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A Model for the Regulation of δ-Aminolaevulinate Synthetase Induction in Rat Liver

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A reciprocal relationship exists between the cytochrome *P*-450 content and δ-aminolaevulinate synthetase activity in adult rats. In young rats the basal δ-aminolaevulinate synthetase activity is higher and the cytochrome *P*-450 content is lower compared with the adult rat liver. Administration of allylisopropylacetamide neither induces the enzyme nor causes degradation of cytochrome *P*-450 in the young rat liver, unlike adult rat liver. Allylisopropylacetamide fails to induce δ-aminolaevulinate synthetase in adrenalectomized–ovariectomized animals or intact animals pretreated with successive doses of the drug, in the absence of cortisol. The cortisol-mediated induction of the enzyme is sensitive to actinomycin D. Allylisopropylacetamide administration degrades microsomal haem but not nuclear haem. Haem does not counteract the decrease in cytochrome *P*-450 content caused by allylisopropylacetamide administration, but there is evidence for the formation of drug-resistant protein-bound haem in liver microsomal material under these conditions. Phenobarbital induces δ-aminolaevulinate synthetase under conditions when there is no breakdown of cytochrome *P*-450. On the basis of these results and those already published, a model is proposed for the regulation of δ-aminolaevulinate synthetase induction in rat liver.

δ-Aminolaevulinate synthetase, the first and rate-limiting enzyme of the haem biosynthetic pathway, is induced in the animal liver by a wide variety of foreign chemicals and physiological steroid metabolites. Haem, the end-product of the pathway, represses the induction of the enzyme (Granick, 1966; Waxman *et al.*, 1966; Marver *et al.*, 1966). Granick (1966) proposed that haem functions as the co-repressor, controlling δ-aminolaevulinate synthetase induction by combining with the apo-repressor and forming the functional repressor. He suggested that drugs and steroids compete with haem, preventing the formation of the functional repressor, and thereby cause enzyme de-repression. However, a wide variety of chemicals with divergent structures are able to induce δ-aminolaevulinate synthetase and it is difficult to visualize how all these chemicals could compete with or prevent haem binding to the apo-repressor. Burnham (1969) therefore suggested that all these foreign chemicals facilitate the accumulation of a common metabolite 'X' that competes with haem. Because some of the physiological steroid metabolites are potent inducers of δ-aminolaevulinate synthetase in the chick embryo liver culture system (Granick & Kappas, 1967; Kappas & Granick, 1968), Burnham (1969) suggested that 'X' could as well be a steroid metabolite. Subsequent studies by Sassa & Granick (1970) with the chick embryo liver culture system have indicated that the porphyrinogenic agent, allylisopropylacetamide, a potent inducer of δ-aminolaevulinate synthetase, as well as haem, the 'co-repressor', probably act at the translational level. Studies by Strand *et al.* (1972) with the chick embryo culture system have supported this contention.

Recent investigations have shown that a primary effect of allylisopropylacetamide is to cause a decrease in liver cytochrome *P* 450 content (De Matteis, 1970; Meyer & Marver, 1971). Cytochrome *P*-450 plays a key role in microsomal electron transport, mediating drug metabolism through the mixed-function oxidase system. The decrease in cytochrome *P*-450 contents caused by allylisopropylacetamide administration involves the degradation of the haem moiety (De Matteis, 1970; Meyer & Marver, 1971). It has been suggested that this primary action of allylisopropylacetamide in causing the destruction of cytochrome *P*-450 haem might be connected with the induction of δ-aminolaevulinate synthetase, haem being the 'co-repressor' for the enzyme (De Matteis, 1970; Meyer & Marver, 1971; Landaw *et al.*, 1970). Satyanarayana Rao *et al.* (1972) examined the inter-relationship between cytochrome *P*-450 degradation and δ-aminolaevulinate synthetase induction and suggested that haem degradation is one of the conditions necessary for the enzyme to be induced by allylisopropylacetamide. In the present report we propose, on the basis of additional experiments as well as published results, that the apo-protein of a microsomal

haemoprotein, which could be a species of apo-cytochrome *P*-450 or a closely associated protein undergoing a similar metabolic fate, facilitates induction of δ-aminolaevulinate synthetase at the translational level. Haem binding to this apo-protein abolishes this positive control. The translation of δ-aminolaevulinate synthetase mRNA provides a trigger for transcription and formation of more mRNA. A hormone metabolite is required for the transcription process.

Experimental

Animals

Female rats (100–110g) as well as male rats (120–130g) of the local Institute strain were used; 5-day-old rats were used in some experiments. The concentrations of the compounds injected are given in the respective tables and figures as well as in the accompanying paper (Satyanarayana Rao & Padmanaban, 1973).

Hormone involvement in δ-aminolaevulinate synthetase induction was studied in adrenalectomized–ovariectomized rats. The animals were maintained on stock diet and saline for 5 days before use. For the first 24h after the operation the animals were also given glucose.

Methods

Determination of cytochrome P-450 content and δ-aminolaevulinate synthetase activity. Cytochrome *P*-450 was estimated in liver microsomal suspensions by the method of Omura & Sato (1964).

