Effect of allylisopropylacetamide on glutathione metabolism in the rat liver

The possible role of glutathione in the induction of 5-aminolaevulinate synthase

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Administration of allylisopropylacetamide to rats caused a marked decline in the concentrations of reduced and oxidized glutathione in the liver. However, this decrease occurred in the presence of uninhibited activities of γ -glutamylcysteine synthase and glutathione reductase, and unaltered activities of glutathione transferases A, B and C. The administration of cysteine, the rate-limiting precursor of glutathione formation, to rats treated with allylisopropylacetamide potentiated the inductive effects of the agent on 5-aminolaevulinate synthase, and markedly decreased the extent of decrease in glutathione concentrations by the agent. Conversely, the administration of diethyl maleate, which depletes the hepatic glutathione concentrations, to allylisopropylacetamide-pretreated rats (1 h) diminished the extent of 5-aminolaevulinate synthase induction and the production of porphyrins by nearly 50%, when measured at 16 h. This treatment did not alter the extent of non-enzymic degradation of liver haem by allylisopropylacetamide. When diethyl maleate was administered to the animals possessing high 5-aminolaevulinate synthase activity (at 3, 7 and 15 h after allylisopropylacetamide), in 1 h the enzyme activity was markedly decreased. Diethyl maleate had no effect on induction of 5-aminolaevulinate synthase by 3,5-diethoxycarbonyl-1,4-dihydrocollidine, also a potent porphyrinogenic agent. Diethyl maleate alone neither inhibited 5-aminolaevulinate synthase activity nor decreased the cellular content of porphyrins and haem. The data suggest that the decreases observed in the glutathione concentrations after allylisopropylacetamide administration are not the result of decreased production of the tripeptide. Rather, they most likely reflect the increased utilization of glutathione. The findings further suggest that the inhibition by diethyl maleate of allylisopropylacetamide-stimulated 5-aminolaevulinate synthase involves the inhibition of induction processes.

A well known property of the barbiturate analogue allylisopropylacetamide is its ability to induce the synthesis of 5-aminolaevulinate synthase and to increase the cellular content of porphyrins in the liver (Schwartz & Ikeda, 1955). The mechanism by which allylisopropylacetamide elicits the induction of the enzyme is not known, although the allyl group of the molecule has been implicated in this action (DeMatteis, 1971; Levin *et al.*, 1973; Marks *et al.*, 1975; Ionnides & Parke, 1976). 5-Aminolaevulinate synthase, a mitochondrial enzyme with a rather short half-life of approx. 60 min, constitutes the rate-limiting enzyme in the pathway of haem biosynthesis (Granick, 1966; Marver *et al.*, 1966)

and its synthesis is believed to be regulated by a feedback repression mechanism. Another property of allylisopropylacetamide is its ability to cause the degradation of hepatic microsomal haem to non-physiological pyrrolic compounds.

Recent studies suggest the involvement of GSH in the induction of 5-aminolaevulinate synthase by allylisopropylacetamide. For example, the addition of diethyl maleate, which severely depletes the cellular GSH concentration (Boyland & Chausseaud, 1970) to cultured avian hepatocytes, caused a decrease in the porphyrinogenic activity of allylisopropylacetamide (Maines & Sinclair, 1977). Also in rats the administration of metal ions known to deplete cellular GSH concentration (Maines & Kappas, 1977) inhibited the induction of 5-aminolaevulinate synthase by allylisopropylacetamide in

Abbreviations used: GSH, reduced glutathione; GSSG, oxidized glutathione.

the liver (Maines *et al.*, 1976). In addition, there have been reports (Edwards *et al.*, 1978; Smith, 1976) on the detection of GSH-conjugated allylisopropylacetamide-derived components in the bile of starved phenobarbital-treated rats, with a concomitant depletion of GSH concentration in the liver of the animals.

The present study was undertaken to investigate the role of cellular GSH in the allylisopropylacetamide-mediated induction of 5-aminolaevulinatase synthase.

Experimental

Fed male Sprague-Dawley rats (130–150g) were used. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. unless otherwise indicated. Allylisopropylacetamide was a gift of Hoffman/La Roche Inc., Nutley, NJ, U.S.A. Diethyl maleate and 3,5-diethoxycarbonyl-1,4-dihydrocollidine were products of Eastman Kodak Co., Rochester, NY, U.S.A. Coproporphyrin III standard was purchased from Porphyrin Products, Salt Lake City, UT, U.S.A.

