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The Relationship between δ-Aminolaevulinate Synthetase Induction and the Concentrations of Cytochrome *P*-450 and Catalase in Rat Liver

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The porphyrinogenic drug 2-allyl-2-isopropylacetamide causes the degradation of microsomal cytochrome *P*-450 and inhibits the synthesis of catalase in rat liver. The inhibition of catalase synthesis follows the induction of δ-aminolaevulinate synthetase and the consequent overproduction of haem. The allylisopropylacetamide-mediated breakdown of cytochrome *P*-450 is a rapid event and has a reciprocal relationship to the pattern of δ-aminolaevulinate synthetase induction. Breakdown of cytochrome *P*-450 appears to be one of the conditions leading to the 'derepression' of δ-aminolaevulinate synthetase.

δ-Aminolaevulinate synthetase, the first and rate-limiting enzyme of the biosynthetic pathway for haem, is induced in mammalian liver by several drugs (Granick, 1966; Marver *et al.*, 1966*a*,*b*). However, whereas porphyrinogenic drugs such as allylisopropylacetamide and diethoxycarbonyl-1,4-dihydrocollidine induce the enzyme markedly in whole animals, compounds such as phenobarbital and hexachlorobenzene cause only a weak to moderate increase in enzyme activities (Schmid *et al.*, 1966; Wada *et al.*, 1968). It has been suggested that the drugs induce δ-aminolaevulinate synthetase to provide more cytochrome *P*-450, eventually leading to a greater metabolism of the drugs. The results obtained with phenobarbital are often cited as an example (Granick, 1966; Baron & Tephly, 1969, 1970). However, allylisopropylacetamide, which causes a striking increase in δ-aminolaevulinate synthetase activity as compared with phenobarbital and which also increases the rate of haem synthesis (Schmid *et al.*, 1955), actually promotes a decrease in cytochrome *P*-450 content (Marver, 1969; De Matteis, 1970). The decrease involves the breakdown of the haem moiety (Meyer & Marver, 1971), leading to the formation of green pigments (De Matteis, 1970; Landaw *et al.*, 1970). The possibility has been raised that the rapid breakdown of cytochrome *P*-450 might have something to do with the derepression of δ-aminolaevulinate synthetase (De Matteis, 1970; Meyer & Marver, 1971; Landaw *et al.*, 1970), haem being the co-repressor for the enzyme (Granick, 1966; Marver *et al.*, 1966*a*). Catalase is another haem protein that shows depressed activity during treatment with allylisopropylacetamide (Schmid *et al.*, 1955; De Matteis, 1967).

In the present study, the possible relationship between decreased amounts of cytochrome *P*-450 and catalase and the induced activity of δ-aminolaevulinate synthetase has been evaluated. A preliminary report has appeared from this laboratory indicating that the overproduction of haem might lead to inhibition of catalase synthesis under conditions of treatment with allylisopropylacetamide (Satyanarayana Rao & Padmanaban, 1971).

Experimental

Materials

Allylisopropylacetamide and [2-^{14}C]allylisopropylacetamide (4.2μCi/mg) were generously supplied by Hoffmann-La Roche Ltd. (Basle, Switzerland). SKF-525A was a gift from Smith, Kline and French Co. (Philadelphia, Pa., U.S.A.). All other biochemicals were purchased from commercial sources.

Methods

Treatment of animals. Female rats (100–110g) were used in all the experiments. In short-term experiments with allylisopropylacetamide, the animals were starved for 48h before administration of the drug. In experiments where the effects of daily administration of allylisopropylacetamide were studied, the animals were fed on stock diet. The stock diet was obtained from Hindustan Lever Co., Bombay, India. Allylisopropylacetamide was given subcutaneously (300 or 400mg/kg) to starved and fed rats respectively. The various other schedules of injections are given in the respective tables and figures. The animals were killed after ether anaesthesia and the livers removed.

Treatment of liver. The liver samples were homogenized in 0.15M-KCl (4ml/g of liver) and a portion of the homogenate was preserved for assay of δ-aminolaevulinate synthetase and catalase. The rest of the homogenate was centrifuged at 15000*g* for

15min and the post-mitochondrial supernatant was centrifuged for 1h at 100000*g* in a Spinco model L ultracentrifuge. The microsomal pellet was washed once with 0.15M-KCl and then suspended in 0.05M-potassium phosphate buffer, pH7.5. The suspension was used for determination of cytochrome *P*-450.

Assay of δ-aminolaevulinate synthetase. The enzyme activity was measured in liver homogenates by the method of Marver *et al.* (1966*a*). The incubation mixture contained, in a total volume of 2ml: 1.0ml of homogenate, 200μmol of glycine, 20μmol of EDTA and 150μmol of tris–HCl buffer at a final pH of 7.2. After incubation for 1h at 37°C, the reaction was stopped with 1ml of 12.5% (w/v) trichloroacetic acid. The protein-free supernatant was used for the determination of δ-aminolaevulinate by using acetyl acetone and modified Ehrlich reagent (Urata & Granick, 1963).