δ-Aminolaevulinate synthetase activity was assayed in liver homogenate, mitochondria and postmicrosomal supernatant as described by Hayashi *et al.* (1969). For this purpose, the livers were homogenized with 0.25M-sucrose in TKM buffer (0.05M-Tris–HCl, pH7.5, 0.025M-KCl and 0.005M-$MgCl_2$) containing 0.1mM-pyridoxal phosphate (4ml/g of liver) in a Potter–Elvehjem homogenizer. After removing the 800*g* sediment, the supernatant was spun at 10000*g* for 15min and the sediment, after washing, was used as the mitochondrial fraction. The postmitochondrial supernatant was spun at 105000*g* for 60 min and the supernatant was used as the soluble fraction. The homogenate incubation mixture in a total volume of 2ml contained: 1.0ml of homogenate, 200μmol of glycine, 20μmol of EDTA and 150μmol of Tris–HCl buffer, pH7.4. The enzyme was assayed in the mitochondrial and soluble fractions by using a succinyl-CoA-generating system in a total volume of 2ml that contained: 0.5ml of mitochondrial suspension or supernatant, 200μmol of glycine, 20μmol of succinate, 20μmol of ATP, 0.3μmol of pyridoxal phosphate, 0.3μmol of CoA, 10μmol of $MgCl_2$, 0.8μmol of EDTA, 120μmol of Tris–HCl buffer, pH7.5, and succinyl-CoA synthetase, catalysing the formation of 10μmol of succinyl-CoA/h. The assay mixtures were incubated for 1h at 37°C and the reaction was stopped with 1ml of 12.5% (w/v) trichloroacetic acid. Aminolaevulinate was determined in the protein-free supernatant by using acetylacetone and column fractionation as described by Hayashi *et al.* (1969) with slight modifications.

Degradation of microsomal and nuclear haem. Female rats were injected with amino[4-^{14}C]laevulinate (3μCi/animal) intraperitoneally. At 6h after the tracer administration, the animals were injected with allylisopropylacetamide (300mg/kg) subcutaneously. The animals were then killed at different times after allylisopropylacetamide administration and the livers were processed for the isolation of haem from microsomal material and nuclei. Nuclei were isolated from liver homogenates by the method of Blobel & Potter (1966). Homogenate (7ml in 0.25M-sucrose–TKM buffer) was mixed with 14ml of 2.3M-sucrose in TKM buffer and layered over 8ml of 2.3M-sucrose in TKM buffer. Centrifugation was carried out by using a Beckman SW 25 rotor for 120min at 24000rev./min. The nuclear pellet was resuspended in 32ml of 2.3M-sucrose in TKM buffer and the run was repeated. The pellet was finally suspended in 0.25M-sucrose in TKM buffer and centrifuged at 800*g* for 10min. The pellet was washed three times by the same procedure.

For the isolation of microsomal material (subsequently termed 'microsomes'), a portion of the 0.25M-sucrose homogenate was centrifuged at 25000*g* for 10min and the supernatant was diluted with 1.15% (w/v) KCl and then centrifuged at 105000*g* for 90min. The pellet was rinsed with KCl and finally suspended in potassium phosphate buffer (0.05M, pH7.5).

Haem was isolated from the microsomal and nuclear suspensions after washing the pellets with organic solvents to remove lipids. Haem was split by using ethyl acetate–acetic acid (3:1, v/v) and the ethyl acetate layer was freed of porphyrins and other possible degradation products of haem. Portions of the ethyl acetate layer were transferred to vials, evaporated to dryness under vacuum and then counted for radioactivity. This method of haem isolation and the radioactive purity of the final product have been standardized and described by Levin *et al.* (1972). Direct determination of the protohaem content in the microsomal suspensions revealed that the recovery of the haem in the final preparation is consistently around 70±2%.

[^{59}Fe]Ferric citrate incorporation into microsomal haem. [^{59}Fe]Ferric citrate (5μCi/animal) was injected intraperitoneally into female rats and allylisopropylacetamide was injected subcutaneously. After 4h the animals were killed and the livers were processed for the isolation of microsomal haem as described above except that 1.15% (w/v) KCl was used for homogenization.

Protein determination was carried out in homogenate and microsomal suspensions by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The suspensions were diluted suitably so as to contain a final concentration of 1% (w/v) deoxycholate.

Radioactivity measurements. ^{14}C radioactivity measurements were made in a liquid-scintillation counter (Beckman LS-100) by using 0.5% of 2,5-diphenyloxazole in toluene as the scintillation fluid. Quench corrections were applied by the channels-ratio method. ^{59}Fe radioactivity measurements were made in a well-type scintillation counter attached to a decade scaler (Nuclear–Chicago).

Results

There is a reciprocal relationship between the content of cytochrome *P*-450 and the extent of induction of δ-aminolaevulinate synthetase in rat liver (De Matteis, 1970; Meyer & Marver, 1971; Satyanarayana Rao *et al.*, 1972). The results in Table 1 indicate another situation where this relationship holds. In young rats the basal hepatic δ-aminolaevulinate synthetase activity is higher than that in adult rats, and the reverse is seen with cytochrome *P*-450 content. Allylisopropylacetamide fails to induce δ-aminolaevulinate synthetase in young rats and neither is there a fall in cytochrome *P*-450 content. The refractory nature of young rats to δ-aminolaevulinate synthetase induction has also been reported by Song *et al.* (1971). The marginal effects of allylisopropylacetamide could be due to the low ability of the young rat liver to metabolize the drug. In young rats the drug-metabolizing enzyme activities are low (Song *et al.*, 1971) and allylisopropylacetamide has to be metabolized before it can lead to degradation of cytochrome *P*-450 (De Matteis, 1971).

