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Studies of the Porphyrin-Inducing Activity of Ethynyl Compounds and Conformationally Restricted and Unrestricted Analogues of Allylisopropylacetamide in Chick Embryo Liver Cell Culture

GERALD S. MARKS, STEPHEN B. ZIMMER, STEPHEN E. DINIZO, BRUCE A. MICO, KENT L. KUNZE, AND PAUL R. ORTIZ DE MONTELLANO

Department of Pharmacology, Queen's University, Kingston, Ontario, Canada, K7L 3N6, and Department of Pharmaceutical Chemistry, School of Pharmacy and Liver Center, University of California, San Francisco, California 94143

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SUMMARY

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A series of conformationally restricted and unrestricted analogues of allylisopropylacetamide (AIA) and a group of ethynyl compounds have been tested for porphyrin-inducing activity in chick embryo liver cell culture. The conformationally restricted analogues, which do not share the ability of AIA to destroy rat and chick embryo hepatic cytochrome P-450, were nevertheless shown to induce porphyrin accumulation. The conformationally unrestricted analogues and the ethynyl compounds which do share the ability of AIA to destroy rat hepatic cytochrome P-450 possessed weak to moderate porphyrin-inducing activity or were inactive. It is concluded that in chick embryo liver cell culture, in contrast to the situation in rat liver, destruction of the heme moiety of cytochrome P-450 does not appear to be a prerequisite for δ-aminolevulinic acid synthetase induction and porphyrin accumulation.

INTRODUCTION

It has been known for many years (1) that a green pigment is formed in rat liver following administration of Sedormid, an AIA[1] analogue. However, neither the structure nor the significance of this green pigment has been understood. In recent years, our knowledge of this green pigment has advanced. In the rat, phenobarbital-induced hepatic cytochrome P-450 is preferentially destroyed by AIA and it has been shown by isotopic labeling techniques that the green pigment consists of iron-free metabolite(s) of the heme group of cytochrome P-450 (2, 3). Ortiz de Montellano *et al.* (4, 5) have shown that, in this process, protoporphyrin IX, the iron-free prosthetic group of cytochrome P-450, is transformed into an abnormal green porphyrin to which AIA is covalently bound. Further study has revealed that the green porphyrin is a 1:1 porphyrin-AIA adduct formed when cytochrome P-450 is destroyed by self-catalyzed addition of AIA to its heme prosthetic group. A series of conformationally restricted analogues of AIA (Fig. 1a–e) do not share the ability of AIA to destroy cytochrome P-450, from which it was concluded that features of the AIA structure affected by conformational changes are important for the destructive action. On the other hand, several conformationally unrestricted analogues of AIA (Fig. 1f–j), as well as several ethynyl compounds (Fig. 2a–d), shared the ability of AIA both to destroy cytochrome P-450 and to form green pigments (6). These analogues offer an interesting tool with which to study the role of the heme moiety of cytochrome P-450 in the control of ALA synthetase. If the destruction of the heme moiety of cytochrome P-450 is an important signal for the induction of increased ALA synthetase activity (7), then conformationally restricted AIA analogues might be expected to be inactive in inducing ALA synthetase activity. On the other hand, one might expect the conformationally unrestricted analogues, as well as the ethynyl compounds, to show activity comparable with that of AIA in inducing ALA synthetase activity. Therefore, the objective of this study was to determine whether this was the case.

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[1] The abbreviations used are: AIA, allylisopropylacetamide; ALA, δ-aminolevulinic acid; PIA, propylisopropylacetamide.

