Kinetic Evidence for Heterogeneous Responsiveness of Mixed Function Oxidase Isozymes to Inhibition and Induction by Allylisopropylacetamide in Chick Embryo Liver*

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Changes in hepatic mixed function oxidase kinetics after administration of allylisopropylacetamide (AIA) to chick embryos indicate that the activities of different cytochrome P-450 isozymes, including those participating in the metabolism of the same substrates, can be simultaneously increased and inhibited by a single xenobiotic. Up to 4 h after administration in ovo, or in vitro, AIA exclusively inhibited mixed function oxidases. At 24 h after administration in ovo, AIA simultaneously decreased the $V_{max}$ of the isozymes active in 7-ethoxycoumarin deethylation and in biphenyl and antipyrine hydroxylations in control liver and caused new isozymes with higher $K_m$ and $V_{max}$ values to appear. At the same time, AIA increased the $V_{max}$ values for isozymes active in aminopyrine demethylation and decreased the $V_{max}$ for benzo(a)pyrene hydroxylation (EC 1.14.14.1). As an inhibitor, AIA did not exhibit substrate selectivity but tended to inhibit isozymes with higher substrate affinity noncompetitively and lower affinity isozymes competitively. Competitive mechanisms and generalized P-450 breakdown could only partially account for the inhibition of mixed function oxidases by AIA. The inhibition at low doses of AIA (0.1 to 0.3 mg/egg) occurred without any decrease in P-450 and at higher doses it exceeded and was more persistent than the decrease in P-450. The data indicate that in addition to the known mechanisms for mixed function oxidase inhibition by AIA there is another noncompetitive mechanism independent of P-450 breakdown. As an inducer, AIA, like phenobarbital rather than $\beta$-naphthoflavone increased the metabolism of aminopyrine and the concentration of $M_i = 50,000$ and 51,000 proteins preferentially. However, unlike either, AIA selectively induced new high $K_m$ and $V_{max}$ isozymes active toward 7-ethoxycoumarin, biphenyl, and antipyrine and increased the concentration of a $M_i = 53,000$ protein. These actions distinguish AIA from either the phenobarbital or polycyclic hydrocarbon class of inducers. The simultaneous inhibition by AIA of higher affinity isozymes with selective induction of low affinity isozymes produced a "crossover effect" in which after AIA administration the rates of 7-ethoxycoumarin deethylation and biphenyl and antipyrine hydroxylases were decreased at low and increased at high substrate concentrations. The findings demonstrate the complexity and selectivity of AIA's actions as a mixed function oxidase inhibitor and induce and illustrate the potential heterogeneity of responses that can occur in the mixed function oxidase system after exposure of an organism to a xenobiotic.

There is evidence that the multiple forms of cytochrome P-450 present in liver in various species are heterogeneous with respect to their function and regulation as well as structure. Thus, P-450s with different electrophoretic mobilities and immunodiffusion properties exhibit differences in substrate specificity (1-3) and can direct the metabolism of substrates to different products (4-7).

In this paper, evidence is presented based on kinetic data, using allylisopropylacetamide as a probe, that a single xenobiotic can concurrently have opposite effects on monooxygenases involved in the metabolism of different substrates and on different monooxygenases involved in the metabolism of the same substrates. Therefore, the diversity of the changes in monooxygenase function that may follow exposure of an organism to a xenobiotic is even more extensive than is generally recognized.

AIA* has recently received much attention because it can form an adduct with the heme of cytochrome P-450, resulting in P-450 breakdown and green pigment formation in the liver (8, 9). AIA, however, is also known to be an inducer of hepatic P-450 (8, 10, 11) as well as of $\delta$-aminolevulinic acid synthetase in chick embryo and mammalian liver (10-13).

The effects of AIA were examined on the kinetics of five model mixed function oxidase reactions and on the cytochrome P-450 and $b_5$ contents of chick embryo liver, at varying doses of AIA and after different periods of exposure. At first, after administration in ovo, and in vitro, AIA inhibited the rate of metabolism of all of the substrates. Later, it simultaneously increased the rate of demethylation of aminopyrine and decreased the rate of hydroxylation of benzo(a)pyrene, (aryl hydrocarbon hydroxylase, EC 1.14.14.1), while concurrently increasing and decreasing the rates of different kinetic components involved in the demethylation of 7-ethoxycoumarin and the hydroxylations of biphenyl and antipyrine.

The studies further revealed selective characteristics of AIA as an inhibitor and an inducer of mixed function oxidases in the chick embryo liver. As an inducer, AIA has more characteristics in common with phenobarbital than with $\beta$-NF, but it cannot be considered a member of either of the major classes of inducers.

**MATERIALS AND METHODS**

*Chemicals and Reagents*—The following were kindly provided as gifts: allylisopropylacetamide, from Hoffmann-La Roche, Inc.; 3-hy-

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*The abbreviations used are: AIA, allylisopropylacetamide; $\beta$-NF, $\beta$-naphthoflavone; MeSO, dimethylsulfoxide; SDS, sodium dodecyl sulfate.
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droxybenzo(a)pyrene, from Dr. Harry Gelboin, National Institutes of Health; and phenobarbital sodium, from Merck Sharp and Dohme. The commercial sources of other chemicals and reagents were as follows: Aldrich Chemical Co.: aminopyrine, 7-ethoxycoumarin (gold label, 99.9% pure), 4-hydroxyantipyrene, isonicotinyl alcohol, β-naphthoflavone, urea (gold label), Allied Chemical: magnesium chloride; Baker Chemical: acetone, benzene, bromphenol blue, cupric sulfate, ethyl acetate, isopropyl alcohol, N,N,N',N'-tetramethylethylenediamine, 2.5 mM NADPH, 0.5 mM NADH, 0.5 mM 7-ethoxycoumarin (added in 0.050 ml of methanol), 60 mM potassium phosphate buffer, pH 7.4, 3.6 mM sodium chloride, 9,000 X g supernatant equivalent to 100 mg, wet weight, in a total volume of 2 ml. After 15 min of incubation, reactions were stopped with 0.5 ml of 1 M sodium hydrochloric acid.