Groups of three to six rats were injected between 07:00h and 14:00h with allylisopropylacetamide (300mg/kg body wt., in iso-osmotic saline subcutaneously), 3,5-diethoxycarbonyl-1,4-dihydrocollidine (300mg/kg body wt. in corn oil subcutaneously), 0.2M-L-cysteine solution in iso-osmotic saline (5ml/kg body wt. orally) or diethyl maleate (0.6ml/kg body wt. intraperitoneally) according to the schedules indicated in the appropriate Figures and Tables. The control animals received iso-osmotic saline or corn oil. At designated times the animals were decapitated and, unless indicated, the livers were perfused *in situ* with 0.9% NaCl until fully blanched; thereafter, they were removed and homogenized in 0.05M-Tris/HCl buffer, pH 7.4, containing 0.25M-sucrose. The microsomal and the mitochondrial fractions were prepared by differential centrifugation as described elsewhere (Maines & Kappas, 1975). The activity of 5-aminolaevulinatase synthase was measured by a modification of the method of Marver *et al.* (1966) as described previously (Maines, 1980). The enzyme activity was measured using the mitochondrial fractions unless otherwise indicated. The total cellular content of porphyrins (uro-, copro- and proto-porphyrin) was determined fluorimetrically (Granick *et al.*, 1975) with aq. 1M-HClO₄ and methanol (1:1, v/v) as extraction mixture, and with coproporphyrin III as standard. The excitation wavelength was 400nm and the emission spectrum was scanned from 500 to 650nm. GSH was measured in cellular homogenate or the cytosol (105000g supernatant) fraction by using the fluorimetric method of Cohn & Lyle (1966). The

concentration of GSSG in the cytosol fraction was measured by the procedure described by Hissin & Hief (1976). The activity of γ -glutamylcysteine synthetase was estimated in the liver cytosol fraction as described by Sekura & Meister (1977) by measuring the formation of P_i (Orlowski & Meister, 1971), from its ATPase activity. The activity of glutathione reductase was measured also in the cytosol by the method described by Massey & Williams (1965), and the activities of glutathione S-transferases A, B and C were assessed with 1-chloro-2,4-dinitrobenzene (A and B) and 1,2-dichloro-4-nitrobenzene (mostly A and C) as substrates by the procedure described by Habig *et al.* (1974).

The microsomal contents of cytochrome P-450 and the total haem were measured as described by Omura & Sato (1964) and Paul *et al.* (1953) respectively. All spectral studies were carried out with an Amino-Chance DW-2 spectrophotometer. The microsomal drug-metabolizing activity was assessed by measuring ethylmorphine N-demethylation. The formaldehyde formed was determined by the method of Nash (1953) with the incubation system described previously (Maines & Kappas, 1975). The activity of 5-aminolaevulinatase dehydratase was measured by the method of Mauzerall & Granick (1958) with dithiothreitol (20mM) as the thiol-containing agent. The protein concentration was measured by the method of Lowry *et al.* (1951). The results are expressed as means $\pm$ S.D. for three to six determinations and one rat was used for each determination. The data were analysed by Student's *t* test and differences were considered significant if the *P* value was ≤ 0.05 .

Results

Effects of allylisopropylacetamide on the contents of GSH and GSSG and the activities of γ -glutamylcysteine synthetase, glutathione reductase and glutathione S-transferases

The effects of allylisopropylacetamide on cellular GSSG and GSH concentrations and the activities of the enzymes of GSH metabolism were investigated. As shown in Table 1, in the liver of untreated rats allylisopropylacetamide significantly depleted the cellular concentration of GSH. However, this effect was manifested after a latent period of at least 2h. Moreover, the decrease in GSH concentration was time-dependent and maximum decreases were observed within 4–8h of allylisopropylacetamide treatment. After 16h the GSH concentration returned to normal values. The addition of allylisopropylacetamide *in vitro* at various concentrations (1, 5, 10 and 15mM) to control cytosol preparations did not alter measured GSH values (results not shown). Table 1 also shows that the time course of depletion of GSH

Table 1. *Time course of effects of allylisopropylacetamide and diethyl maleate on GSH concentrations in the liver*
Groups of three rats (130–150 g) were injected with allylisopropylacetamide (300 mg/kg body wt. subcutaneously), diethyl maleate (0.6 ml/kg body wt. intraperitoneally) or saline. All injections were made between 07:00 h and 08:00 h. At the indicated times the animals were killed, the whole cellular homogenate of unperfused livers was prepared and the GSH concentrations were measured (Cohn & Lyle, 1966). The decreases observed in the GSH concentrations in the livers of saline-treated animals are due to the diurnal variations in the GSH concentrations. The results are presented as means $\pm$ s.d.