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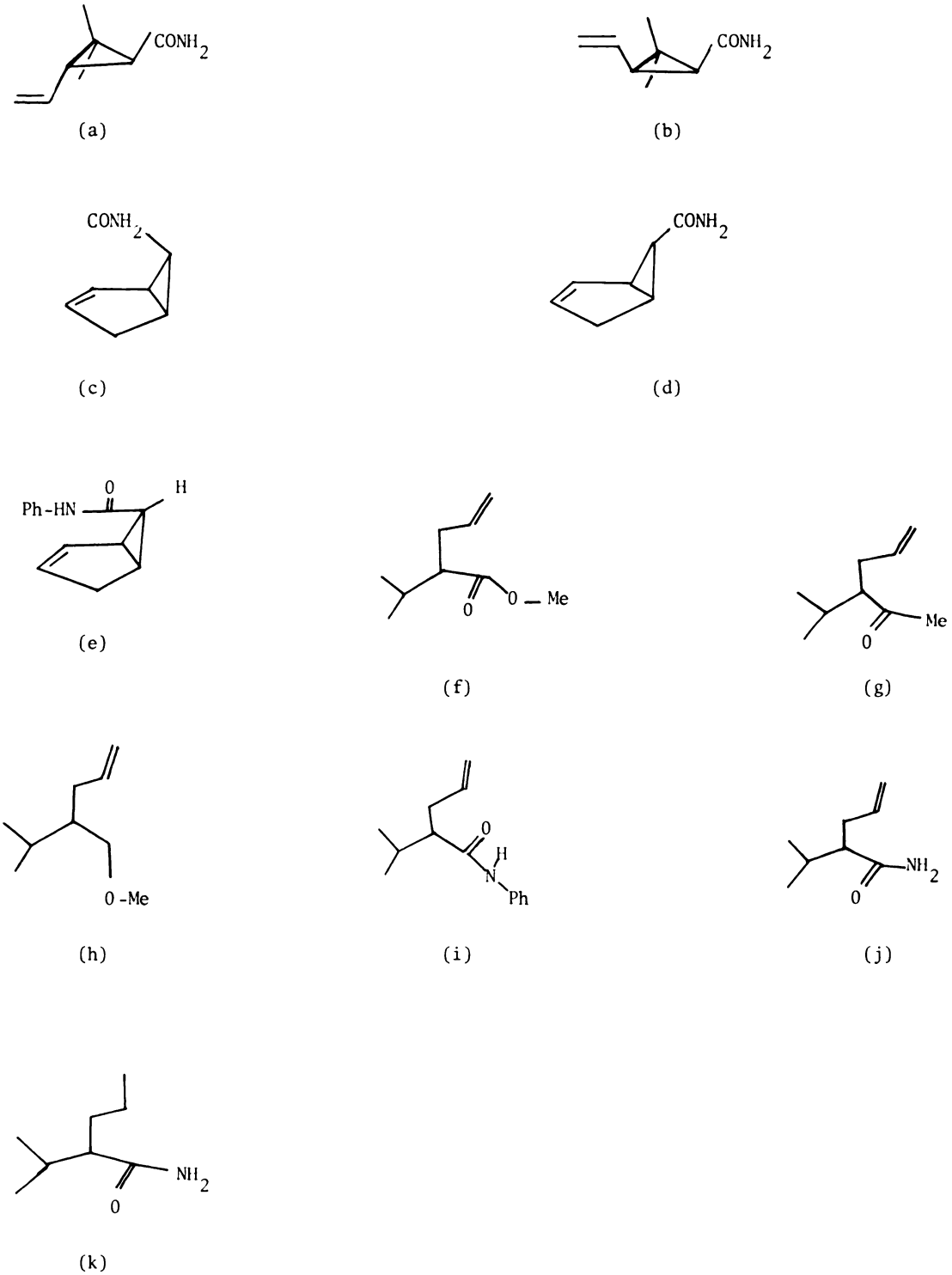
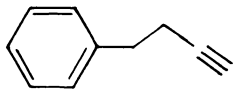
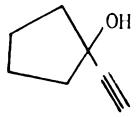


