Spectroscopic evidence of interaction between 2-allyl-2-Isopropylacetamide and cytochrome P-450 of rat liver microsomes.

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SUMMARY

2-allyl-2-isopropylacetamide (AIA) causes marked induction of heme synthesis in rats and other species, degrades cytochrome P-450 in the presence of NADPH and causes experimental porphyria. Using difference spectroscopy we sought evidence of an interaction between AIA and P-450 in microsomes prepared from rat liver. AIA alone caused small and variable changes in the spectral properties of liver microsomes but markedly inhibited the Type I spectral change due to hexobarbitone. Phenobarbitone exhibited behaviour qualitatively similar to AIA. It is concluded that AIA binds to cytochrome P-450 without much altering its spectral properties but in such a way as to prevent the change induced by the Type I substrate hexobarbitone.

INTRODUCTION

Large doses of 2-allyl-2-isopropylacetamide (AIA) cause experimental porphyria in rats and other species (1). Initially a biochemical curiosity, interest in experimental porphyria has increased considerably since the demonstration of Granick (2,3) that δ-aminolevulinic acid synthetase (ALA-S) is induced in avian and mammalian liver following exposure to porphyrinogenic drugs. The mechanism whereby this occurs in unknown but Granick (3) has suggested that regulation of hepatic microsomal cytochrome P-450 might be linked to induction of heme synthesis by various drugs and steroids.

The first suggestive evidence of a direct interaction between AIA and hepatic cytochrome P-450 was the demonstration by de Matteis (4) that the concentration of P-450 in rat liver microsomes decreased rapidly following a single dose of AIA. Further (5), it was shown that destruction of P-450 occurred in vitro when rat liver microsomes and AIA were incubated in the presence of NADPH. Levin et al (6) have suggested that this destruction can be attributed to the allyl group of AIA, perhaps via epoxide formation. Metabolism of AIA has been demonstrated in rats by Kaufman

et al (7); the metabolite demonstrated in plasma was not identified but evidence was advanced suggesting that the effects of AIA are due to the parent molecule and not a metabolite. Clearly, considerable interest attaches to the mechanism of interaction of AIA with cytochrome P-450 and spectroscopic evidence of such interaction is reported in this communication.

MATERIALS AND METHODS

Male Long-Evans rats (100-150g) were obtained from the McMaster University animal facility and housed on corn-cob bedding for at least one week before use. Animals pretreated with phenobarbital received drinking water containing 0.1% w/v of the sodium salt of this drug for 3 days; plain water was substituted 36 hours before sacrifice. Rats were decapitated and the livers perfused with 0.15 M sodium chloride, minced, and a 25% homogenate prepared in 0.1 M KCl buffered with 0.05 M Tris (pH 7.4). After centrifuging at 9,000 x g for 20 min, the centrifugate was recentrifuged at 100,000 x g for 1 hour to sediment a microsomal fraction which was then resuspended in 0.1 M phosphate buffer at pH 7.4. Microsomal protein was measured by a modified Lowry method (8), and cytochrome P-450 content was determined according to Omura and Sato (9).

AIA was a generous gift of Hofmann-la Roche, (Canada) Ltd; hexobarbitone (B.P.C.) (HB) was obtained from May and Baker (Canada) Ltd. and phenobarbitone sodium from BDH Pharmaceuticals (Canada). Aniline (A.C.S. reagent) was from Fisher Scientific Co. (Fairlawn, N.J.). All drugs, with the exception of aniline, were prepared as aqueous solutions to avoid any effects of organic solvents on spectral properties of P-450; HB was dissolved in an equimolar amount of sodium hydroxide; aniline was used without dilution or as a 25% (v/v) solution in ethanol.