Eggs injected with Me2S0 alone were included as controls in each experiment. Livers from six to 10 embryos in each treatment group were pooled. The livers were homogenized in 0.1 M potassium phosphate buffer, pH 7.4 (25%, w/v) and centrifuged for 10 min at 9,000 x g. The 9,000 x g supernatants were used to measure mixed function oxidase activities. Microsomes were prepared by centrifuging the 9,000 x g supernatants at 105,000 X g for 1 h. For measurements of P450, b5, and E1, microsomes were suspended in 0.1 M potassium phosphate buffer, pH 7.4, in 100 mg, wet weight, in a total volume of 2.5 ml. After incubation for 10 min, reactions were stopped by transferring 1 ml of the reaction mixture, in duplicate, to tubes containing 8 ml of chloroform and approximately 2.5 g of sodium chloride. Six millimolar sodium hydroxide was added and the mixture was extracted with 25 ml of heptane containing isoamyl alcohol gel thin layer chromatography plates (20 x 20 cm), 0.25 mm thick (Merck 60 F-254). The chromatograms were developed in a solvent system of ethyl acetate (85%), v/v, methanol (10%, v/v), and ammonium hydroxide (5%, v/v). The plates were scanned using a Schoeffel spectrodensitometer. Peak areas were measured graphically by triangulation. A standard curve for 4-hydroxyantipyrine was included in each assay to measure the amount of 4-hydroxyantipyrine formed.

Enzyme Assays—Benzo(a)pyrene hydroxylase was assayed by formation of phenolic fluorescent products from benzo(a)pyrene (18). The reaction mixtures contained 5 mM magnesium chloride, 0.1 mM benzo(a)pyrene (added in 0.025 ml of methanol), 55 mM Tris-sucrose, pH 7.5, and 9,000 x g supernatant equivalent to 100 mg, wet weight, in a total volume of 2.5 ml. After incubation for 10 min, reactions were terminated with 0.5 ml of acetone and 1.6 ml of hexane. The organic phase (1.0 ml) was added to 2 ml of 0.1 M sodium hydroxide and the fluorescence of the aqueous phase was measured in a Hitachi MPF 3 spectrophotofluorimeter, at excitation and emission wavelengths of 396 and 522 nm, respectively. The amount of fluorescent phenolic metabolites produced was measured using a quinine sulfate standard, previously standardized against 3-hydroxybenzo(a)pyrene.

Aminopyrine demethylase was measured by production of formaldehyde using a modification of the method of Kato and Onoda (15). Reaction mixtures contained 5 mM magnesium chloride, 10 mM sodium bicarbonate, 5 mM isocitric acid, 0.5 mM NADPH, 5 mM sodium bicarbonate, 10 mM magnesium chloride, 0.1 mM benzo(a)pyrene (added in 0.025 ml of methanol), 55 mM Tris-sucrose, pH 7.5, and 9,000 x g supernatant equivalent to 125 mg, wet weight, in a total volume of 1.25 ml. After incubation for 30 min, reactions were stopped with 0.3 ml of trichloroacetic acid (25%, w/v). The formaldehyde formed was measured using the method of Nash as modified by Cochin and Axelrod (16). Allylisopropylacetamide, phenobarbital sodium, or β-naphthoflavone in 0.1 ml of dimethylsulfoxide were injected through a 23-gauge 1-inch needle inserted its full length through a hole made in the shell. Eggs injected with Me2S0 alone were included as controls in each experiment.

Enzyme Kinetics—To measure the kinetic constants, the reaction rates were measured using the following ranges of substrate concentrations: benzo(a)pyrene, 0.8 μM to 2 mM; aminopyrine, 0.01 to 4 mM; 7-ethoxycoumarin, 0.02 μM to 1.0 mM; benzyln, 5 μM to 1.0 mM; and antipyrine, 0.03 to 8 mM. The data were plotted on Lineweaver-Burk paper and the kinetic constants were determined using a weighted regression analysis of the data (21). When the plots were nonlinear, but were resolvable into two or more components, the Eadie-Hofstee plots were used as described by McPherson et al. (19) using benzyln, 2- and 4-hydroxybenzophenone as standards.

Biphenyl 4-hydroxylase was assayed by formation of 4-hydroxyantipyrine. Incubation mixtures included 5 mM magnesium chloride, 0.5 mM NADP, 5 mM glucose 6-phosphate, 76 μM potassium phosphatase buffer, pH 7.4, 4 mM antipyrine (in 0.1 ml water), and 9,000 x g supernatant equivalent to 100 mg, wet weight, in a total volume of 2.5 ml. After incubation for 10 min, reactions were stopped by transferring 1 ml of the reaction mixture, in duplicate, to tubes containing 8 ml of chloroform and approximately 2.5 g of sodium chloride. Six millimolar sodium hydroxide was added and the mixture was extracted with 25 ml of heptane containing isoamyl alcohol gel thin layer chromatography plates (20 x 20 cm), 0.25 mm thick (Merck 60-F-254). The chromatograms were developed in a solvent system of ethyl acetate (85%), v/v, methanol (10%, v/v), and ammonium hydroxide (5%, v/v). The plates were scanned using a Schoeffel spectrodensitometer. Peak areas were measured graphically by triangulation. A standard curve for 4-hydroxyantipyrine was included in each assay to measure the amount of 4-hydroxyantipyrine formed.