FIG. 1. *A series of conformationally restricted and unrestricted analogues of AIA (see text for details.)*
a. Structure of *trans*-2,2-dimethyl-3-vinyl-cyclopropanecarboxamide.
b. Structure of *cis*-2,2-dimethyl-3-vinyl-cyclopropanecarboxamide.
c. Structure of bicyclo-[3.1.0]-hex-2-ene-6-endo-carboxamide.
d. Structure of bicyclo-[3.1.0]-hex-2-ene-6-exo-carboxamide.
e. Structure of *N*-phenyl-bicyclo-[3.1.0]-hex-2-ene-6-endo-carboxamide.
f. Structure of methyl-2-isopropyl-4-pentenoate.
g. Structure of 3-isopropyl-5-hexene-2-one.
h. Structure of methyl-2-isopropyl-4-pentenyl ether.
i. Structure of *N*-phenyl-2-isopropyl-4-pentenamide.
j. Structure of 2-isopropyl-4-pentenamide (AIA).
k. Structure of 2-isopropyl-4-pentanamide (PIA).



(a)



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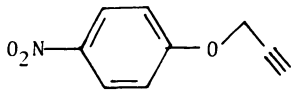
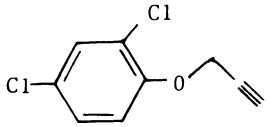


(c)



(d)

FIG. 2. *Ethynyl compounds*
a. Structure of 4-phenyl-1-butyne.
b. Structure of 1-ethynylcyclopentanol.
c. Structure of 3-(4-nitrophenoxy)-1-propyne.
d. Structure of 3-(2,4-dichlorophenoxy)-1-propyne.

METHODS

Source of compounds. Waymouth MD 705/1 medium was purchased in powder form from Grand Island Biological Company (Grand Island, N. Y.). Insulin (bovine pancreas, 24 IU/mg), L-thyroxine sodium pentahydrate, bovine serum albumin (Fraction V powder), bovine serum albumin (crystallized and lyophilized, for protein standard), collagenase (Type I, 460 NF units/mg), penicillin G sodium, and streptomycin sulfate were purchased from Sigma Chemical Company (St. Louis, Mo.). Folin and Ciocalteau phenol reagent was purchased from Fisher Scientific Company (Ottawa, Ont.). AIA was obtained as a gift from Hoffmann La Roche (Montreal, Canada). *Cis-* and *trans*-2,2-dimethyl-3-vinylcyclopropane carboxamide were synthesized by methods described previously (8). Oxidation of *endo*-bicyclo-[3.1.0]-hex-2-ene-6-carboxaldehyde (Aldrich Chemical Company, Inc., Milwaukee, Wisc.) with silver oxide, as reported by Meinwald *et al.* (9), provided the corresponding acid. Reaction of the acid with 1.1 Eq of oxalychloride in pyridine/benzene at 0°, followed by treatment with concentrated ammonium hydroxide, provided *endo*-bicyclo-[3.1.0]-hex-2-ene-6-carboxamide (m.p. 116–117°) in 48% yield. The starting *endo* aldehyde was isomerized to the *exo* isomer by refluxing with 2.5 Eq of sodium methoxide in dry methanol for 30 min. The *exo*-bicyclo-[3.1.0]-hex-2-ene-6-carboxamide (m.p. 171.5–172.5°) was then prepared as outlined for the *endo* isomer. Aniline was used instead of ammonium hydroxide in the preparation of *endo-N*-phenylbicyclo-[3.1.0]-hex-2-ene-6-carboxamide. All structures were consistent with elemental analyses and with IR, NMR, and mass spectrometric data. The syntheses of methyl 2-isopropyl-4-pentenoate, 3-isopropyl-5-hexene-2-one, methyl 2-isopropyl-4-pentenyl ether and *N*-phenyl-2-isopropyl-4-pentenamide have been described previously (4). 1-Ethynylcyclopentanol and 4-phenyl-1-butyne were purchased from Farchan Division, Story Chemical Corporation (Muskegon, Mich.). The compounds 3-(2,4-dichlorophenoxy)-1-propyne and 3-(4-nitrophenoxy)-1-propyne were prepared according to established procedures (10).