Time (h)	Treatment	[Glutathione] (mM)		
		Allylisopropylacetamide	Diethyl maleate	Saline
0		4.09 $\pm$ 0.35	3.87 $\pm$ 0.30	3.80 $\pm$ 0.41
1		4.45 $\pm$ 0.47	0.95 $\pm$ 0.56*	3.95 $\pm$ 0.29
2		4.11 $\pm$ 0.50	1.85 $\pm$ 0.48*	4.02 $\pm$ 0.36
4		2.84 $\pm$ 0.25*	1.72 $\pm$ 0.35*	3.68 $\pm$ 0.23
8		1.79 $\pm$ 0.42*	3.24 $\pm$ 0.29	3.23 $\pm$ 0.20
16		2.91 $\pm$ 0.50	4.01 $\pm$ 0.63*	2.70 $\pm$ 0.40

* $P \leq 0.05$, when data are compared with the saline-treated animals.

Table 2. *Effect of allylisopropylacetamide treatment on the cellular glutathione concentrations and the activities of γ -glutamylcysteine synthetase, glutathione reductase and glutathione S-transferases in the rat liver*

Rats were treated with allylisopropylacetamide (300 mg/kg body wt. subcutaneously) or saline. After 6 h the animals were killed, the liver cytosol fraction (105 000 g supernatant) was prepared as described in the Experimental section and used for the measurement of the indicated parameters. Measurements were made as described in the Experimental section. The results are expressed per mg of protein. The results are means $\pm$ s.d. for four rats. Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene.

Treatment	GSH (nmol/mg)	GSSG (nmol/mg)	γ -Glutamylcysteine synthase (nmol/mg per min)	GSSG reductase (units/mg per min)*	CDNB-GSH S-transferase (units/mg per min)*	DCNB-GSH S-transferase (units/mg per min)*
Saline	41.9 $\pm$ 7.7	1.69 $\pm$ 0.10	22.2 $\pm$ 3.1	129 $\pm$ 11	1340 $\pm$ 132	67 $\pm$ 9
Allylisopropylacetamide	26.0 $\pm$ 4.1†	1.21 $\pm$ 0.21†	20.3 $\pm$ 2.5	115 $\pm$ 15	1180 $\pm$ 99	56 $\pm$ 7

* nmol of substrate used.

† $P < 0.05$.

by allylisopropylacetamide was quite distinct from that of diethyl maleate, which depletes GSH concentrations by direct binding to the tripeptide (Boyland & Chausseaud, 1970). As shown, 1 h after the injection of diethyl maleate the cellular concentrations of GSH were decreased by nearly 75% and within 8 h the values approximated those of the controls. Although allylisopropylacetamide when injected subcutaneously is readily absorbed (Doedens, 1971), its depleting effects on the GSH concentrations were not expressed promptly. The delayed GSH-depleting action of allylisopropylacetamide plus the differential pattern of depletion of GSH by allylisopropylacetamide and diethyl maleate are consistent with the possibility that intact allylisopropylacetamide did not deplete the cellular GSH concentrations, and suggest the likelihood that the observed depletion was attributable to a derivative(s) of the compound.