The results in Table 2 indicate that in adrenalectomized–ovariectomized rats allylisopropylacetamide leads to cytochrome *P*-450 breakdown, but δ-aminolaevulinate synthetase is not induced. However, administration of cortisol with the drug results in induction of the enzyme without in any way altering the breakdown of cytochrome *P*-450. The effect of cortisol on enzyme induction is blocked by actinomycin D. The results in Table 3 indicate that cortisol is again required for the induction of δ-aminolaevulinate synthetase by allylisopropylacetamide when the animal has received repeated administrations of the drug. It has previously been shown (De Matteis, 1971) that the initial decrease in cytochrome *P*-450 content after allylisopropylacetamide administration is a temporary phenomenon and this is followed by a regeneration phase when more than normal amounts of cytochrome *P*-450 are reached 24h after the drug administration. The results in Table 3 indicate that each administration of allylisopropylacetamide at 24h intervals causes a fresh decrease in cytochrome *P*-450 content, but δ-aminolaevulinate synthetase is poorly induced after the fifth administration of the drug unless cortisol is also injected simultaneously. The cortisol-mediated effect is blocked by actinomycin D.

The actinomycin D sensitivity of the cortisol-mediated effect indicates that possibly the hormone metabolite acts at the transcriptional level. Haematin has been reported to act at the translational level in the chick embryo liver culture system (Sassa & Granick, 1970; Strand *et al.*, 1972). To further resolve this question, the fate of labelled nuclear and microsomal haem was investigated after allylisopropylacetamide administration. The results in Fig. 1 show that, whereas allylisopropylacetamide causes loss of radioactivity from microsomal haem, the nuclear haem radioactivity remains constant. Nuclear membranes have been reported to contain haemoproteins. The contribution of the endoplasmic reticulum to the nuclear membranes in this regard has been recognized, although it is thought that

Table 1. *Effect of allylisopropylacetamide on δ-aminolaevulinate synthetase induction and cytochrome P-450 content in young rats*

Rats (5 days old; 7–8g) as well as fed adult female rats (100–110g) were injected with allylisopropylacetamide (400mg/kg body wt.) subcutaneously. The animals were killed after 4h and the livers were processed for the assay of δ-aminolaevulinate synthetase activity and cytochrome *P*-450 content. The results represent the average of two experiments in which five livers of young rats and two livers of adult rats were pooled in each experiment.

Animal used	Treatment	δ-Aminolaevulinate synthetase activity (nmol of aminolaevulinate/g of liver)	Cytochrome *P*-450 content (nmol/mg of microsomal protein)
Young rat	Saline control	32.2	0.38
Young rat	Allylisopropylacetamide	35.8	0.37
Adult rat	Saline control	15.3	0.77
Adult rat	Allylisopropylacetamide	90.0	0.59

Table 2. *Effect of cortisol on allylisopropylacetamide-mediated induction of δ-aminolaevulinate synthetase and destruction of cytochrome P-450 in adrenalectomized–ovariectomized rats*

Adrenalectomized–ovariectomized adult rats (110–120g) were maintained on saline and stock diet for 5 days before use. Allylisopropylacetamide was injected subcutaneously at a dose of 400mg/kg body wt. Cortisol and actinomycin D were injected intraperitoneally at the same time as allylisopropylacetamide at concentrations of 10mg/animal and 0.15mg/animal respectively. The animals were killed 5h after drug administration and the livers processed for δ-aminolaevulinate synthetase assay and cytochrome *P*-450 content. The results represent the means ±S.D. from three experiments and two livers were pooled in each experiment.

Animal used	Treatment	Aminolaevulinate synthetase activity (nmol of aminolaevulinate/g of liver)	Cytochrome *P*-450 content (nmol/mg of microsomal protein)
Normal	Saline control	16.2 ± 1.3	0.81 ± 0.04
Normal	Allylisopropylacetamide	84.6 ± 3.7	0.62 ± 0.01
Normal	Allylisopropylacetamide + cortisol	87.3 ± 2.9	0.60 ± 0.01
Normal	Cortisol	17.1 ± 2.3	0.84 ± 0.05
Normal	Allylisopropylacetamide + cortisol + actinomycin D	20.4 ± 1.3	0.63 ± 0.02
Adrenalectomized–ovariectomized	Saline control	12.2 ± 2.4	0.72 ± 0.05
Adrenalectomized–ovariectomized	Allylisopropylacetamide	17.2 ± 4.6	0.59 ± 0.06
Adrenalectomized–ovariectomized	Allylisopropylacetamide + cortisol	75.3 ± 2.7	0.61 ± 0.03
Adrenalectomized–ovariectomized	Cortisol	18.1 ± 3.4	0.70 ± 0.06
Adrenalectomized–ovariectomized	Allylisopropylacetamide + cortisol + actinomycin D	22.1 ± 2.1	0.64 ± 0.02

Table 3. *Effects of cortisol on allylisopropylacetamide-mediated induction of δ-aminolaevulinate synthetase and destruction of cytochrome P-450 in continuously drug-treated rats*

Fed female rats (100–110g) were given subcutaneous injections of allylisopropylacetamide (400mg/kg body wt.) every 24h. Cortisol (10mg/animal) and actinomycin D (0.15mg/animal) were injected intraperitoneally into rats along with the first or fifth injection of allylisopropylacetamide. The animals were killed 5h after the last drug injection and the livers were processed for δ-aminolaevulinate synthetase assay and cytochrome *P*-450 contents. The results represent the means±S.D. obtained from three experiments in which two livers were pooled in each experiment.