Cell culture technique. The details of the cell culture technique have been previously described (11, 12). The cells were maintained in 6-cm diameter disposable plastic Petri dishes (Falcon Plastics, Oxnard Calif.) containing 5 ml of Waymouth MD 705/1 medium supplemented with 60 mg of penicillin G, 100 mg of streptomycin sulfate, 1.0 mg of insulin, and 1.0 mg of L-thyroxine sodium pentahydrate per litre. After an initial incubation period of 24 hr, the medium was discarded and replaced with fresh medium. Chemicals dissolved in 95% ethanol (10 μl) were added to the cell culture and the dishes were reincubated. The porphyrin content of cells and medium and the protein content of cells were measured quantitatively 24 hr later (13). Porphyrins were measured by a fluorometric procedure. For this purpose, a standard fluorescence curve was constructed using 1, 2, 3, 4, and 5 ng/ml of standard of coproporphyrin I in 1 M perchloric acid-ethanol (1:1 v/v). Results are expressed as nanograms of porphyrins per milligram of protein.

Isolation of microsomes. Thirty-five chick embryos were treated for 3 days (days 16–18 of embryonic development) with once-daily injections of sodium phenobarbital (5 mg/0.1 ml of distilled water). The drug was injected through the chorioallantois into the fluids surrounding the embryo. Twenty-four hours after the last injection, the embryos were killed and the livers were removed. Liver microsomes were prepared in 0.05 M Tris buffer (pH 7.5) containing 1.15% potassium chloride, according to the procedure of Levin and Kuntzman (14) and Levin *et al.* (15).

Effect of AIA and analogues on microsomal cytochrome P-450 (4). A solution (4 ml) was prepared containing microsomal protein (0.5 mg/ml), NADPH (1 mM), KCl (150 mM), and EDTA (1.5 mM) in 0.1 N phosphate buffer, pH 7.4. AIA or the *cis-* or *trans*-cyclopropyl analogue was added to a concentration of 10 mM. Incubations were terminated after 30 min and cytochrome P-450 was measured spectrophotometrically (14, 15).

RESULTS AND DISCUSSION

After incubation of chick embryo microsomes with NADPH for 0.5 hr, the cytochrome P-450 content was shown to be 1.544 nmoles/mg of protein ± 0.022 (n = 5, mean ± SEM). When AIA was included in the incubation medium, a significant drop in cytochrome P-450 levels to 1.257 ± 0.027 (n = 5, mean ± SEM) was noted. When the *cis-* analogue of AIA (Fig. 1b) was included in the incubation medium, the cytochrome P-450 levels were not significantly changed (1.587 ± 0.032, n = 5, mean ± SEM). In a second experiment, the control level of cytochrome P-450 was found to be 1.172 nmoles/mg of protein ± 0.021 (n = 5, mean ± SEM). Although AIA caused a significant drop in these levels to 0.857 ± 0.018 (n = 5, mean ± SEM), the *trans* analogue was without effect, yielding levels of 1.150 ± 0.024 (n = 5, mean ± SEM). Thus, the results with AIA and the *cis* and *trans* analogues in chick embryo liver paralleled those reported in rat liver (4).

The potency of a porphyrin-inducing chemical in chick embryo liver cells can be determined either by measuring the level of ALA synthetase or by measuring porphyrin accumulation. In general, our experience supports the suggestion by Granick (13) that porphyrin accumulation is an indirect measure of the level of ALA synthetase in the cells. Therefore, in the present study, porphyrin accumulation has been measured and used as an index of ALA synthetase activity. The activity of the conformationally restricted analogues is compared in Fig. 3 with the activity of AIA at doses of 3, 10, and 30 μg/ml of medium. The *cis-* and *trans*-cyclopropyl analogues (Fig. 1a and b) differ from AIA (Fig. 1j) only by the presence of the darkened bond. The *trans*-isomer (Fig. 1a) exhibited higher potency than AIA, whereas the *cis*-isomer did not differ significantly in potency when compared with AIA. A considerable drop in activity relative to AIA was noted in the conformationally restricted analogues containing two-ring systems (Fig. 1c and d). The *endo* analogue (Fig. 1c) exhibited activity at 10 and 30 μg/ml, whereas the *exo* analogue (Fig. 1d) showed activity at 30 μg/ml; the phenylbicyclo analogue was inactive.