The mechanism by which allylisopropylacetamide treatment depleted the cellular GSH concentrations was investigated by measuring the activities of various enzymes of glutathione meta-

bolism. As shown in Table 2, 6 h after allylisopropylacetamide treatment, the cellular concentrations of GSH and GSSG were significantly decreased. However, the activity of γ -glutamylcysteine synthetase, the initial and rate-limiting enzyme in the synthesis of GSH (Orlowski & Meister, 1971), remained unaltered. Also the activity of the enzyme glutathione reductase was unchanged. Accordingly it can be asserted that the effects of allylisopropylacetamide on glutathione concentrations most likely were not due to the inhibition of the synthesis of the tripeptide, and/or its formation from GSSG. Moreover, the finding that the activities of glutathione transferases A, B and C were not affected in response to allylisopropylacetamide treatment (Table 2) suggests that alterations in transferase utilization of GSH apparently were not contributing factors to the decreases observed in the cellular concentrations of the tripeptide in the liver in response to allylisopropylacetamide treatment. The results shown in Table 3 further support the assertion that allylisopropylacetamide did not interfere with the synthesis of

Table 3. *Effect of cysteine treatment on the allylisopropylacetamide-mediated induction of 5-aminolaevulinate synthase and the depletion of glutathione in the liver*

Groups of four rats (130–150 g) were used. One group was given L-cysteine (0.5 ml/100 g of a 0.2 M solution orally) 1 h before, and 1 and 3 h after the injection of allylisopropylacetamide (300 mg/kg body wt. subcutaneously). The other groups received cysteine, allylisopropylacetamide or saline. After 6 h the animals were killed and subcellular fractions of the liver were prepared as described in the Experimental section. The activity of 5-aminolaevulinate synthase was measured in the mitochondrial fraction and the glutathione measurements were made by utilizing the cytosol fraction (105 000 g supernatant). The assay procedures are described in the Experimental section. Results are means $\pm$ S.D.

Treatment	5-Aminolaevulinate synthase (pmol/mg per h)	GSH (mmol/mg)	GSSG (mmol/mg)
Saline	210 $\pm$ 23	49.2 $\pm$ 4.9	1.70 $\pm$ 0.14
Allylisopropylacetamide	1612 $\pm$ 102	26.8 $\pm$ 3.7	1.19 $\pm$ 0.13
Allylisopropylacetamide + cysteine	2373 $\pm$ 164*	43.4 $\pm$ 6.9*	1.73 $\pm$ 0.23*
Cysteine	260 $\pm$ 31	51.4 $\pm$ 6.0	2.01 $\pm$ 0.30

* $P \leq 0.05$, when allylisopropylacetamide + cysteine results are compared with allylisopropylacetamide results.

Table 4. *Effects of diethyl maleate treatment on cellular parameters subject to the porphyrinogenic and haem-degrading properties of allylisopropylacetamide in the liver*

Groups of six rats (130–150 g) were used. One group was injected with diethyl maleate (0.6 ml/kg body wt. intraperitoneally) 1 h after the administration of allylisopropylacetamide (300 mg/kg body wt. subcutaneously). The other groups received the same doses of allylisopropylacetamide, diethyl maleate or saline. At 16 h after allylisopropylacetamide injection the animals were killed, and the subcellular fractions were prepared as described in the Experimental section. 5-Aminolaevulinate synthase activity was measured using the mitochondrial fraction (Maines, 1980). Microsomal fractions were used for the measurements of cytochrome P-450 (Omura & Sato, 1964) and haem (Paul *et al.*, 1953) contents as well as the activities of ethylmorphine N-demethylase (Nash, 1953) and haem oxygenase (Maines & Kappas, 1975). The cellular content of porphyrins was measured in the whole homogenate of the liver (Granick *et al.*, 1975). The results are expressed as means $\pm$ S.D.. All measurements are presented per mg of protein.

Treatment	5-Aminolaevulinate synthase (pmol/mg per h)	Total porphyrins (pmol/mg)	Cytochrome P-450 (nmol/mg)	Microsomal haem (nmol/mg)	Ethylmorphine N-demethylase (nmol of formaldehyde/ mg per h)	Haem oxygenase (nmol of bilirubin/ mg per h)
Saline	178 $\pm$ 21	2.41 $\pm$ 0.34	0.70 $\pm$ 0.06	1.27 $\pm$ 0.18	297 $\pm$ 31	1.52 $\pm$ 0.21
Allylisopropylacetamide	1754 $\pm$ 194	5.14 $\pm$ 0.60	0.37 $\pm$ 0.08	0.81 $\pm$ 0.20	55 $\pm$ 14	1.34 $\pm$ 0.21
Allylisopropylacetamide + diethyl maleate	1138 $\pm$ 137*	2.80 $\pm$ 0.10*	0.35 $\pm$ 0.09	0.82 $\pm$ 0.19	42 $\pm$ 15	2.09 $\pm$ 0.40
Diethyl maleate	210 $\pm$ 29	2.52 $\pm$ 0.49	0.73 $\pm$ 0.05	1.31 $\pm$ 0.20	206 $\pm$ 45	3.18 $\pm$ 0.39

