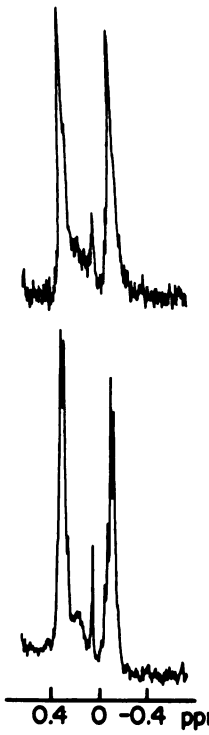


FIG. 6. *Signals of the two isopropyl methyls in the 240-MHz NMR spectrum of zinc-complexed isomer II of the AIA adduct*

The upper spectrum shows the result of irradiating the tertiary isopropyl proton at 1.35 ppm. The collapse of both methyl doublets to singlets identifies them as the methyls of one isopropyl function. The small peaks between the doublets are due to the isopropyl group of the other isomer.

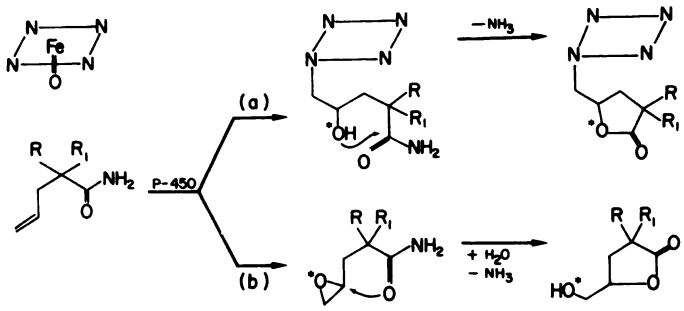
and ring D in the other. The lactone substructures of the two isomers therefore differ in the stereochemistry either of the carbon bearing H_b or of that bearing the isopropyl function. Four possible isomeric structures are thus possible for the *N*-alkyl moiety (**2a–2d**, Fig. 3). The stereochemistry of H_b is determined by the catalytic reaction, whereas that of the isopropyl group is governed by the stereochemistry of the AIA enantiomer (of the two in the racemic mixture employed) bound in the active site. Even though the results imply that the isopropyl group and the porphyrin are *cis* to each other in one isomer (either **2a** or **2b**) and *trans* (either **2c** or **2d**) in the other, little can be said about which of the two structures in each pair is the correct one or about the possibility that pyrrole ring D is alkylated in one isomer but pyrrole ring C in the other.

Origin of the lactone oxygen in the AIA adduct. The origin of the oxygen atom incorporated into the lactone ring of the AIA adduct has been determined by the procedure used to establish the origin of the oxygen atom in the ethylene adduct (18). The monoprotonated molecular ion of the adduct isolated from an incubation of AIA with hepatic microsomes under $^{18}O_2$ appears in a clean field desorption mass spectrum at *m/e* 733 (data not shown). Peaks are not observed at *m/e* 730 or 731, the values for the unprotonated and monoprotonated molecular ions observed in the mass spectrum of the unlabeled AIA adduct. One atom of molecular oxygen therefore is introduced into the substrate moiety by the catalytic action of the enzyme in the process that alkylates the heme.

DISCUSSION

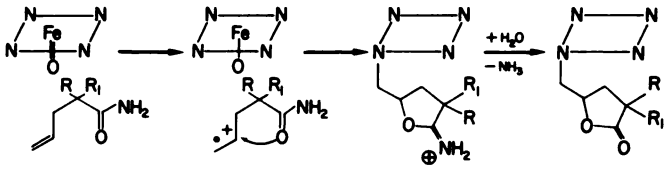
The inactivation of cytochrome P-450 by AIA and novonal (5–7, 19, 20), known for AIA to involve prosthetic heme alkylation (9, 10), has been shown here to also involve heme alkylation in the case of novonal. As might be expected from the structural similarity of AIA and novonal, the hepatic porphyrins which they engender are also very similar. Both reflect reaction of a prosthetic heme nitrogen with the terminal carbon and addition of catalytically activated oxygen to the internal carbon of the π-bond, followed by intramolecular reaction of the new hydroxyl group with the amide to give a lactone (Scheme 1, Path *a*). Alkylation by AIA and novonal thus differs from alkylation by other olefins in that the initial alcohol product undergoes a secondary internal lactonization reaction. An analogous mechanism, in which lactonization occurs with elimination of methanol, readily rationalizes the finding that the gross structure of the heme adduct obtained with the methyl ester analogue of AIA is indistinguishable from that obtained with AIA itself (10).