Assay of catalase. Catalase was assayed in the liver homogenates after treating them with sodium deoxycholate as described by Ganschow & Schimke (1968). The enzyme activity was assayed by the permanganate-titration method of Ramachandran & Sarma (1954). The liver preparation was diluted such that not more than 20% of H_2O_2 was decomposed in 5min at 30°C. The validity of the method was also checked by using the spectrophotometric method of Beers & Sizer (1952).

Determination of cytochrome P-450. This was done by measuring the CO-difference spectrum of dithionite-reduced microsome suspensions. The cytochrome *P*-450 concentration was calculated with the use of an extinction coefficient of $91 cm^{-1} \cdot mM^{-1}$ for E_{450}-E_{490} (Omura & Sato, 1964).

Determination of half-life of δ-aminolaevulinate synthetase. Animals that had received two injections of allylisopropylacetamide (400mg/kg) or three injections of phenobarbital (80mg/kg) at 12h intervals were used. At 6h after the last injection, the animals were injected with cycloheximide (10mg/kg), and then killed at different time-intervals. δ-Aminolaevulinate synthetase was assayed in the different liver homogenates as described above.

Effect of SKF-525A on uptake of [2-^{14}C]allylisopropylacetamide. SKF-525A was given intraperitoneally at 50mg/kg, 30min before administration of allylisopropylacetamide. [2-^{14}C]Allylisopropylacetamide was given intraperitoneally at two doses: 150mg/kg or 300mg/kg. The amount of radioactivity given was adjusted to 2.5μCi in both doses. The uptakes of radioactivity in total liver and the microsomal pellet were measured.

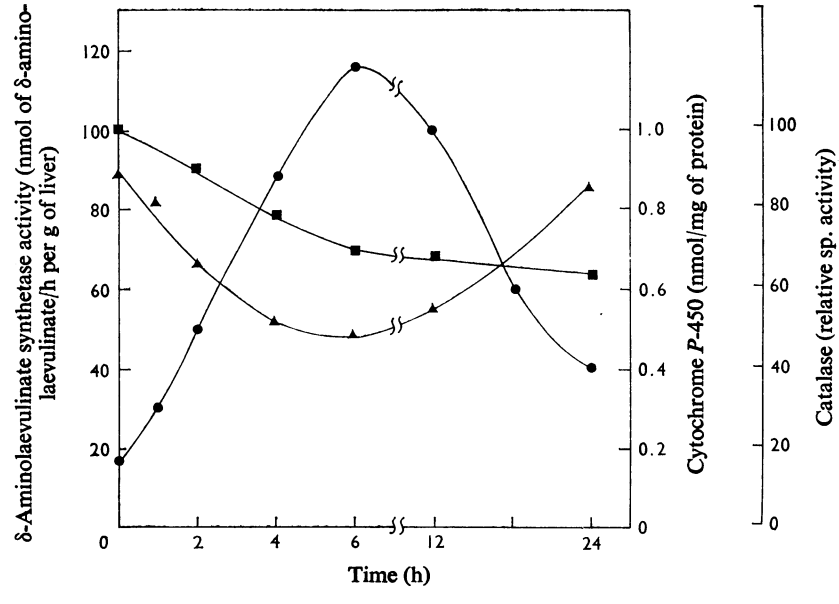

Fig. 1. *Effect of allylisopropylacetamide on activities of δ-aminolaevulinate synthetase and catalase and concentration of cytochrome P-450*

The experimental conditions are as described in Table 1. Each point represents an average of two experiments where three livers have been pooled in each experiment. ●, δ-Aminolaevulinate synthetase; ▲, cytochrome *P*-450; ■, catalase.

Measurements of radioactivity. These were made in a Beckman LS-100 liquid-scintillation counter. A portion of the liver or the microsomal pellet was dissolved in a known volume of formic acid and samples were placed on Whatman no. 3 filter-paper discs. The discs were dried and the radioactivity was counted in vials containing 5ml of 0.5% 2,5-diphenyloxazole in toluene.

Determination of protein. This was done by the method of Lowry *et al.* (1951) by using bovine serum albumin as the standard.

Results

Fig. 1 shows that a single injection of allylisopropylacetamide to starved rats leads to a fall in the amount of cytochrome *P*-450 and catalase activity and an increase in δ-aminolaevulinate synthetase activity in liver. The decrease in cytochrome *P*-450 concentration is rapid and there is an inverse relationship between the activity of δ-aminolaevulinate synthetase and the concentration of cytochrome *P*-450. The decrease in catalase activity is slower and the depression persists even at 24h after treatment with allylisopropylacetamide, when δ-aminolaevulinate synthetase activity and cytochrome *P*-450 concentration have returned to normal values.