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When the plots were nonlinear, they could be resolved into two or more components. The Eadie-Hofstee plots were used as described by McPherson et al. (19) using benzyln, 2- and 4-hydroxybenzophenone as standards.
if the reconstructed and observed curves closely approximated each other.

To determine $K$ values, aminopyrine demethylase and 7-ethoxycoumarin deethylase activities were measured at both low and high ranges of substrate concentrations in the absence and presence of AIA at several concentrations in the range $10^{-4}$ to $10^{-3}$ M. The data were plotted on double reciprocal plots, and the slopes of the resulting lines (if the inhibition was competitive) or the intercepts and the slopes (if the inhibition was noncompetitive) were replotted as a function of AIA concentration. The slopes and abscissa intercepts for those lines were, in turn, used to calculate the $K$ values.

Microsomal Cytochromes—Microsomal cytochromes P-450 and b$_v$ were measured according to the method of Omura and Sato (25). Cytochrome P-450 was measured using the carbon monoxide difference spectrum of microsomes reduced with dithionite and an extinction coefficient of 91 mm$^{-1}$ cm$^{-1}$ for the difference in absorbance at 450 nm relative to 490 nm. Cytochrome b$_v$ was measured using the NADH difference spectrum of air-saturated microsomes and an extinction coefficient of 185 mm$^{-1}$ cm$^{-1}$ for the difference in absorbance at 424 nm relative to 409 nm. Protein was measured by the method of Lowry et al. (26) using bovine serum albumin as a standard.

RESULTS

Effects of AIA in Ovo on Hepatic Mixed Function Oxidase Activities—Fig. 1 shows the mean changes in hepatic mixed function oxidase activities (measured under standard $V_{max}$ conditions as described under “Materials and Methods”) and cytochrome P-450 and b$_v$ concentrations at various intervals after injection of 3 mg of AIA into 18-day-old chick embryos. One hour after injection of AIA, the rates of the mixed function oxidase reactions were decreased by 55 to 75%, cytochrome P-450 concentrations were decreased by a mean of 28%, and cytochrome b$_v$ was not decreased as compared to controls. By 4 h, the rates of all of the mixed function oxidase reactions remained depressed, but the rate of aminopyrine demethylase was less depressed than at 1 h, and cytochrome P-450 levels were no longer significantly depressed. At 24 h, the rate of aminopyrine demethylase and cytochrome P-450 content were increased above control levels. The rate of benzo(a)pyrene hydroxylase remained decreased. The mean rates of 7-ethoxycoumarin deethylation and biphenyl 4-hydroxylation were close to control values. However, the rates of the latter two reactions were altered variably; they were increased over control in about half of the experiments and decreased in half. In contrast, 24 h after phenobarbital or $\beta$-NF pretreatment, the rates of all of the mixed function oxidases were increased above control rates, although as in other species, the amount of increase for the different reactions differed for the two inducers.

The Effect of AIA in Ovo on Mixed Function Oxidase Activities as a Function of Substrate Concentration—The observed experimental variation in the effects of AIA on 7-ethoxycoumarin deethylase and biphenyl hydroxylase at 24 h after injection became understandable when their rates were measured at varying substrate concentrations. The rate of 7-ethoxycoumarin deethylation was lower than in control livers at low substrate concentrations, but was higher than in control liver at higher substrate concentrations, a phenomenon henceforth referred to here as the “crossover effect” (Fig. 2). The crossover effect was not observed in livers from phenobarbital- or $\beta$-NF-pretreated embryos, where 7-ethoxycoumarin deethylase activity was increased over the full substrate range (Fig. 3), or 4 h after injection of AIA, when 7-ethoxycoumarin deethylase activity was decreased over the same substrate range (Fig. 4).

The crossover point for the AIA-treated livers occurred at about 500 $\mu$m 7-ethoxycoumarin, the substrate concentration routinely used to measure the reaction ($V_{max}$ condition for livers from control and phenobarbital- and $\beta$-NF-pretreated embryos). Therefore, given normal biological variation, it was predictable that, at a substrate concentration of 500 $\mu$m, on some occasions small increases in 7-ethoxycoumarin deethylase would be observed and on other occasions small decreases.
FIG. 2. Effect of AIA on 7-ethoxycoumarin deethylase 24 h after administration, as a function of substrate concentration. AIA, 3 mg/egg, or solvent was injected into 18-day-old chick embryos, using 12 eggs for each group. 24 hours later, livers were removed and pooled, 9000 x g supernatants were prepared, and 7-ethoxycoumarin deethylase was measured as described under “Materials and Methods.” Each point represents the mean of triplicate determinations.

The crossover effect was also observed for biphenyl hydroxylase 24 h after injection of AIA (Fig. 5). Again, the crossover point occurred at about 500 µM, the biphenyl concentration routinely used to measure the rate of the reaction. Therefore, the observed experimental variability again became explainable. As with 7-ethoxycoumarin deethylase, the crossover effect was not observed for livers from phenobarbital- or β-NF-pretreated embryos (Fig. 6, a and b).

Antipyrine hydroxylase also exhibited the crossover effect, with the crossover occurring at about 1.5 mM antipyrine. The effect was not observed for livers from phenobarbital-pretreated embryos (Fig. 7).

In contrast, the crossover effect did not occur for aminopyrine demethylase or benzo(a)pyrene hydroxylase after AIA pretreatment. Twenty-four hours after AIA administration, aminopyrine demethylase activity was increased and benzo(a)pyrene hydroxylase activity was decreased over the full substrate ranges for which those activities could be measured.

Effects of AIA in Ovo on Kinetics of Mixed Function Oxidase Reactions—The kinetic parameters of 7-ethoxycoumarin deethylase, biphenyl hydroxylase, and antipyrine hydroxylase 24 h after drug administration are shown in Tables I–III.