To record difference spectra, 3.0 ml aliquots of the diluted microsomal suspension was pipetted into matched 1 cm cuvettes and placed in the primary sample compartment of a Perkin Elmer model 356 dual wavelength spectrophotometer operating in the split beam mode with $\lambda_1 = \lambda_2$. A

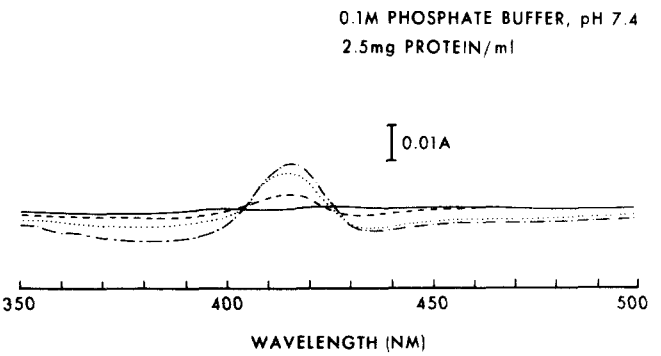

FIGURE 1

3.0 ml aliquots of microsomes (2.0 mg protein/ml) from livers of rats pretreated with phenobarbital were pipetted into matched quartz cuvettes (1.0 cm light path). Increasing concentrations of AIA were added to the sample cuvette with equal volumes of solvent (water) to the reference cuvette. Difference spectra recorded for 3 of the concentrations studied are shown —·—·— 2.5 mM AIA; ····· 0.5 mM AIA; ------ 0.1 mM AIA and ——— baseline of equal light absorbance.

25-turn potentiometer attached to the wavelength drive and suitably energized allowed repeated scans from 500 to 350 nm to be superimposed using a Hewlett Packard model 7035B X-Y recorder. The precise procedure followed to record a set of difference spectra was as follows: with the cuvettes containing microsomes only in position, the spectrophotometer was set at 500 nm and absorbance adjusted to the mid-point of the range in use (0 - 0.1 A.U. or 0 - 0.3 A.U.); the baseline of equal light absorbance was scanned at 120 nm/minute and the analogue signal corresponding to this baseline recorded on magnetic tape using a Hewlett Packard 3960 instrumentation recorder. The baseline was then re-scanned from 500-350 nm and recorded on the X-Y recorder with the signal on magnetic tape (corresponding to deviations of the baseline from a horizontal trace) subtracted using a simple analogue computer. Successive scans of a single sample used the same technique and a new baseline correction signal was recorded each time the microsomal suspension was changed.

Analyses of the changes in absorbance noted follow the same assumptions set out by Schenkman (10). Slopes and intercepts of double reciprocal plots were determined by linear regression analysis.