* $P \leq 0.05$, when allylisopropylacetamide + diethyl maleate results are compared with allylisopropylacetamide results.

glutathione. As shown, when allylisopropylacetamide-treated rats were given cysteine, the rate-limiting precursor for the biosynthesis of glutathione, the cellular ability to utilize the precursor for the production of glutathione remained unaltered. Moreover, cysteine treatment further augmented induction of 5-aminolaevulinate synthase activity of allylisopropylacetamide.

The effects of diethyl maleate on allylisopropylacetamide induction of 5-aminolaevulinate synthase, the degradation of cellular haem and drug metabolism

As shown in Table 4, when diethyl maleate was

injected 1 h after allylisopropylacetamide, a significant decline in the magnitude of the induction of liver 5-aminolaevulinate synthase occurred in the subsequent 16 h compared with that observed with allylisopropylacetamide alone. Also, the administration of diethyl maleate to allylisopropylacetamide-treated rats inhibited porphyrin accumulation in the liver tissue. However, diethyl maleate treatment did not alter the deleterious effects of allylisopropylacetamide on the microsomal haem and cytochrome P-450. As shown, in the presence of diethyl maleate, allylisopropylacetamide treatment decreased the cellular contents of cytochrome P-450 and the microsomal haem by approx. 40%. The magnitude of reductions in these cellular consti-

tuents was the same when allylisopropylacetamide alone was injected. Moreover, the administration of diethyl maleate to allylisopropylacetamide-treated rats did not significantly change the extent of severe inhibitory effect of allylisopropylacetamide on the haemoprotein-dependent biotransformation of ethylmorphine. Conversely, allylisopropylacetamide did not significantly modify the diethyl maleate-mediated increase in haem oxygenase activity. As shown in Table 4, in response to diethyl maleate treatment in the presence or absence of allylisopropylacetamide, a 2-fold increase in the activity of microsomal haem oxygenase was observed. The stimulation of haem oxygenase activity by diethyl maleate has been reported also by Burk & Correia (1979).

It is generally accepted that allylisopropylacetamide induction of liver 5-aminolaevulinate synthase is directly related to the ability of the compound to degrade cellular haem; haem is believed to regulate the synthesis of the enzyme by a feedback repression mechanism (Granick, 1966; Marver *et al.*, 1966). However, the results presented in Table 4 showing the differential effects of diethyl maleate on the porphyrinogenic and the haem-degrading properties of allylisopropylacetamide suggest that diethyl maleate did not inhibit the porphyrinogenic property of allylisopropylacetamide by preventing the destruction of cellular haem by allylisopropylacetamide. Rather, it appears to involve the inhibition of the induction of 5-aminolaevulinate synthase. The results shown in Fig. 1 are in agreement with this possibility. As Fig. 1 shows, when diethyl maleate was administered after 5-aminolaevulinate synthase had been induced by allylisopropylacetamide, within 1 h a marked decline in the enzyme activity occurred. This rapid decrease in the synthase activity was observed when diethyl maleate was injected 3, 7 or 15 h after allylisopropylacetamide administration, and in all instances the magnitude of the decrease was in the range 40–50%. The possibility that these observations were due to diethyl maleate interference with allylisopropylacetamide-mediated induction of 5-aminolaevulinate synthase by altering cellular constituents necessary for increased enzyme synthesis was investigated. For these studies, 3,5-diethoxycarbonyl-1,4-dihydrocollidine, another well known inducer of hepatic 5-aminolaevulinate synthase, was used. As shown in Fig. 1 the administration of diethyl maleate to rats treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine for 3, 7 or 15 h did not cause any decrease in the synthase activity. These findings are in contrast with the observations made in the allylisopropylacetamide-treated rats.