Treatment	δ-Aminolaevulinate synthetase activity (nmol of aminolaevulinate/g of liver)	Cytochrome *P*-450 content (nmol/mg of microsomal protein)
Normal	15.3±1.2	0.76±0.03
Allylisopropylacetamide (one injection)	89.6±3.4	0.61±0.02
Allylisopropylacetamide (two injections)	140.6±2.9	0.59±0.03
Allylisopropylacetamide (one injection)+cortisol	72.4±2.6	0.62±0.01
Allylisopropylacetamide (five injections)	25.2±2.4	0.58±0.04
Allylisopropylacetamide (five injections)+cortisol	58.9±1.2	0.56±0.03
Allylisopropylacetamide (five injections)+cortisol+actinomycin D	22.3±122	0.61±0.03
Cortisol	18.2±3.6	0.78±0.02

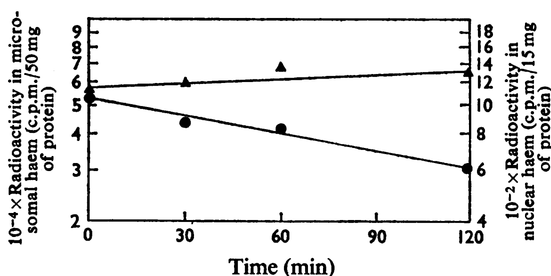

Fig. 1. *Effect of allylisopropylacetamide injection on the loss of radioactivity from microsomal and nuclear haem*

The experimental details are given in the text. The radioactivity results in the figure are the actual counts recorded in a typical experiment and correspond to the haem isolated from 15mg of nuclear protein and 50mg of microsomal protein. ●, Microsomal haem radioactivity; ▲, nuclear haem radioactivity.

cytochrome *P*-450 is not present in purified nuclear membrane preparations (Kasper, 1971; Berezney & Crane, 1971). In the present study purified whole nuclear preparations were used. Although contribution of haem radioactivity by the microsomal membrane fragments to the nuclear haem radioactivity is not ruled out, the fact that the bulk of nuclear haem radioactivity remains constant when the microsomal haem radioactivity decreases significantly owing to allylisopropylacetamide administration indicates that the contamination is not sufficiently significant to vitiate the results. We could not detect cytochrome *P*-450 in the whole nuclear preparations.

The effect of haematin on cortisol-mediated induction of δ-aminolaevulinate synthetase by allylisopropylacetamide was studied in adrenalectomized–ovariectomized rats, to understand the level of interaction of these two regulators. The results in Fig. 2 indicate that haematin blocks the enzyme induction when given with allylisopropylacetamide and cortisol in adrenalectomized–ovariectomized rats. When haematin was given 4h after enzyme induction it was still effective and caused a significant decrease in the δ-aminolaevulinate synthetase activities of the homogenate and mitochondria. The soluble enzyme activity increased slightly initially but subsequently decreased. Similar results were obtained when the effect of haematin on enzyme induction by allylisopropylacetamide was studied in intact animals (Fig. 2). These results are quantitatively different from those reported by Hayashi *et al.* (1972). They reported that haematin, when given after the enzyme has been

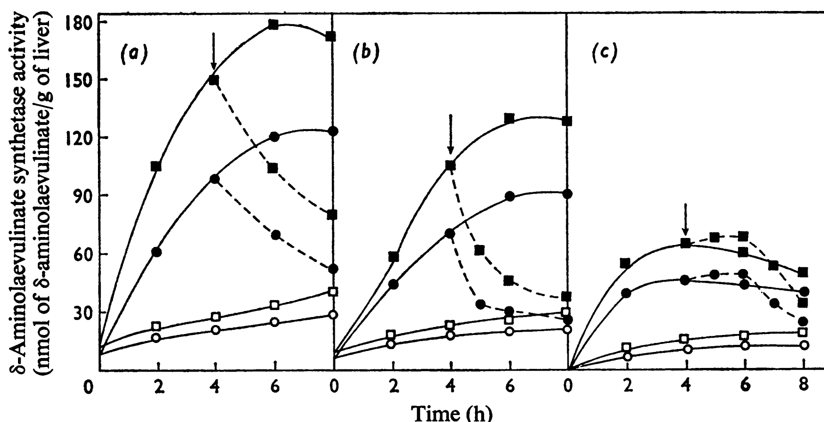

Fig. 2. *Effect of simultaneous and delayed administration of haem on the induction of δ-aminolaevulinate synthetase by allylisopropylacetamide*

Female rats were starved for 24h and allylisopropylacetamide was given at a concentration of 300mg/kg body wt. (*a*) Induction pattern in the homogenate; (*b*) induction pattern in mitochondria; (*c*) induction pattern in cytosol. ■, Normal animals treated with allylisopropylacetamide; □, normal animals treated with allylisopropylacetamide +haem (at zero time); ●, adrenalectomized–ovariectomized animals treated with allylisopropylacetamide+ cortisol (at zero time); ○, adrenalectomized–ovariectomized animals treated with allylisopropylacetamide+ cortisol+haemin (at zero time). The arrow indicates the time at which haemin was injected after the induction of the enzyme. The dashed lines represent the activity of δ-aminolaevulinate synthetase when haemin was administered 4h after enzyme induction.