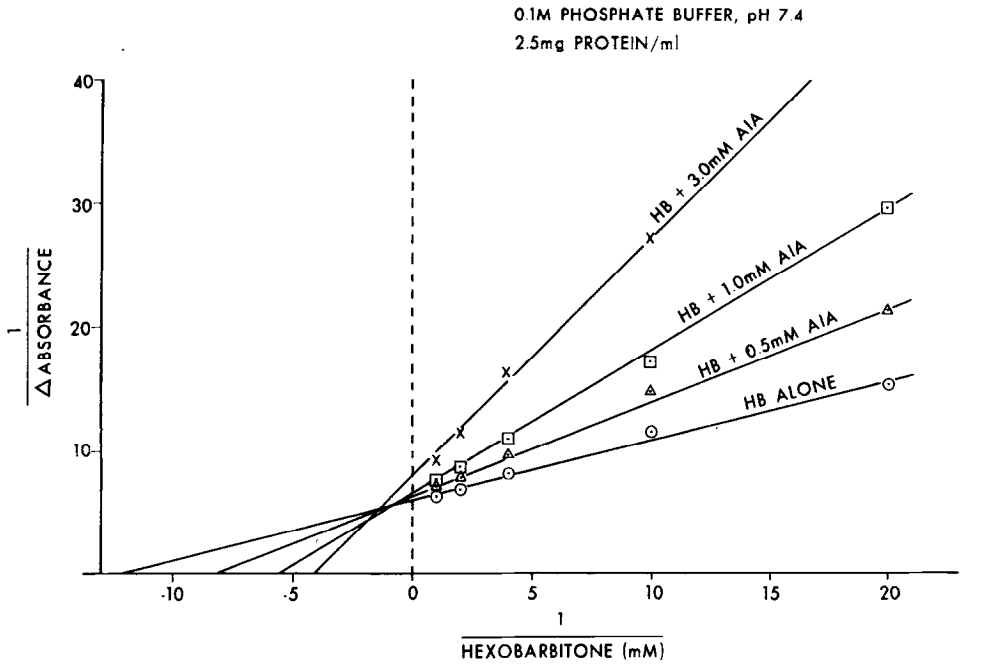

FIGURE 2

3.0 ml aliquots of microsomes (2.0 mg protein/ml) from livers of rats pretreated with phenobarbital were pipetted into matched quartz cuvettes (1.0 cm light path). If AIA was to be studied it was added to both cuvettes in the concentration shown. Difference spectra were then recorded as the concentration of hexobarbital was increased. "ΔA" indicates the magnitude of the difference spectrum measured from peak (390 nm) to trough (422 nm).

RESULTS

Difference spectra have been recorded following addition of AIA to the sample cuvette using five separate samples of microsomes prepared from untreated animals or after pretreatment with PB. Observations were made using a range of AIA concentrations from 5 μM to 5 mM. On one occasion a small Type I difference spectrum was recorded (ΔA_{max} 388-426 = .0046 A/nmole P-450/ml; the apparent affinity constant determined by a double reciprocal plot of this titration was K_s = 0.25 mM AIA). On two occasions the difference spectrum recorded resembled that referred to as Reverse Type I (11) with a rather ill-defined trough at 380-390 nm, intersection with the baseline of equal light absorbance at 404 nm and a peak in the range 414 to 416 nm (Figure 1).

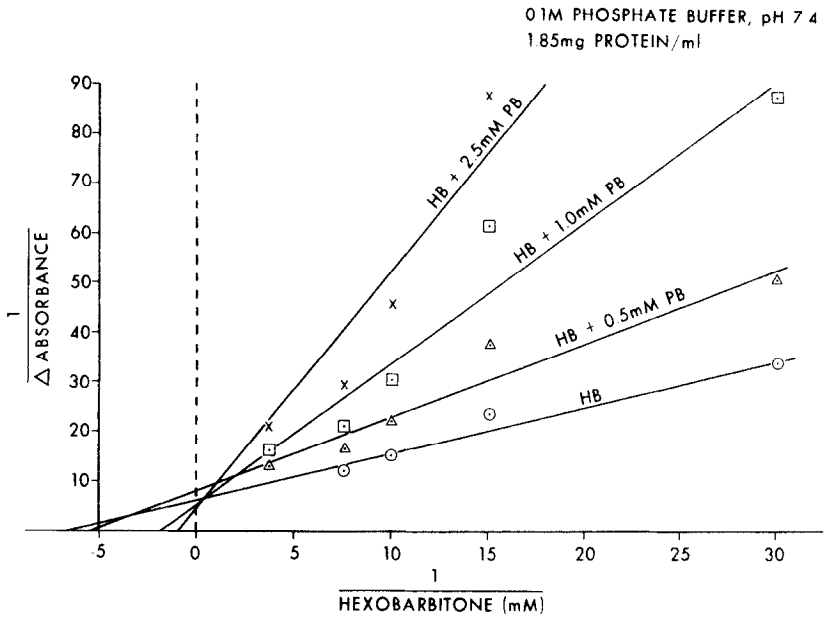

FIGURE 3

3.0 ml aliquots of microsomes prepared from livers of rats pretreated with PB and containing 1.85 mg protein per ml were pipetted into matched quartz cuvettes. Phenobarbital 0, 0.5, 1.0 and 2.5 mM was added to both cuvettes as shown. Other details of procedure were as for Figure 2.