The study described in Table 4 was repeated, with 3,5-diethoxycarbonyl-1,4-dihydrocollidine in place of allylisopropylacetamide and the mitochondrial 5-aminolaevulinate synthase activity was measured

Fig. 1. Time course of the effect of diethyl maleate on the 5-aminolaevulinate synthase activity induced by allylisopropylacetamide and diethoxycarbonyl-1,4-dihydrocollidine

Groups of six fed male Sprague-Dawley rats (130–150 g) were treated with allylisopropylacetamide or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (300 mg/kg body wt. subcutaneously). At 3, 7 or 15 h, as indicated by the arrows, diethyl maleate was injected into three rats in each group (0.6 ml/kg body wt. intraperitoneally). After 1 h the animals were killed and liver 5-aminolaevulinate synthase activity was determined using the mitochondrial fraction. The activity of the synthase is expressed per mg of protein. Results presented are the means of experiments. ▲—▲, Allylisopropylacetamide; ▲—---▲, allylisopropylacetamide + diethyl maleate; ●—●, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; ●—---●, 3,5-diethoxycarbonyl-1,4-dihydrocollidine + diethyl maleate.

16 h after 3,5-diethoxycarbonyl-1,4-dihydrocollidine treatment. Again in contrast with the allylisopropylacetamide-treated rats the extent of enzyme induction was not decreased by diethyl maleate and the following values were obtained for rats treated with 3,5-diethoxycarbonyl-1,4-dihydrocollidine, 3,5-diethoxycarbonyl-1,4-dihydrocollidine + diethyl maleate and the controls respectively: 621 ± 105 , 718 ± 119 and 191 ± 35 pmol of 5-aminolaevulinate/mg of protein per h. The time course of the effect

of 3,5-diethoxycarbonyl-1,4-dihydrocollidine on the liver GSH concentrations was also investigated, and at no time was there a detectable decrease in the GSH concentration. These findings indicated that diethyl maleate was not a general inhibitor of 5-aminolaevulinate synthase production. Rather, the data suggest that the inhibition of allylisopropylacetamide-mediated 5-aminolaevulinate synthase induction by diethyl maleate was specific for the inducer.

Studies in vitro

The effect of addition of diethyl maleate *in vitro* to 5-aminolaevulinate synthase using an enzyme preparation obtained from allylisopropylacetamide or 3,5-diethoxycarbonyl-1,4-dihydrocollidine-treated animals was studied to investigate whether the allylisopropylacetamide-induced enzyme exhibits an increased degree of sensitivity to diethyl maleate in comparison with the enzyme obtained from control- or 3,5-diethoxycarbonyl-1,4-dihydrocollidine-treated animals.

The results presented in Table 5 demonstrate that the direct addition of diethyl maleate (25, 50 or 100 μM final concentration) to the assay medium for 5-aminolaevulinate synthase containing whole cellular homogenate obtained from allylisopropylacetamide- or 3,5-diethoxycarbonyl-1,4-dihydrocollidine-treated (both for 16h) animals did not differentially affect the activity of the enzyme. In all instances the activity was not substantially inhibited at 25 and 50 μM -diethyl maleate concentrations, but at a concentration of 100 μM -diethyl maleate a nearly

90% inhibition in the enzyme activity was observed. The inhibitory effect of high concentrations of diethyl maleate may involve various non-specific components such as the alteration of the permeability of the mitochondrial membrane to the components of the assay system constituents, or the enhancement of lipid peroxidation activity in the assay system leading to the destruction of various cellular constituents including 5-aminolaevulinate synthase.

Addition of an allylisopropylacetamide/GSH or allylisopropylacetamide/diethyl maleate mixture (1:1, molar ratio), at a final concentration of 100 μM , to the synthase assay system produced effects similar to those seen with allylisopropylacetamide, diethyl maleate or GSH alone. As shown, allylisopropylacetamide and GSH were essentially ineffective in altering the enzyme activity, whereas diethyl maleate, when added in a mixture with allylisopropylacetamide, produced an inhibitory effect similar to that observed with diethyl maleate alone. The effect of 3,5-diethoxycarbonyl-1,4-dihydrocollidine *in vitro* on cellular GSH concentrations was also studied. This agent, when added at 12.5, 25 and 50 mM concentrations, had no effect on the concentration of GSH in the liver.