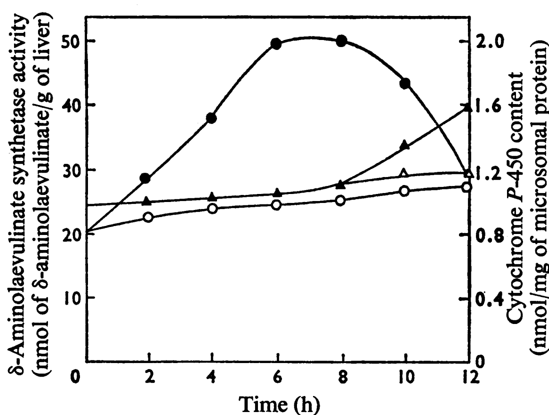

Fig. 3. *Effect of phenobarbital on δ-aminolaevulinate synthetase activity and cytochrome P-450 content in starved male rats*

Male rats (120–130g) were starved for 24h and phenobarbital was administered at a concentration of 80mg/kg body wt. ●, δ-Aminolaevulinate synthetase activity in phenobarbital-treated rats; ○, δ-aminolaevulinate synthetase activity in phenobarbital +actinomycin D-treated rats; ▲, cytochrome *P*-450 content in phenobarbital-treated rats; △, cytochrome *P*-450 content in phenobarbital+actinomycin D-treated rats.

induced, failed to suppress homogenate enzyme activity significantly. They also reported a striking decrease in mitochondrial enzyme activity that was compensated for by a corresponding increase in the cytosol enzyme activity. In the present study, the homogenate δ-aminolaevulinate synthetase activity was found to decrease significantly. The mitochondrial enzyme activity decreased significantly, but the increase in cytosolic enzyme activity was marginal and transient. The quantitative differences observed could be due to the concentration of the drug and haematin employed in the different studies. Strand *et al.* (1972) and Sassa & Granick (1970) found a significant decrease in homogenate enzyme activity with the chick embryo liver culture system even when haematin was administered after the enzyme had been induced.

The obligatory requirement for cytochrome *P*-450 breakdown to induce δ-aminolaevulinate synthetase has been examined with respect to another inducer, phenobarbital. The results in Fig. 3 indicate that phenobarbital induces the enzyme to about 2.5-fold in starved male rats. The enzyme activity reaches a maximum in about 6–8h. There is no evidence for any decrease in cytochrome *P*-450 content. A detectable increase in cytochrome *P*-450 content is seen at 10h after phenobarbital administration. The increases in δ-aminolaevulinate synthetase activity and cytochrome *P*-450 content owing to

phenobarbital administration are sensitive to actinomycin D. Similar results have been reported by Baron & Tephly (1970), except that they observed very high amounts of enzyme induction by phenobarbital. However, in most studies phenobarbital was found to be a weak or moderate inducer of δ-aminolaevulinate synthetase in female as well as starved male rats (Marver *et al.*, 1968; Marver, 1969; Meyer & Marver, 1971; Satyanarayana Rao *et al.*, 1972).

Discussion

Evidence for the involvement of cytochrome P-450 degradation in δ-aminolaevulinate synthetase induction

The ability of allylisopropylacetamide to cause a decrease in cytochrome *P*-450 content involving the degradation of the haem moiety, the 'co-repressor' of δ-aminolaevulinate synthetase, has already been mentioned. In addition, a reciprocal relationship exists between the changes in the content of cytochrome *P*-450 and δ-aminolaevulinate synthetase activity after allylisopropylacetamide administration (De Matteis, 1970; Meyer & Marver, 1971; Satyanarayana Rao *et al.*, 1972). We have shown (Satyanarayana Rao *et al.*, 1972) that when the degradation of cytochrome *P*-450 owing to allylisopropylacetamide is prevented by the simultaneous administration of SKF 525A (Smith, Kline and French Co., Philadelphia, Pa., U.S.A.), there is very little induction of the enzyme. Although the degradation of cytochrome *P*-450 could lead to δ-aminolaevulinate synthetase induction, we have ruled out the reverse situation, namely the possibility that the induction of the enzyme could cause overproduction of haem, which in turn might catalyse its own breakdown by the induction of haem oxygenase (Satynarayana Rao *et al.*, 1972). In addition, the results obtained with young rats in the present investigation clearly substantiate the involvement of cytochrome *P*-450 degradation in δ-aminolaevulinate synthetase induction.

Evidence for the participation of a hormone metabolite in δ-aminolaevulinate synthetase induction

Granick & Kappas (1967) and Kappas & Granick (1968) have shown that certain physiological steroid metabolites are potent inducers of δ-aminolaevulinate synthetase in the chick embryo liver culture system. Matsuoka *et al.* (1968) have shown that in the intact rat, cortisol has a permissive effect on the induction of the enzyme. In adrenalectomized animals they have shown that the enzyme is not induced by allylisopropylacetamide unless the animal is pretreated with cortisol. The present experiments with adrenalectomized–ovariectomized rats, as well as with animals receiving successive doses of the inducer, indicate that the enzyme is not induced by allylisopropylacetamide in the absence of cortisol administration. However, cortisol has no effect on the drug-mediated breakdown of cytochrome *P*-450. These results indicate that cytochrome *P*-450 breakdown, as well as the availability of a hormone metabolite, are necessary requirements for enzyme induction. These results substantiate our suggestion (Satyanarayana Rao *et al.*, 1972) that the decreased ability of allylisopropylacetamide to induce δ-aminolaevulinate synthetase after repeated administrations of the drug may be caused by the unavailability of the active hormone metabolite owing to its increased metabolism.

Site of action of haem, allylisopropylacetamide and the hormone metabolite

Although Granick (1966) originally proposed that haem and the drug act at the transcriptional level, Sassa & Granick (1970) favoured the translational site of action in the chick embryo liver culture system. Strand *et al.* (1972) obtained results supporting this contention by using the same experimental set-up. The present results indicate that in rat liver allylisopropylacetamide-mediated degradation of haem is restricted to the microsomes and is not detectable in nuclei (Fig. 1). Marver (1969) has shown that exogenously administered haematin is localized mainly in microsomal fractions and is not detectable in purified nuclear preparations. We have not been able to detect any radioactivity in purified nuclear preparations when the animals are injected with [2-^{14}C]-allylisopropylacetamide. Significant amounts are localized only in the cytosol and microsomal fractions. Exogenous administration of haematin has no effect on the distribution pattern of radioactivity (Satyanarayana Rao & Padmanaban, 1973). Although these results do not eliminate the possibility that a minute quantity of exogenously administered haematin or allylisopropylacetamide is localized in the nuclei, the bulk of the evidence indicates that the primary events leading to the repression of the enzyme by the exogenous administration of haematin, as well as the de-repression of the enzyme by the degradation of endogenous haem, are not likely to be taking place in the nuclei.