Titration of the interaction shown in this figure gave ΔA_{max} = 416-385 nm = .0058A/nmole P-450/ml and an apparent affinity constant K_s = 0.14 mM AIA. On the remaining two occasions intermediate results were obtained; low concentrations of AIA (< 0.2 mM) induced a small Type I spectral change, but above 0.5 mM a peak at about 415 nm apparently similar to that in Figure 1 appeared changing the difference spectrum towards Schenkman's reverse Type I. These results are reported in detail to emphasize the small variable spectral changes produced by the interaction of AIA with microsomes.

Having established the nature of difference spectra obtained when AIA is added to hepatic microsomes, we proceeded to seek other evidence of direct interaction of AIA with cytochrome P-450. Titration of the Type I spectral change produced by hexobarbitone (HB) was performed adding HB to

the sample cuvette after having recorded the baseline of equal light absorbance and with 0, 0.5, 1.0 and 3.0 mM AIA present as "modifier" in both sample and reference cuvettes. HB produces large Type I spectral changes; we argued that if AIA were interacting with the Type I site without producing either very significant or consistent spectral changes such interaction might be shown in its effect on other ligands. Marked inhibition of the Type I spectral change produced by HB was observed in the presence of AIA. Double reciprocal plots of the Type I absorbance changes produced by HB in the concentration range 0.05 to 1.0 mM are shown in Figure 2 for HB alone and in the presence of varying concentrations of AIA. This experiment has been repeated and the results are consistent, unlike the difference spectra obtained on adding AIA alone to hepatic microsomes. Lines were fitted to the points obtained using linear regression analysis; regression coefficients exceeded 0.998 suggesting that the mathematic model used provides a valid analytic tool. Apparent affinity constants varied from 0.08 mM for HB alone to 0.12 mM, 0.18 mM and 0.24 mM in the presence of 0.5, 1.0 and 3.0 mM AIA respectively.

A similar study was made of the effect of PB as a modifier on the magnitude of the Type I change produced by HB. The result of this study is shown in Figure 3, again with the reciprocal of the Type I absorbance change plotted against the reciprocal of HB concentration at various concentrations of modifier. Although PB, unlike AIA, reduced the size of the Type I spectral change induced by HB, the double reciprocal plots appear curvilinear and the straight lines drawn by linear regression analysis provide a poor fit. Again, this experiment has been repeated without more linear data being obtained.

Finally, we have investigated the effect of AIA as modifier on the Type II spectral change induced in hepatic microsomes by aniline but were unable to demonstrate any significant change.

DISCUSSION

This study was undertaken because of the unusual effects of AIA on the

liver, some of which seem to be caused by direct interaction with cytochrome P-450 in hepatic endoplasmic reticulum. It was felt that if spectroscopic evidence of P-450-substrate interaction has any predictive value regarding the possible fate of an AIA-substrate complex, AIA induced difference spectra would be worthy of study. Clearly no consistent spectral interaction has been identified using AIA alone except that whatever change is produced is small. Equally clearly, while AIA itself produces little change in the spectral properties of P-450 of rat liver, it modifies the HB binding site in two ways. First, the affinity for HB alters as shown by the progressive increase in apparent K_s with increasing AIA concentration. Second, the presence of AIA either alters the number of sites available to bind HB and contribute to spectral change or it alters the magnitude of the spectral change produced by binding of HB to a given number of sites, or both. These alternative explanations for the AIA concentration-dependent change in Y axis intercept (Figure 2) are not resolved in this study. If the action of Type I substrates such as HB is to cause a shift of low spin cytochrome P-450 to the high spin form, AIA can be described as binding to cytochrome P-450 to inhibit this induced spin change both competitively and non-competitively. Qualitatively, PB appears to exert a similar effect and the non-linearity of double reciprocal plots we have observed may be attributable to the fact that PB, unlike AIA, contributes significantly to the total Type I signal recorded.

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