The demonstration that the lactone oxygen in the AIA adduct derives from molecular oxygen is consistent with the origin of the oxygen incorporated (18) into the ethylene adduct and strengthens our conclusion that heme alkylation by olefins is set in motion by oxygen transfer to the π-bond of the substrate. The possibility that the lactone in the case of AIA and novonal might involve interception by the *amide* oxygen of a species *prior* to the alkylation event is ruled out by the $^{18}O_2$ experiment. The results show that, if the radical cation of the π-bond intervenes in the reaction trajectory (Scheme 2), it is intercepted by the iron-bound oxygen at a substantially faster rate than by the amide oxygen. The present result contrasts with recent work on the mechanism of formation of the hydroxy lactone metabolite of AIA (Scheme 1, Path *b*). ^{18}O Studies have shown that the metabolite probably arises by intramolecular addition of the amide oxygen to the epoxide metabolite rather than from lactonization of the diol obtained by hydrolysis of the epoxide (26).



SCHEME 1. *Proposed mechanisms for formation of the isolated heme adduct (Path a) and of the principal AIA-metabolite (Path b)*

Protoporphyrin IX is denoted by the four nitrogens: for AIA, R = isopropyl and R_1 = H; for novonal, R = R_1 = ethyl.



SCHEME 2. *Mechanism for the formation of the AIA adduct excluded by the ^{18}O experiment*

Protoporphyrin IX is denoted by the four nitrogens: R = isopropyl and R_1 = H.

The regiochemistry of heme alkylation observed with novonal is the same as that found for unbranched hydrocarbon olefins (18). As before, alkylation by olefins seems to focus on the nitrogen of pyrrole ring D. Although we have not differentiated between rings C and D in the case of the AIA adduct, the fact that it must be one of these two is consistent with the other results. The finding that these relatively complicated, branched structures react with the same nitrogen as do simple linear olefins supports the postulate that the active site of the phenobarbital-inducible isozyme(s) that metabolize these drugs has a lipophilic binding site over pyrrole ring C and a steric impediment to reaction with the nitrogen of pyrrole ring B (18). Recent work by Waxman and Walsh (27) indicates that AIA inactivates cytochrome P-450_{PB-4}, one of the closely related isozymes induced by phenobarbital in Sprague-Dawley rats, with high selectivity. The topological information provided by the heme adducts with AIA and other olefins thus bears on the active site of at least this isozyme.

If the two unresolved isomers of the novonal adduct differ, as concluded here, in the absolute stereochemistry

of the carbon to which activated oxygen is added, an interesting difference exists between the reactions of novonal and octene. Octene was found to react exclusively on the side of the π-bond presented to the iron-bound oxygen if the hydrophobic tail is bound over pyrrole ring C and the terminal carbon of the olefin points toward the pyrrole ring D (28). The addition of oxygen from both sides of the π-bond in novonal with (still) exclusive alkylation of pyrrole ring D requires, if the proposed active site topology is valid, that sufficient space exist above the heme for the congested amide terminus of the substrate to bend away from pyrrole ring B toward pyrrole ring C or A. We have found previously with ethchlorvynol, a branched acetylenic sedative hypnotic, that congestion adjacent to the acetylenic function loosens the alkylation specificity (29). It is possible that the active site is conformationally flexible and adjusts to the insertion of branched substrates in a way that decreases steric constraints on the reaction. We cannot at this time exclude the possibility, however, that the major isomer stems from alkylation of the heme in one cytochrome P-450 isozyme (presumably PB-4) and that the second reflects alkylation of a second phenobarbital-inducible isozyme (perhaps PB-5) (27).

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