There were two kinetic components for 7-ethoxycoumarin deethylase in control liver (Table I). After AIA pretreatment, their Vmax values were decreased. The Ks of the second component showed a small increase. Thus, AIA appeared to inhibit the highest affinity component for 7-ethoxycoumarin deethylase noncompetitively and the second component by a mixed competitive and noncompetitive mechanism. AIA also caused a third kinetic component with higher Ks and Vmax than those of either of the components in control liver to appear. The effects of AIA differed from those of phenobarbital or β-NF, both of which increased the Vmax values for both kinetic components participating in 7-ethoxycoumarin deethylase and did not cause any new component to appear. Phenobarbital and β-NF produced comparable increases in the Ks of the higher affinity component, but phenobarbital increased the Ks for the second component more than had β-NF.

For biphenyl hydroxylase, there was a single component in control liver (Table II). After AIA treatment, that component was inhibited noncompetitively. AIA also caused a new lower affinity component with higher Ks and Vmax, than the component in control liver to appear. Phenobarbital and β-NF produced similar increases in the Ks and Vmax of the higher
In contrast, for those reactions for which the crossover effect had not been observed, aminopyrine demethylase and benzo(a)pyrene hydroxylase, AIA pretreatment did not change the number of components nor was there any evidence

for new components with higher $K_a$ values (Table IV). For aminopyrine demethylase, AIA increased the $V_{\text{max}}$ of both components and increased the $K_a$ values, particularly the $K_a$ of the higher affinity component. The kinetics for benzo(a)pyrene hydroxylase were linear for livers of both control and AIA-pretreated embryos. AIA pretreatment decreased the $V_{\text{max}}$ and increased the $K_a$, suggesting that AIA inhibited benzo(a)pyrene hydroxylase by a mixed mechanism. 

**Inhibition of Mixed Function Oxidase Activities by AIA in Vitro—In vitro**, AIA exclusively inhibited and did not activate any of the mixed function oxidase reactions (Table V).

For aminopyrine demethylase, AIA at $10^{-5}$ M could eliminate the higher affinity component completely. It increased the $K_a$ without affecting the $V_{\text{max}}$ of the lower affinity component, suggesting that it inhibited the lower affinity component by a competitive mechanism.

For 7-ethoxycoumarin deethylase, AIA decreased the $V_{\text{max}}$ but did not affect the $K_a$ of the higher affinity component while it increased the $K_a$ and decreased the $V_{\text{max}}$ of the lower affinity component. These findings suggest that AIA inhibited the higher affinity component mainly noncompetitively and the lower affinity component by a mixed, competitive and noncompetitive mechanism.

AIA increased the $K_a$ and decreased the $V_{\text{max}}$ for benzyphenyl hydroxylase, suggesting that it inhibited benzyphenyl hydroxylase by a mixed, competitive and noncompetitive mechanism.

These findings established that AIA could inhibit mixed function oxidases by competitive and noncompetitive mechanisms. Noncompetitive mechanisms appeared to operate preferentially toward higher affinity isozymes, and competitive mechanisms toward lower affinity isozymes. Competitive inhibition was more evident after *in vitro* than *in vivo* administration of AIA, probably because lower concentrations of AIA were likely to be present in the assays performed after *in vivo* administration. Thus, for example, assuming homogenous distribution of AIA in the 50-g egg, after administration of a 3-mg dose of AIA, the concentrations of AIA in the reaction mixtures would be about $1 \times 10^{-4}$ M for benzo(a)pyrene hydroxylase and 7-ethoxycoumarin deethylase and $2 \times 10^{-5}$ M for aminopyrine demethylase and biphenyl hydroxylase.

**Evidence That Differences in the Effects of AIA in Vivo on**

4 It is understood that our designation of kinetic components as isozymes is heuristic but provisional and that physical separation of the isozymes would be required for definitive demonstration that different isozymes are present.
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**Table I**

| Drug           | K_m | V_max | K_m | V_max | K_m | V_max |
|---------------|-----|-------|-----|-------|-----|-------|
| Control       | 1.2 x 10^-1 | 72   | 4.1 x 10^-4 | 1199 | 5.0 x 10^-4 | 2671 |
| AIA           | 1.3 x 10^-2 | 31   | 7.0 x 10^-4 | 615  | 2.75 x 10^-4 | 1624 |
| Phenobarbital | 2.4 x 10^-1 | 262  | 8.7 x 10^-5 | 2986 | —          | —    |
| β-NF          | 3.1 x 10^-3 | 178  | 5.0 x 10^-6 | 2671 | —          | —    |

* K_m [μM]; V_max, nanomoles of umbelliferone/g of liver/h.
* —, only two components present.

**Table II**

Effect of drugs injected in vivo on apparent kinetic constants for 7-ethoxycoumarin deethylase

Procedure was the same as in Table I. Biphenyl hydroxylase was assayed at substrate concentrations of 0.005 to 1.0 mM. The data shown represent the means from two to three experiments.

| Drug           | K_m | V_max | K_m | V_max |
|---------------|-----|-------|-----|-------|
| Control       | 1.5 x 10^-5 | 829 | 1.2 x 10^-3 | 5140 |
| AIA           | 1.1 x 10^-5 | 430 | 6.5 x 10^-3 | — |
| Phenobarbital | 3.5 x 10^-5 | 1467| —     | — |
| β-NF          | 3.7 x 10^-5 | 1498| 7.0 x 10^-4 | 1207 |

* K_m [μM]; V_max, nanomoles of 4-hydroxybenzyl/g of liver/h.
* —, only one component present.