The status of cytochrome *P*-450 and catalase under conditions of block in the induction of δ-aminolaevulinate synthetase was examined by using cycloheximide, glucose and haem. The results (Table 1) show that cycloheximide and glucose, which inhibit induction of δ-aminolaevulinate synthetase by allylisopropylacetamide, do not prevent the degradation of cytochrome *P*-450. However, under these conditions the depressing effect of the drug on catalase activity is counteracted. Haem on its own or in combination with allylisopropylacetamide leads to depressed catalase activity.

It has been shown earlier that catalase activity is depressed by exogenous haemin as well as bilirubin and it has been suggested that this might involve inhibition of catalase synthesis (Satyanarayana Rao & Padmanaban, 1971). Allylisopropylacetamide itself has been shown to inhibit catalase synthesis on the basis that it prevents the regain in catalase activity after treatment with aminotriazole. Aminotriazole forms complexes with the existing catalase molecules, rendering them inactive, and the subsequent regain in catalase activity has been shown to be due to fresh synthesis (Price *et al.*, 1962). Fig. 2 indicates that haemin and bilirubin prevent the regain in catalase activity after treatment with aminotriazole, indicating that they inhibit the enzyme synthesis.

Daily administration of allylisopropylacetamide to rats for over 3 days leads to a stage when the animals fail to respond to the drug by showing increased

Table 1. *Effect of cycloheximide, glucose and haem on allylisopropylacetamide-mediated changes in δ-aminolaevulinate synthetase, catalase and cytochrome P-450*

Female rats (100–110g) were starved for 48h and then treated with the different compounds. Allylisopropylacetamide was injected subcutaneously (300mg/kg) in a volume of 1.5ml. Glucose was given by stomach intubation (2g/animal) 6h before and at the time of injection of allylisopropylacetamide. Cycloheximide and haem were injected intraperitoneally (10 and 20mg/kg respectively) at the time of injection of allylisopropylacetamide. Haem was dissolved in 0.05ml of M-NaOH and then made up to the required volume with buffer, maintaining a pH of 7.8. The animals were killed 6h after the injection and the liver was processed as described in the text. δ-Aminolaevulinate synthetase was assayed in liver homogenates. The results are expressed as per cent of the value (100) for the control (absolute values are given in parentheses). The units of the enzyme activities are: δ-aminolaevulinate synthetase, nmol of δ-aminolaevulinate/g of liver per h; catalase, μmol of H_2O_2 decomposed/mg of protein per min; cytochrome *P*-450, nmol/mg of microsomal protein. The control values represent the mean±S.E.M. from three experiments, where two livers have been pooled in each experiment.

Treatment	δ-Aminolaevulinate synthetase	Catalase	Cytochrome *P*-450
Control	100 (16.6±2.4)	100 (350.0±11.4)	100 (0.86±0.02)
Allylisopropylacetamide	520	69	62
Glucose	98	98	99
Cycloheximide	86	95	95
Haem	75	70	100
Allylisopropylacetamide+glucose	225	94	51
Allylisopropylacetamide+cycloheximide	152	92	55
Allylisopropylacetamide+haem	112	65	—

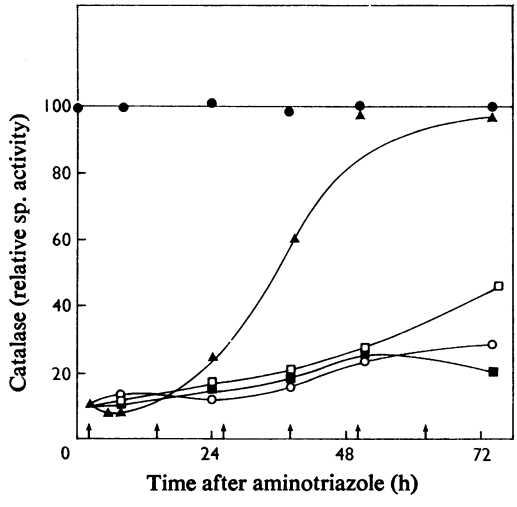

Fig. 2. *Effect of allylisopropylacetamide, haem and bilirubin on catalase synthesis*

Aminotriazole (1 g/kg) was injected intraperitoneally into female rats (100–110 g). At 2 h after treatment with aminotriazole the animals were divided into four groups. One group received no treatment and the other three received allylisopropylacetamide (400 mg/kg), haem (20 mg/kg) and bilirubin (10 mg/kg) respectively. These three compounds were injected every 12 h, as indicated by the arrows in the figure. The animals were killed at different time-intervals and the catalase activities were assayed in liver homogenates as described in the text. Each point represents an average of two experiments, where three livers have been pooled in each experiment. ●, Control; ▲, aminotriazole; □, aminotriazole plus bilirubin; ○, aminotriazole plus haem; ■, aminotriazole plus allylisopropylacetamide.