The possibility that diethyl maleate itself might cause a differentially increased rate of degradation of 5-aminolaevulinate synthase enzyme protein by allylisopropylacetamide was investigated. Diethyl maleate at a concentration of 50 μM was added to the 5-aminolaevulinate synthase assay system in combination with allylisopropylacetamide (15 mM) or

Table 5. *Effect of diethyl maleate in vitro on the induced liver 5-aminolaevulinate synthase activity*

The tissue source for the assays was the pooled livers of three rats treated with allylisopropylacetamide, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (300mg/kg body wt. subcutaneously, 16h) or saline. The activity of 5-aminolaevulinate synthase was measured in whole liver homogenate (Maines, 1980). The results are averages of three determinations. The variations between the determinations were less than 10%. Abbreviations used: AIA, allylisopropylacetamide; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DEM, diethyl maleate.

Treatment <i>in vivo</i>	Addition <i>in vitro</i>	5-Aminolaevulinate synthase (pmol/mg of protein per h)	Change (%)
—	—	105	100
—	DEM (25 μM)	115	+9
—	DEM (50 μM)	86	-18
—	DEM (100 μM)	15	-86
AIA	—	905	100
AIA	DEM (25 μM)	900	-1
AIA	DEM (50 μM)	720	-20
AIA	DEM (100 μM)	75	-92
DDC	—	340	100
DDC	DEM (25 μM)	320	-6
DDC	DEM (50 μM)	287	-15
DDC	DEM (100 μM)	42	-88
—	AIA/GSH (100 μM)	100	-5
—	AIA/DEM (100 μM)	20	-81
—	AIA (100 μM)	115	+10
—	GSH (100 μM)	95	-10

3,5-diethoxycarbonyl-1,4-dihydrocollidine (50 mM). A decrease of 15–20% in the enzyme activity with both agents was observed. This decrease was of the same magnitude as that produced by diethyl maleate alone (Table 5). The possibility of the diethyl maleate-mediated enhancement of the degradation by allylisopropylacetamide of 5-aminolaevulinate was also explored. In these experiments allylisopropylacetamide (15 mM) or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (50 mM) in the presence or absence of diethyl maleate (50 μ M) were added to the 5-aminolaevulinate dehydratase assay system along with 5-aminolaevulinate (150 mM) used as the substrate in the system. In the presence of diethyl maleate the amount of porphobilinogen formed by the enzyme, through the condensation of two 5-aminolaevulinate molecules, was not significantly different from that produced in the absence of diethyl maleate (results not shown). Moreover, the addition of allylisopropylacetamide or 3,5-diethoxycarbonyl-1,4-dihydrocollidine alone to the assay system did not alter the activity of the dehydratase. These experiments indicate that diethyl maleate treatment did not differentially promote the destruction of 5-aminolaevulinate synthase or 5-aminolaevulinate induced by allylisopropylacetamide.

Discussion

The present study reports on the action of allylisopropylacetamide on cellular glutathione concentration. Allylisopropylacetamide was found to markedly decrease the concentrations of GSH in the untreated rat liver in a time-dependent manner (Table 1). The decrease did not appear to involve the inhibition of the formation of the tripeptide by allylisopropylacetamide (Tables 2 and 3). Moreover, the data in the present paper demonstrate that the two-known properties of allylisopropylacetamide, the degradation of cellular haem and the induction of 5-aminolaevulinate synthase in the liver, can be independently altered. As shown here (Table 4) only the latter, i.e. the porphyrinogenic dimension of allylisopropylacetamide, was altered in response to treatment with the thiol-reactive reagent, diethyl maleate, a potent depletor of cellular GSH (Boyland & Chausseaud, 1970).

The mechanism by which diethyl maleate decreased the measured activity of 5-aminolaevulinate synthase appears to involve the inhibition of the induction process. The prompt decline of induced liver 5-aminolaevulinate synthase activity after the administration of diethyl maleate (Fig. 1), the short half-life of the enzyme (Granick, 1966) and the marked interference by diethyl maleate of the induction of 5-aminolaevulinate synthase activity by allylisopropylacetamide (Table 4) suggest the inhibition of the formation of the enzyme as a con-