Aminopyrine Demethylase and 7-Ethoxycoumarin Deethylase Are Not Attributable to Differences in Sensitivity of the Isozymes for Each Reaction to Inhibition by AIA—A principal finding of these studies was that the crossover effect was observed for some mixed function oxidases such as 7-ethoxycoumarin deethylase and not for others such as aminopyrine demethylase. A greater sensitivity to inhibition by AIA of the isozymes involved in 7-ethoxycoumarin demethylase than of the isozymes involved in aminopyrine demethylation could explain this difference. However, the in vitro data indicated that AIA could inhibit both sets of kinetic components participating in 7-ethoxycoumarin deethylase and aminopyrine demethylation. Further, the K_m values for the inhibition by AIA of both the low and high affinity components for both reactions were in the same range (for high affinity component, K_a (intercept) and K_a (slope): 5.8 x 10^-4 and 4.3 x 10^-3 M for 7-ethoxycoumarin deethylase and 4.2 x 10^-4 and 1.2 x 10^-3 M for aminopyrine demethylase; for low affinity component, K_a: 4.2 x 10^-4 and 2.0 x 10^-3 M for 7-ethoxycoumarin deethylase and aminopyrine demethylase, respectively.) Further, after preincubation of AIA with 9000 x g supernatant, the noncompetitive inhibition of aminopyrine demethylase and 7-ethoxycoumarin deethylase was enhanced (data not shown). The data suggest that AIA is about equally effective as an inhibitor of the isozymes for both reactions. Consequently, the occurrence of the crossover effect for 7-ethoxycoumarin deethylase, but not for aminopyrine demethylase, after AIA administration in vivo, could not be attributed to differences in responsiveness of the sets of isozymes for each reaction to inhibition by AIA. AIA’s selectivity as an inducer of mixed function oxidase enzymes better explains the findings, as discussed below.

Evidence that P-450 Breakdown does not Account for Inhibition of Mixed Function Oxidase Activity—One hour after administration of 3 mg of AIA in vivo (Fig. 1), the rates of the mixed function oxidase reactions were decreased by means of 57-78%, but cytochrome P-450 concentrations were decreased by a mean of 28 ± 4% (S.E.). By 4 h, although all of the mixed function oxidase activities remained decreased, concentrations from 0.02 to 1000 μM. The apparent kinetic constants were derived from computer analysis as described under “Materials and Methods.” The data shown represent the means from four experiments each for control and AIA and two experiments each for phenobarbital and β-NF treatment.

| Drug           | K_m | V_max | K_m | V_max |
|---------------|-----|-------|-----|-------|
| Aminopyrine demethylase | Control | 7 x 10^-6 | 0.46 | 3.8 x 10^-4 | 1.40 |
| AIA           | 1.5 x 10^-4 | 1.78 | 5.9 x 10^-4 | 6.08 |
| Benzo(a)pyrene hydroxylase | Control | 1.8 x 10^-6 | 1.40 | — | — |
| AIA           | 1.3 x 10^-6 | 0.63 | — | — |

* K_m [μM]; V_max, nanomoles of formaldehyde/g of liver/h for aminopyrine demethylase; micromoles of phenols/g of liver/h for benzo(a)pyrene hydroxylase.
* —, only one component present.

**Table III**

Effect of AIA in vivo on kinetics of aminopyrine demethylase and benzo(a)pyrene hydroxylase

Procedures were the same as for Tables I-III. Aminopyrine demethylase was measured over a substrate range of 0.01 to 4 mM. Benzo(a)pyrene hydroxylase was measured over a substrate range of 0.008 to 2 mM. The data for aminopyrine demethylase represent the means from four experiments and data for benzo(a)pyrene hydroxylase the means from two experiments.

**Table IV**

Effect of AIA in vivo on kinetics of cytochrome P-450

Cytochrome P-450 levels were no longer depressed. Higher doses of AIA did not produce any greater or more persistent depression of cytochrome P-450. At lower doses, mixed function oxidase activities were decreased without any depression of P-450. Thus, at 1 h after administration of AIA at 0.1 and 0.3 mg/egg, mixed function oxidases were decreased to 47 to 68%, and to 40 to 60% of control levels, respectively, while P-450 was not decreased.

After incubation of the 9000 x g supernatant of liver in vitro with AIA for 30 min, the maximum destruction of P-450 observed was 25%, even at high concentrations of AIA (1-3 mM) (Fig. 8). There was a parallel but lesser decrease in cytochrome b5. At the same time, greater decreases in mixed function oxidase activity occurred. At 3 mM AIA, aminopyrine demethylase was decreased by 65%. Thus, the degree of inhibition of mixed function oxidase activity in vivo or in vitro always exceeded the degree of decrease of cytochrome P-450.

The Inhibition of Mixed Function Oxidase Activity by AIA is Not Attributable Either to Metabolism of the Substrates to Other Products or to Protoporphyrin Accumulation in the
Methods with substrate concentrations as in Tables I, II, and III. AIA in water was added to the reaction mixtures prior to the initiation of the reactions with substrate. The $K_m$ and $V_{max}$ values were derived as described under "Materials and Methods." The data shown are the means from two to three experiments.

| Reaction                          | Drug added to reaction mixture | $K_m$ | $V_{max}$ | $K_{cat}$ | $V_{max}$ |
|----------------------------------|--------------------------------|------|----------|----------|----------|
| Aminopyrine demethylase          | None                           | $5.1 \times 10^{-4}$ | 469      | $4.3 \times 10^{-4}$ | 1400     |
| 7-Ethoxycoumarin deethylase      | AIA (10$^{-5}$ M)              | $2.6 \times 10^{-7}$ | 225      | $3.9 \times 10^{-5}$ | 986      |
| Biphenyl hydroxylase             | None                           | $1.7 \times 10^{-5}$ | 1020     | $13.6 \times 10^{-6}$ | 896      |
|                                  | AIA (4 $\times 10^{-3}$ M)     | $9.0 \times 10^{-8}$ | 775      | $3.9 \times 10^{-6}$ | $3.9 \times 10^{-6}$ |

* $K_m$, [M]; $V_{max}$, nanomoles of product/g of liver/h.