The fact that the cortisol-mediated induction of δ-aminolaevulinate synthetase by allylisopropylacetamide is sensitive to actinomycin D indicates that the hormone metabolite may act at the transcriptional level. The results proving the efficiency of haematin to block cortisol-mediated induction of the enzyme when given at zero time or 4h after the hormone administration (Fig. 2) also substantiate the contention that the hormone metabolite and haematin act at transcriptional and translational sites respectively. Even though there is a marginal increase in the cytosolic enzyme activity when

haematin is administered 4h after enzyme induction, the fact that this is a transient phenomenon indicates that it is only a question of time before the effect of haematin is felt at this step. Irrespective of the mechanisms proposed to explain the differential effect of haematin *in vivo* on pre-induced mitochondrial and cytosolic δ-aminolaevulinate synthetase activities (Hayashi *et al.*, 1972; Gayathri *et al.*, 1973), the results are not against a translational site of action for haematin and a transcriptional site for the hormone metabolite.

Finally, the implicated site of action of allylisopropylacetamide has to be reconciled with the finding that δ-aminolaevulinate synthetase induction owing to the drug in the intact animal is sensitive to actinomycin D (Marver *et al.*, 1966). We therefore propose that a limited amount of mRNA exists for the enzyme in normal liver. Soon after allylisopropylacetamide administration the translation of this mRNA provides a trigger for transcription and formation of more mRNA. These observations are, however, not compatible with the results obtained in the chick embryo liver culture system where the RNA for the enzyme appears to accumulate in presence of allylisopropylacetamide and cycloheximide (Sassa & Granick, 1970; Tyrrell & Marks, 1972). More direct experiments on the synthesis of mRNA for the enzyme only may resolve this problem.

Translational regulation of δ-aminolaevulinate synthetase messenger by positive control

The absence of cytochrome *P*-450 degradation in phenobarbital-mediated induction of δ-aminolaevulinate synthetase is not compatible with the microsomal haem being the 'co-repressor' for the enzyme at the translational level. Previously we have shown that in fed female rats phenobarbital is a weak inducer of the enzyme compared with allylisopropylacetamide and the increase in enzyme activity is detectable only after two injections of the drug at 12h intervals. Under these conditions phenobarbital decreased the rate of enzyme degradation *in vivo* (Satyanarayana Rao *et al.*, 1972). It is clear, however, that this explanation is not feasible in the case of starved male rats where a single injection of phenobarbital induces δ-aminolaevulinate synthetase to a moderate extent within 6–8h, the induction process being sensitive to actinomycin D (Fig. 3). We propose that a drug such as phenobarbital primarily increases the rate of apo-cytochrome *P*-450 synthesis, which in turn promotes the translation of δ-aminolaevulinate synthetase messenger, leading to more enzyme formation and haem synthesis. This would result in subsequent increases in cytochrome *P*-450 content. Thus, an alternative possible mode of haem action would be to annul the positive control exerted by the apo-protein on the translation of the enzyme.

The mode of action of phenobarbital suggested here requires rethinking of the idea that drugs primarily induce δ-aminolaevulinate synthetase, which causes increased haem synthesis, and the haem in turn causes increased cytochrome *P*-450 synthesis (Granick, 1966; Marver, 1969; Baron & Tephly, 1969*a*,*b*, 1970). We have discussed (Satyanarayana Rao & Padmanaban, 1973) recent studies which indicate that the availability of haem is not rate-limiting for cytochrome *P*-450 synthesis, at least under certain conditions. In these cases the increase in cytochrome *P*-450 content owing to phenobarbital administration is thus necessarily due to a direct effect of the drug on the rate of apo-protein synthesis, since Greim *et al.* (1970) have shown that phenobarbital does not affect the rate of cytochrome *P*-450 degradation. Availability of haem could become rate-limiting if the rate of apo-protein synthesis is considerably enhanced, which may occur in the starved male rat. We propose that the extra haem is made available by the promotion of δ-aminolaevulinate synthetase synthesis by the apo-protein at the translational level. It is believed that more than one species or form of cytochrome *P*-450 could exist in liver microsomes (Mannering *et al.*, 1969; Atkin *et al.*, 1972). As such the apo-cytochrome *P*-450 involved in δ-aminolaevulinate synthetase induction could be one of the several species or it could even be the apo-protein of a minor haemoprotein component that undergoes a metabolic fate similar to that of cytochrome *P*-450.