sequence of diethyl maleate treatment. Alternatively, diethyl maleate may affect the activity of the synthase by mechanisms involving the acceleration of the degradation of allylisopropylacetamide-induced enzyme, and/or the enhancement of the rate of degradation of allylisopropylacetamide by the microsomal mixed-function oxidase system. However, the latter possibilities are not supported by the findings presented in Tables 4 and 5. The finding that diethyl maleate alone neither significantly affected the oxidative metabolism of the prototype drug ethylmorphine nor decreased the microsomal contents of cytochrome *P*-450 and haem (Table 4) would imply that the oxidative biotransformation of allylisopropylacetamide was not accelerated as a result of diethyl maleate treatment. In addition, as noted in Table 4, the presence of diethyl maleate did not modify the deleterious effects of allylisopropylacetamide on parameters associated with microsomal drug-metabolizing activities, and the degradation of haem. Moreover, as shown in Table 5, low concentrations of diethyl maleate *in vitro* did not inhibit the activity of allylisopropylacetamide-induced 5-aminolaevulinate synthase, and high concentrations of the reagent did not differentially inhibit the activity of allylisopropylacetamide-induced enzyme over that of the control or the 3,5-diethoxycarbonyl-1,4-dihydrocollidine-induced enzyme. Therefore, the possibility of the increased rate of degradation of the enzyme protein by diethyl maleate appears rather unlikely.

Previous studies (Maines & Sinclair, 1977) have shown that the addition of diethyl maleate to avian cultured liver cells leads to a decreased porphyrinogenic effect of allylisopropylacetamide. Accordingly, it may be asserted that the inhibition by diethyl maleate of the induction of the synthase (Table 4 and Fig. 1) did not involve extrahepatic factors as intermediates in the repression of the enzyme activity. However, it remains unclear whether diethyl maleate inhibition of the allylisopropylacetamide-induced 5-aminolaevulinate synthase involves the direct action of diethyl maleate at the regulatory site(s) for the formation of enzyme protein, or that it reflects the GSH-depleting property of this thiol-reactive reagent and the role of GSH in the induction of the enzyme. The presently observed (Fig. 1) pattern and the magnitude of decrease in allylisopropylacetamide-induced 5-aminolaevulinate synthase activity in response to diethyl maleate very closely resembles those previously observed with known direct inhibitors of the synthesis of the enzyme such as Co^{2+} , Co-haem and Fe-haem (Granick, 1966; Marver *et al.*, 1966; Maines & Sinclair, 1977; Nakamura *et al.*, 1975; Maines *et al.*, 1976; Igarashi *et al.*, 1978; Tephly *et al.*, 1978; Sinclair *et al.*, 1979). For example, it has been observed that such agents in cultured

avian liver cells, as well as in intact rat liver, promptly (within 1h) decrease the activity of allylisopropylacetamide-induced 5-aminolaevulinate synthase by about 50%. These effects closely approximate those observed in the present study (Fig. 1). On the other hand the evidence discussed below is supportive of an indirect mode of diethyl maleate action in the inhibition of the induction of synthase by allylisopropylacetamide, and suggests a role for GSH in this process.

The finding that allylisopropylacetamide *in vivo* (Table 1) but not *in vitro* decreased the cellular concentrations of GSH in the presence of an uninhibited GSH biosynthetic activity (Tables 2 and 3) alludes to the likelihood of increased utilization of the tripeptide in the course of induction of 5-aminolaevulinate synthase by allylisopropylacetamide. It is also possible that intact allylisopropylacetamide was not solely responsible for the induction of the synthase and that the enzyme induction in part may reflect the activity of derivative(s) of the compound. Accordingly it can be postulated that conjugation with GSH was required for expression of enzyme-inducing property of the putative derivative(s). It follows that the depletion of the tripeptide by diethyl maleate would prevent the formation of such conjugates and the increased availability of GSH would facilitate the formation of such derivatives. The recent reports by Edwards *et al.* (1978) and Smith (1976) on the formation of several structurally ill-defined GSH-conjugated allylisopropylacetamide metabolites in the rat liver is in agreement with such a possibility. It should be noted that the concept of allylisopropylacetamide biotransformation to derivatives possessing a 5-amino-laevulinate synthase-inducing property was originally proposed by Doedens (1971). Alternatively it is possible that on the depletion of the GSH pool by allylisopropylacetamide, diethyl maleate could exhibit its activity to inhibit the formation of 5-aminolaevulinate synthase and/or its activity. This possibility is compatible with the present finding that diethyl maleate was not effective in altering 3,5-diethoxycarbonyl-1,4-dihydrocollidine-induced 5-aminolaevulinate synthase activity. As shown in the present report 3,5-diethoxycarbonyl-1,4-dihydrocollidine did not decrease cellular GSH concentrations.

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