**There was no evidence for a higher affinity component for aminopyrine demethylase.

* There was only one component evident for biphenyl hydroxylase.

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**Fig. 8. Effect of AIA in vitro on microsomal cytochromes and aminopyrine demethylase activity.** Livers were pooled from 18-day-old chick embryos and 9000 × g supernatant was prepared. Another set of flasks was prepared containing the same reaction mixture, but without aminopyrine. One hour later, cycloheximide, 10 µg/egg in 0.1 ml of water, was injected into 12 of the eggs. Control eggs received 0.1 ml of MeSO and 0.1 ml of water. Twenty-four hours later, livers were removed. The livers from each treatment group were pooled and assayed for 7-ethoxycoumarin deethylase. Cycloheximide alone did not depress 7-ethoxycoumarin deethylase activity.

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**Fig. 9. Modification by cycloheximide of AIA's substrate-dependent effects on 7-ethoxycoumarin deethylase.** AIA, 3 mg/egg in 0.1 ml of MeSO, was injected into 24 18-day-old chick embryos. One hour later, cycloheximide, 10 µg/egg in 0.1 ml of water, was injected into 12 of the eggs. Control eggs received 0.1 ml of MeSO and 0.1 ml of water. Twenty-four hours later, livers were removed. The livers from each treatment group were pooled and assayed for 7-ethoxycoumarin deethylase. Cycloheximide alone did not depress 7-ethoxycoumarin deethylase activity.

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**Liver**—In other experiments, AIA in vitro did not increase the rate of hepatic metabolism of umbelliferone, nor, as determined by thin layer chromatography, did it change the pattern of metabolite production from biphenyl. Therefore, the inhibition of mixed function oxidase activity observed after AIA administration in vivo was not merely an artifact of the assay methods used.

Also, it could not be attributed to porphyrin accumulation. The decrease in mixed function oxidase activity preceded any increase in hepatic porphyrin accumulation by the chick embryo liver. Further, protoporphyrin at more than twice the concentration found in the liver 24 h after administration of 3 mg of AIA, did not inhibit 7-ethoxycoumarin deethylase activity in vitro, whether the activity was measured at high or low substrate levels. Therefore, porphyrin accumulation did not appear to be contributing to the early or late inhibition of mixed function oxidase activity by AIA.

**Evidence That AIA Increased Mixed Function Oxidase Activity by an Induction Mechanism**—The findings that AIA in vitro exclusively inhibited and did not increase mixed function oxidase activity in vitro and that several hours were required for the increase in aminopyrine demethylase and the crossover effect to appear after in vivo exposure to AIA suggested that induction rather than activation was responsible for the increase in mixed function oxidase activity.

In support of an induction mechanism, cycloheximide, at 10 µg/egg (a dose which does not decrease 7-ethoxycoumarin deethylase activity in chick embryo liver), injected into chick embryos 1 h before AIA, prevented the increase in deethylase activity at high substrate concentrations, but not the decrease at low substrate concentrations (Fig. 9). Therefore, new protein synthesis, as required for induction, is also required for the increase by AIA in 7-ethoxycoumarin deethylase activity at high substrate concentrations.

Dose-response characteristics for AIA's effects also support an induction mechanism. Thus, aminopyrine demethylase activity, and 7-ethoxycoumarin deethylase activities when measured at high substrate levels, were increased only at
doses of AIA which also increased cytochrome P-450 concentration (3 mg/egg and higher).

**Gel Electrophoresis of Microsomes after AIA Pretreatment**—Electrophoretic data further supported an induction mechanism. Fig. 10 shows a photograph of an SDS-polyacrylamide gel, following electrophoresis of microsomes from embryos pretreated with solvent alone or with phenobarbital, β-NF, or AIA. At least five bands were affected by the drug treatments. Two bands in the $M_r$ = 50,000 and 51,000 regions were increased by phenobarbital in agreement with the findings of Althaus et al. (29). β-NF increased the intensity of two other bands, in the $M_r$ = 56,000 and 57,000 regions. AIA increased the intensity of both of the phenobarbital-inducible bands and also of another band of $M_r$ = 53,000 similar to that previously reported (30).

To learn whether there were concurrent increases in the AIA-inducible bands and mixed function oxidase activity and whether there were differences in the sequence of appearance of the various AIA-inducible bands, electrophoresis was performed on microsomes at various intervals up to 24 h after injection of 3 mg of AIA (Fig. 11). Mixed function oxidase activity and cytochrome P-450 and $b_{5}$ concentrations in the same microsomes are shown in Table VI. It was not possible to discern from the gels the small increase in P-450 identifiable spectrophotometrically 5 h after AIA administration. There was, however, an increase in all the bands at 15 h after AIA administration, when increases in cytochrome P-450 and amipyrine demethylase were also clearly apparent. The staining intensity of the bands increased at 24 h, concurrent with further increases in P-450 concentrations and in the rates of aminopyrine demethylase and 7-ethoxy-coumarin deethylase. There were no differences in the time of appearance of the three bands. In this gel, there appeared to be concurrent increases in the staining of a band in the $M_r$ region increased by AIA: (1) $M_r$ = 50,000 to 60,000 region increased by P-NF 50,000; (2) 51,000; (3) 53,000.