The results obtained with the conformationally unrestricted analogues of AIA (Fig. 1f–j) are shown in Fig. 4. *N*-phenyl AIA (Fig. 1i) displayed considerable activity, although it was less potent than AIA. The ketone (Fig. 1g) displayed slight activity, whereas the ether (Fig. 1h) and the ester (Fig. 1f) were inactive. The results obtained with the ethynyl compounds are shown in Fig. 5. The compound 3-(2,4-dichlorophenoxy)-1-propyne (Fig. 2d) showed moderate porphyrin-inducing activity, whereas 3-(4-nitrophenoxy)-1-propyne (Fig. 2c) showed slight activity. The remaining ethynyl compounds (Fig. 2a and b) were essentially inactive.

The conformationally restricted analogues of AIA, several of which show marked porphyrin-inducing activity (Fig. 3), do not share the ability of AIA to destroy the heme moiety of rat hepatic cytochrome P-450. The most potent of these conformationally restricted analogues,

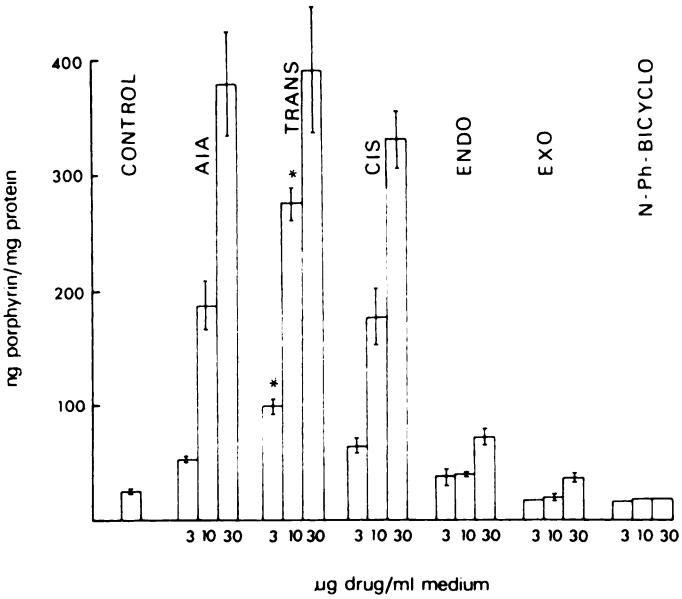


FIG. 3. *Porphyrin-inducing activity in chick embryo liver cell culture of solvent control (10 μl of ethanol); AIA; trans-2,2-dimethyl-3-vinyl-cyclopropanecarboxamide (TRANS); cis-2,2-dimethyl-3-vinyl-cyclopropanecarboxamide (CIS); bicyclo-[3.1.0]-hex-2-ene-6-endo-carboxamide (ENDO); bicyclo-[3.1.0]-hex-2-ene-6-exo-carboxamide (EXO); and N-phenyl-bicyclo-[3.1.0]-hex-2-ene-6-endo-carboxamide (N-Ph-BICYCLO)*

Each *bar* represents the mean of five determinations ± standard error of the mean. An *asterisk* denotes a significantly higher value than AIA tested at the same dose.