De Matteis & Gibbs (1972) have proposed an alternative explanation for the inductive effects of 3,5-diethoxycarbonyl-1,4-dihydrocollidine and phenylbutazone, which resemble allylisopropylacetamide and phenobarbital respectively in their effects on δ-aminolaevulinate synthetase and cytochrome *P*-450. 3,5-Diethoxycarbonyl-1,4-dihydrocollidine is an inhibitor of haem synthesis and also decreases cytochrome *P*-450 content initially. Thus δ-aminolaevulinate synthetase could be induced as a result of the lack of haem to 'repress' it. In the case of phenylbutazone De Matteis & Gibbs (1972) proposed that it could either fix haem as cytochrome *P*-450, thus rendering it unavailable for repression, or the effects of the drug could be due to its general effects on liver protein metabolism. For allylisopropylacetamide, there is no inhibition of haem synthesis and the effect is primarily one of cytochrome *P*-450 haem degradation, suggesting that this haem is normally available for repression. To propose that, for phenobarbital, haem might be fixed as cytochrome *P*-450 would appear inconsistent with the assumption made to explain the effects of allylisopropylacetamide. However, this situation is not inconceivable, because of the possible existence of several species or forms of closely related haemoproteins in liver microsomes (Mannering *et al.*, 1969; Atkin *et al.*, 1972). Thus, whereas one form is susceptible to degradation by allylisopropylacetamide, drugs

Table 4. [^{59}Fe]*Ferric citrate incorporation into microsomal haem under conditions of repression of δ-aminolaevulinate synthetase by endogenous haem*

Adult female rats (100–110g) were starved for 48h, which included the duration of the experiment. Allylisopropylacetamide was injected subcutaneously at a concentration of 300mg/kg. [^{59}Fe]Ferric citrate (5μCi/animal) was injected intraperitoneally immediately after allylisopropylacetamide. The animals were killed after 4h and the radioactivity incorporated into microsomal haem and the cytochrome *P*-450 content were measured as described in the Experimental section. δ-Aminolaevulinate synthetase activity was assayed in the liver homogenate. The results represent the averages of two experiments where two livers were pooled in each experiment.

Treatment	δ-Aminolaevulinate synthetase activity (nmol of aminolaevulinate/g of liver)	Cytochrome *P*-450 content (nmol/mg of microsomal protein) (A)	Microsomal haem radioactivity (c.p.m./mg of microsomal protein) (B)	Ratio of B/A
Saline control	16.8	0.75	296	395
Allylisopropylacetamide	171.5	0.55	228	414
Aminolaevulinic acid	17.5	0.70	326	466
Allylisopropylacetamide +aminolaevulinic acid	102.3	0.49	301	614

such as phenobarbital and phenylbutazone might bind haem in the form of a species of cytochrome *P*-450, or closely related haemoproteins with slow turnover rates, consequently rendering haem unavailable for repression. Ours is an alternative model, which in addition to the ones already mentioned can also explain other relevant observations as follows.

(1) The inductive effect of allyisopropylacetamide can be explained on the basis that it causes haem degradation, making the apo-protein available to promote translation of δ-aminolaevulinate synthetase messenger.

(2) When haematin is given with allylisopropylacetamide, it may bind to the apo-protein (regulator) and thus prevent δ-aminolaevulinate synthetase induction. However, it is known that haematin does not counteract the decrease in cytochrome *P*-450 contents owing to allylisopropylacetamide administration (Sweeney *et al.*, 1972; Satyanarayana Rao & Padmanaban, 1973). It is still possible that haem could combine with the apo-protein, but the newly formed haemoprotein may not show the spectral properties of cytochrome *P*-450. It is also possible that the regulator of δ-aminolaevulinate synthetase is not cytochrome *P*-450, but a closely associated haemoprotein undergoing a similar metabolic fate. We have investigated whether there is evidence for the formation of a microsomal haemoprotein under conditions when the induction of δ-aminolaevulinate synthetase is blocked by haem. Since exogenously administered haematin can bind non-specifically to different proteins, we have looked for protein-bound microsomal haem when the induction of δ-aminolaevulinate synthetase by allylisopropylacetamide is blocked by haem synthesized endogenously from δ-aminolaevulinate (Song *et al.*, 1971). The results in Table 4 show that under conditions of enzyme repression by aminolaevulinate a significant amount of ^{59}Fe-labelled protein-bound haem is present in the microsomes. The higher ratio of microsomal haem radioactivity to the cytochrome *P*-450 content under repressed conditions as compared with induced conditions indicates that at least part of the haem synthesized from aminolaevulinate is bound to microsomal protein in a form that is not susceptible to allylisopropylacetamide-mediated degradation.

(3) Pretreatment of rats with phenobarbital decreases the inductive effect of allylisopropylacetamide (Satyanarayana Rao & Padmanaban, 1971). This could be because of the unavailability of the active hormone metabolite owing to increased metabolism. However, when phenobarbital and allylisopropylacetamide are administered together a striking induction of δ-aminolaevulinate synthetase is observed as compared with that observed with allylisopropylacetamide only (Table 5). These results can be explained on the basis that under these conditions the net amount of apo-cytochrome *P*-450 would be high because phenobarbital would accelerate apo-cytochrome *P*-450 synthesis, whereas allylisopropylacetamide would degrade the haem moiety. Pretreatment with phenobarbital increases cytochrome *P*-450 content as a result primarily of an increase in the rate of apo-protein synthesis followed by an increase in the rate of haem synthesis. This causes an increase in the activity of drug-metabolizing enzymes, for which the endogenous steroids appear to be the natural substrates. The increased metabolism of the steroids

Table 5. *Effect of phenobarbital on δ-aminolaevulinate synthetase induction by allylisopropylacetamide*

Fed female rats (100–110g) were given simultaneous injections of phenobarbital (80mg/kg) and allylisopropylacetamide (400mg/kg) or allylisopropylacetamide after phenobarbital pretreatment. Phenobarbital pretreatment involved three injections of the drug at 24h intervals. The animals were killed 6h after allylisopropylacetamide injection and δ-aminolaevulinate synthetase activity was assayed in liver homogenate. The results represent the averages of two experiments in which two livers were pooled in each experiment.