**Fig. 11.** SDS-polyacrylamide gel electrophoresis of microsomes from livers of embryos exposed to AIA for 1 to 24 h. AIA, 3 mg in 0.2 ml of water, was injected into 17- to 18-day-old chick embryos at 24, 15, 5, and 1 h before dissection using 14 eggs in each group. Controls were uninjected. Livers were removed and two groups with seven livers each were pooled for each time point. 9000 × g supernatants and microsomes were prepared and assayed for mixed function oxidase and cytochrome content as described under “Materials and Methods.” The data are shown in Table VI. A portion of the microsomes was prepared for electrophoresis as described under “Materials and Methods.” The electrophoretic pattern for one of the two duplicate groups at each time point is shown. Microsomal protein, 100 µg/well, was added to each sample well and electrophoresis was performed for 25.5 h at 10 mA. The molecular weight (MW) standards, shown in the right lane × 1000 (K) were the same as for Fig. 10. A, control; B, AIA, 1-h exposure; C, AIA, 5-h exposure; D, AIA, 15-h exposure; E, AIA, 24-h exposure. Lines 1–3 indicate electrophoretic bands in the $M_r$ = 50,000 to 60,000 region increased by AIA: (1) $M_r$ = 50,000; (2) 51,000; (3) 53,000.

**Fig. 10.** SDS-polyacrylamide gel electrophoresis of liver microsomes from allylisopropylacetamide-, phenobarbital-, and β-naphthoflavone-treated chick embryos. Chick embryos (18 days old) were injected with 0.1 ml of MeSO (control, Cont), AIA (3 mg in 0.1 ml of MeSO), phenobarbital (PB) (9 mg in 0.1 ml of MeSO), or β-naphthoflavone (β-NF) (6.7 mg in 0.1 ml of MeSO), using seven livers per group. Twenty-four hours later, the livers from each group were removed and pooled, and microsomes were prepared for electrophoresis. Electrophoresis was performed by the method of Ficcion et al. (28) using an 11% acrylamide gel, as described under “Materials and Methods.” Electrophoretic migration was from top to bottom. 65 µg of protein were added to each sample well and electrophoresis was performed for 20 h at 10 mA. Molecular weight standards, shown in the left lane, included bovine serum albumin, 67,000, catalase, 57,000, ovalbumin, 43,000, and aldolase, 40,000. Lines 1–5 indicate electrophoretic bands at (1) $M_r$ = 50,000 and (2) 51,000 (increased by phenobarbital and AIA), (3) 53,000 (increased by AIA), (4) 56,000, and (5) 57,000 (increased by β-naphthoflavone).

**DISCUSSION**

This paper presents kinetic evidence supporting the heterogeneity of function and regulation of mixed function oxidases in intact liver. Sequential inhibition and induction of hepatic mixed function oxidase activity are well known occurrences following xenobiotic exposure (10, 31). These data show that a single xenobiotic can also simultaneously increase and decrease the rates of metabolism of monoxygenases that are kinetically distinguishable, thereby leading to simultaneous increases and decreases of the reaction rates for different monoxygenases active toward the same substrate and to increases and decreases in the rates of metabolism of different substrates. Different monoxygenase isozymes may, therefore,
be susceptible to regulation independently of each other.

Following exposure to certain drugs, the liver may be able to metabolize some mixed function oxidase substrates at faster rates than normal and others at slower rates than normal. Further, for some substrates, the concentration of the substrate in the liver may determine whether induction or inhibition will be observed. Thus, 24 h after exposure to AIA, the chick embryo liver metabolized biphenyl, 7-ethoxycoumarin, and antipyrine at an increased rate as compared to the rates in livers of unexposed embryos when high concentrations of those substrates (above 0.5 mM for biphenyl or 7-ethoxycoumarin and above 1.5 mM for antipyrine) were presented to the P-450 system, and at a decreased rate at lower substrate concentrations. Thus, the substrate concentration is a potential determinant of the response of the P-450 system.

In contrast to AIA, neither phenobarbital nor β-NF had concurrent opposite effects on any of the mixed function oxidases examined. Therefore, not all inducers of mixed function oxidase activity in chick embryo liver inhibit certain of those reactions at the same time as they are inducing others. Conversely, AIA is not unique in this regard. 3,5-Diethoxy-1,4-dihydrocollidine (11) and trimethadione, for example, also selectively increase and decrease the rates of different mixed function oxidase reactions in chick embryo liver, although neither of those drugs produced a crossover effect or increased the density of a $M_i = 53,000$ band on electrophoresis, as did AIA.

The phenomenon also does not appear to be unique to the chick embryo. Thus, in the rat, 3-methylcholanthrene treatment has been found to increase the N-demethylation but to inhibit the 4'-hydroxylation of $N,N$-dimethyl-4-aminobenzene (32); pyrazole treatment increases the N-demethylation of dimethylnitrosamine while decreasing the N-demethylation of benzphetamine (33). Thus, simultaneous increases and decreases of different monooxygenases following xenobiotic exposure may be a more widespread phenomenon than is generally recognized.

These studies also illustrate complexities in the effects of AIA as an inhibitor and inducer of mixed function oxidase activity. The inhibition of mixed function oxidase activity by AIA preceded the induction, but persisted into the induction phase. As an inhibitor, AIA did not evidence any distinctive activity. The inhibition of mixed function oxidase activities by AIA which decreased P-450 at 1 h after AIA administration, before any compensatory induction would have occurred, the degree of inhibition of mixed function oxidase activity was more than twice as great as the decrease of P-450. At 4 h, all of the mixed function oxidases were depressed, and at 24 h, some were selectively depressed; yet at neither time was there any accompanying decrease in P-450. At lower doses of 0.1 and 0.3 mg of AIA/egg, at which there was no breakdown of P-450 observed, or any subsequent induction that might have obscured a decrease, mixed function oxidase activities were regularly depressed at 1 and 4 h after administration of AIA.