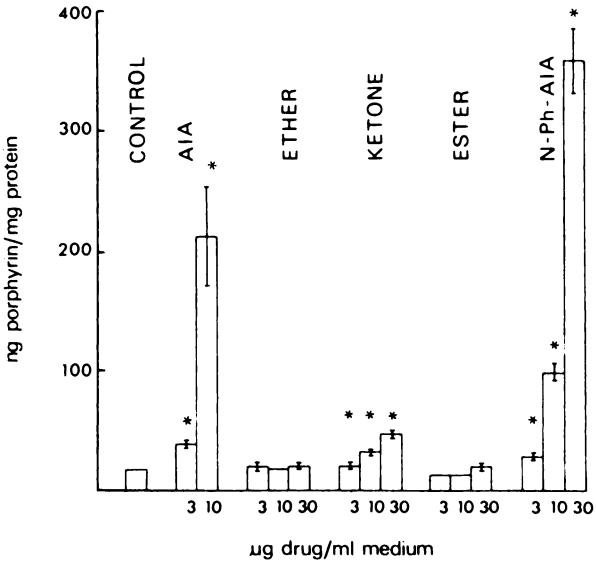


FIG. 4. *Porphyrin-inducing activity in chick embryo liver cell culture of solvent control (10 μl of ethanol); AIA; methyl-2-isopropyl-4-pentenyl ether (ETHER); 3-isopropyl-5-hexene-2-one (KETONE); methyl-2-isopropyl-4-pentenoate (ESTER); and N-phenyl-2-isopropyl-4-pentenamide (N-Phenyl AIA)*

Each *bar* represents the mean of five determinations ± standard error of the mean. *Asterisks* denote values significantly higher than those of solvent controls.

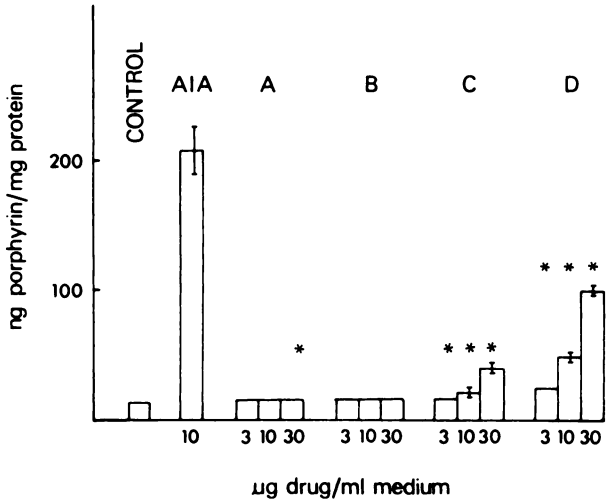


FIG. 5. *Porphyrin-inducing activity in chick embryo liver cell culture of solvent control (10 µl of ethanol); 4-phenyl-2-butyne (A); 1-ethynylcyclopentanol (B); 3-(4-nitrophenoxy)-1-propyne (C); and 3-(2,4-dichlorophenoxy)-1-propyne (D)*

Each *bar* represents the means of five determinations ± standard error of the mean. *Asterisks* denote values significantly higher than those of solvent controls.

viz., the *cis* and *trans* analogues (Figs. 1a and b), also do not destroy the heme moiety of chick embryo hepatic cytochrome P-450, in contrast to AIA. Conversely, the conformationally unrestricted AIA analogues (Fig. 1f–i) and the ethynyl compounds (Fig. 2a–d), which are either inactive or show moderate porphyrin-inducing activity (Figs. 4 and 5), do in fact share the ability of AIA to destroy rat hepatic cytochrome P-450. Therefore, it follows that in chick embryo liver cell culture, unlike rat liver (7), destruction of the heme moiety of cytochrome P-450 is not a requirement for ALA synthetase induction and porphyrin accumulation.

These results are consistent with previously reported differences between rat and chick embryo liver. Thus, in the rat, substituted amides lacking allyl groups, e.g. PIA (Fig. 1k), fail to promote destruction of the heme moiety of cytochrome P-450 (15, 16) and fail to induce ALA synthetase activity and porphyrin accumulation (16). In the chick embryo, on the other hand, despite the fact that PIA does not promote destruction of the heme moiety of cytochrome P-450, it is an inducer of ALA synthetase activity and porphyrin accumulation (17). Therefore, these original studies led to the conclusion that destruction of the heme moiety of cytochrome P-450 with concomitant lowering of cytochrome P-450 levels was not a prerequisite for ALA synthetase induction and porphyrin accumulation in chick embryo. The present studies therefore strengthen the conclusions previously reached by comparison of the properties of AIA and PIA.

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Send reprint requests to: Dr. Gerald S. Marks, Department of Pharmacology, Queen's University, Kingston, Ontario K7L 3N6, Canada.