Treatment	Aminolaevulinate synthetase activity (nmol of aminolaevulinate/g of liver)
Normal	18.1
Phenobarbital (one injection)	20.2
Phenobarbital (three injections)	49.3
Allylisopropylacetamide (one injection)	89.3
Allylisopropylacetamide + phenobarbital (one injection)	140.9
Phenobarbital pretreated + allylisopropylacetamide (one injection)	59.3

would possibly deplete the pool of active steroid metabolite involved in the induction of δ-aminolaevulinate synthetase. This situation is the same as that for repeated administration of allylisopropylacetamide. However, a single simultaneous administration of phenobarbital and allylisopropylacetamide elicits a striking induction of the enzyme and presumably the active hormone metabolite is available endogenously under these conditions.

In summary, we propose that the apo-protein of a microsomal haemoprotein, which could be a species of apo-cytochrome *P*-450 or a closely associated protein undergoing a similar metabolic fate, facilitates induction of δ-aminolaevulinate synthetase at the translational level. Haem binding to this apoprotein abolishes this positive control. The translation of δ-aminolaevulinate synthetase mRNA provides a trigger for transcription and formation of more mRNA. A hormone metabolite is required for the transcription.

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References

Atkin, S. D., Palmer, E. D., English, P. D., Morgan, B., Cawthorne, M. A. & Green, J. (1972) *Biochem. J.* **128**, 237–242

Baron, J. & Tephly, T. R. (1969*a*) *Mol. Pharmacol.* **5**, 10–20

Baron, J. & Tephly, T. R. (1969*b*) *Biochem. Biophys. Res. Commun.* **36**, 526–532

Baron, J. & Tephly, T. R. (1970) *Arch. Biochem. Biophys.* **139**, 410–420

Berezney, R. & Crane, F. L. (1971) *Biochem. Biophys. Res. Commun.* **43**, 1017–1023

Blobel, G. & Potter, V. R. (1966) *Science* **154**, 1662–1665

Burnham, B. F. (1969) *Metab. Pathways* **3**, 403–537

De Matteis, F. (1970) *FEBS Lett.* **6**, 343–345

De Matteis, F. (1971) *Biochem. J.* **124**, 767–777

De Matteis, F. & Gibbs, A. (1972) *Biochem. J.* **126**, 1149–1160

Gayathri, A. K., Rao, M. R. S. & Padmanaban, G. (1973) *Arch. Biochem. Biophys.* **155**, 299

Granick, S. (1966) *J. Biol. Chem.* **241**, 1359–1375

Granick, S. & Kappas, A. (1967) *Proc. Nat. Acad. Sci. U.S.* **57**, 1463–1467

Greim, H., Schenkman, J. B., Klotzbucher, M. & Remmer, H. (1970) *Biochem. Biophys. Acta.* **201**, 20–25

Hayashi, N., Yoda, B. & Kikuchi, G. (1969) *Arch. Biochem. Biophys.* **131**, 83–91

Hayashi, N., Kurashima, Y. & Kikichi, G. (1972) *Arch. Biochem. Biophys.* **148**, 10–21

Kappas, A. & Granick, S. (1968) *J. Biol. Chem.* **243**, 346–351

Kasper, C. B. (1971) *J. Biol. Chem.* **246**, 577–581

Landaw, S. A., Callahan, C. W. & Schmid, R. (1970) *J. Clin. Invest.* **49**, 914–925

Levin, W., Jacobson, M. & Kuntzman, R. (1972) *Arch. Biochem. Biophys.* **148**, 262–269

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Mannering, G. J., Sladek, N. E., Parli, C. J. & Shoeman, D. N. (1969) in *Microsomes and Drug Oxidations* (Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R. & Mannering, G. J., eds.), pp. 303–330, Academic Press, New York

Marver, H. S. (1969) in *Microsomes and Drug Oxidations* (Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R. & Mannering, G. J., eds.), pp. 495–515, Academic Press, New York

Marver, H. S., Collins, A., Tschudy, D. P. & Rechcigl, M., Jr. (1966) *J. Biol. Chem.* **241**, 4323–4329

Marver, H. S., Schmid, R. & Schutzel, H. (1968) *Biochem. Biophys. Res. Commun.* **33**, 969–974

Matsuoka, T., Yoda, B. & Kikuchi, G. (1968) *Arch. Biochem. Biophys.* **126**, 530–538

Meyer, U. S. & Marver, H. S. (1971) *Science* **171**, 64–66

Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370–2378
Sassa, S. & Granick, S. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 517–522
Satyanarayana Rao, M. R. & Padmanaban, G. (1971) *Biochem. J.* **122**, 593–595
Satyanarayana Rao, M. R. & Padmanaban, G. (1973) *Biochem. J.* **134**, 859–868
Satyanarayana Rao, M. R., Malathi, K. & Padmanaban, G. (1972) *Biochem. J.* **127**, 553–559
Song, C. S., Harold, H. L., Rosenthal, A. S., Gelb, N. A. & Kappas, A. (1971) *J. Exp. Med.* **124**, 1349–1371
Strand, L. J., Manning, J. & Marver, H. S. (1972) *J. Biol. Chem.* **247**, 2820–2827
Sweeney, G., Freeman, K. B., Rothwell, D. & Lai, H. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1366–1371
Tyrrell, D. L. J. & Marks, G. S. (1972) *Biochem. Pharmacol.* **21**, 2977–2093
Waxman, A. D., Collins, A. & Tschudy, D. P. (1966) *Biochem. Biophys. Res. Commun.* **24**, 675–683