Competitive inhibition may account for some of the discrepancy between P-450 inhibition and mixed function oxidase inhibition at the higher doses of AIA. However, it is unlikely to play a major role at the lower doses of 0.1 and 0.3 mg/egg, which are likely to achieve concentrations at least an order of magnitude lower than those necessary to induce the P-450. Nevertheless, it is likely that AIA which decreased P-450 at 1 h after AIA administration, before any compensatory induction would have occurred, the degree of inhibition of mixed function oxidase activity was more than twice as great as the decrease of P-450. At 4 h, all of the mixed function oxidases were depressed, and at 24 h, some were selectively depressed; yet at neither time was there any accompanying decrease in P-450. At lower doses of 0.1 and 0.3 mg of AIA/egg, at which there was no breakdown of P-450 observed, or any subsequent induction that might have obscured a decrease, mixed function oxidase activities were regularly depressed at 1 and 4 h after administration of AIA.

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magnitude lower than the  \( K \) for competitive inhibition (37). While the concentrations of AIA achieved in the liver at these doses are also likely to be lower than the  \( K \) values for noncompetitive inhibition observed in vitro, preincubation of AIA with liver was found to increase the potency of AIA as a noncompetitive inhibitor.

Possible explanations of these findings are that AIA selectively breaks down only certain forms of P-450 in control livers, or that in addition to breaking down some P-450, it can also impair P-450 function without destroying its carbon monoxide binding characteristics or its electrophoretic mobility. The data tend to support the second mechanism more than the first. Thus, if AIA selectively broke down the heme of only certain forms of P-450 in control liver, then AIA might be expected to have greater substrate selectivity as an inhibitor of mixed function oxidase activity than is observed. Further, given that, at 0.3 mg of AIA/egg, there was up to 60% inhibition of mixed function oxidase activity, a small decrease in total P-450 would be expected even if only certain P-450s were destroyed, but none was observed. To fit the findings, the selected forms of P-450 destroyed at low doses of AIA would have to constitute a tiny fraction of the total P-450 be responsible for a major fraction of the mixed function oxidase activity of control liver. It seems more likely that, in addition to causing some P-450 breakdown, AIA can also impair the function of P-450s without physically destroying them. This could reflect an early phase in the established process of suicidal destruction of P-450 by AIA (9) or it could occur by an independent mechanism. The data suggest that there may be other significant ways for AIA to impair mixed function oxidase activity than by actually destroying P-450.

AIA exhibited greater selectivity as an inducer of mixed function oxidase activity than as an inhibitor. Its selectivity as an inducer, in fact, proved to be responsible for the cross-over effect. Thus, AIA had similar  \( K \) values for the inhibition of 7-ethoxycoumarin deethylation and aminopyrine demethylase, indicating a similar potential for inhibiting both reactions. However, AIA, but not phenobarbital or  \( \beta\)-NF, increased the activity of low affinity high velocity isozymes active toward 7-ethoxycoumarin, biphenyl, and antipyrine while not inducing any higher affinity isozymes. AIA did not induce any new lowest affinity isozymes for aminopyrine demethylase or benzo(a)pyrene hydroxylase. In agreement with these findings, the crossover effect was observed for 7-ethoxycoumarin deethylation, biphenyl hydroxylase, and antipyrine hydroxylase, after AIA but not after phenobarbital or  \( \beta\)-NF pretreatment and not for aminopyrine demethylase or benzo(a)pyrene hydroxylase after AIA. Thus, the differential effects of AIA as an inducer of mixed function oxidase isozymes were reflected in its differential production of the crossover phenomena for different substrates.

While AIA shared more features as an inducer with phenobarbital than with  \( \beta\)-NF, it also had features that distinguished it from either of the classic prototype inducers. Like phenobarbital, AIA increased the overall rate of aminopyrine demethylation, more than the rate of metabolism of other substrates. AIA also increased the concentration of electrophoretic bands with  \( M_1 = 50,000 \) and 51,000 as did phenobarbital. However, AIA alone, and neither phenobarbital nor  \( \beta\)-NF, increased the staining intensity of an electrophoretic band at  \( M = 53,000 \). AIA, and neither phenobarbital nor  \( \beta\)-NF, selectively increased the activity of the new low affinity isozymes active toward biphenyl, 7-ethoxycoumarin, and antipyrine. It is tempting to speculate but purely conjectural that the new band induced by AIA may correspond to the new low affinity kinetic components induced by AIA. Whether or not the two are related, AIA’s special kinetic and electrophoretic characteristics distinguish it from phenobarbital and  \( \beta\)-NF. They indicate that AIA cannot be considered a member of either the phenobarbital or polycyclic hydrocarbon class of inducers in the chick embryo.

There are increasing examples of chemicals whose inducing effects differ from those of the phenobarbital or polycyclic hydrocarbon classes and from each other. As more mixed function oxidase reactions are being examined, more inducers are identified that do not fit into a particular class. Among these deviants, which AIA joins, are pregnenolone-16α-carbonitrile (38), isosafrole (39), imizonid (40), mirex (41), kepone (41), and pyrazole (33). The lengthening list suggests that the heterogeneity of induction effects may be comparable to the heterogeneity of mixed function oxidases present in liver. The conception of inducers as belonging with only a few exceptions to two major classes may be as erroneous as the earlier view that there were only two forms of cytochrome P-450.

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An egg weighs about 50 g. If it is assumed that the AIA will distribute equally throughout the egg and not be metabolized, then for doses of 0.3 and 0.1 mg/egg (2.1 and 0.7 μmol), the concentration of AIA in the egg would be about 4.2 × 10^{-4} and 1.4 × 10^{-5} M.
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