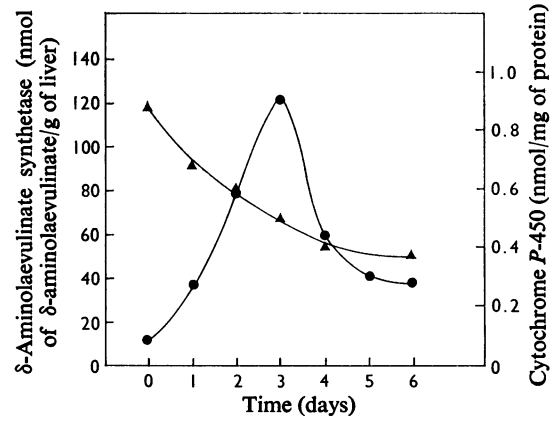

Fig. 3. *Effect of daily injections of allylisopropylacetamide on δ-aminolaevulinate synthetase activity and cytochrome P-450 concentration*

Fed animals were given daily subcutaneous injections of allylisopropylacetamide (400 mg/kg). The animals were killed 6 h after injection and the δ-aminolaevulinate synthetase activity and cytochrome *P*-450 concentration were determined in the liver. Each point represents an average of two experiments where two livers have been pooled in each experiment. ●, δ-Aminolaevulinate synthetase; ▲, cytochrome *P*-450.

enzyme activity (Satyanarayana Rao *et al.*, 1971). It was of interest to see whether allylisopropylacetamide can still bring about the breakdown of cytochrome *P*-450 at a stage when δ-aminolaevulinate synthetase does not respond to the drug. Rats were given daily subcutaneous injections of allylisopropylacetamide, and 6 h after each injection the amounts of cytochrome *P*-450 and δ-aminolaevulinate synthetase were determined. Fig. 3 shows that administration of allylisopropylacetamide after day 3 does not cause an increase in δ-aminolaevulinate synthetase activity. However, cytochrome *P*-450 is maintained at a decreased concentration. It was found that 24 h after each injection of allylisopropylacetamide the amount of cytochrome *P*-450 had returned to normal or even higher than normal values, and thus the depressed concentrations indicated in Fig. 3 represent fresh breakdown after each injection, as well as that each injection is progressively more effective in decreasing cytochrome *P*-450 concentration. These results are in accord with the recent findings of De Matteis (1971) that the metabolism of allylisopropylacetamide is necessary to cause degradation of cytochrome *P*-450. The metabolism of allylisopropylacetamide could also be enhanced by pretreating the animals with phenobarbital (De Matteis, 1970). The results in Table 2 indicate that pretreatment with phenobarbital results in a decreased induction of δ-aminolaevulinate synthetase by allylisopropylacetamide. However, there is a striking fall in the concentration of cytochrome *P*-450 under these conditions. The depressing effect of allylisopropylacetamide on catalase is partially counteracted by pretreatment with phenobarbital. De Matteis (1970) has also shown with isolated microsomes that metabolism of allylisopropylacetamide is necessary to bring about breakdown of cytochrome *P*-450. SKF-525A was shown to prevent the breakdown of cytochrome *P*-450 under these conditions. The effect of SKF-525A was studied in the present investigation *in vivo*, when it was given 30 min before administration of allylisopropylacetamide, and the activity of δ-aminolaevulinate

Table 2. *Effect of pretreatment with phenobarbital on allylisopropylacetamide-mediated changes in δ-aminolaevulinate synthetase, catalase and cytochrome P-450*

Phenobarbital (80mg/kg) was injected intraperitoneally at intervals of 24h. Allylisopropylacetamide was given subcutaneously 24h after the third injection of phenobarbital and the animals were killed 6h afterwards. The animals were starved for 24h before injection of allylisopropylacetamide. The results represent the mean±S.E.M. from three experiments where two livers were pooled in each experiment. The absolute units are the same as given in Table 1.

Treatment	δ-Aminolaevulinate synthetase	Catalase	Cytochrome *P*-450
Control	100 (15.6±1.4)	100 (320.0±9.4)	100 (0.91±0.06)
Allylisopropylacetamide	625	64	60
Phenobarbital	220	102	322
Phenobarbital+allylisopropylacetamide	312	87	96

Table 3. *Effect of SKF-525A on allylisopropylacetamide-mediated changes in δ-aminolaevulinate synthetase, catalase and cytochrome P-450*

Allylisopropylacetamide was given intraperitoneally at two concentrations (150 or 300mg/kg) to rats starved for 48h. SKF-525A was also given intraperitoneally (50mg/kg), 30min before injection of allylisopropylacetamide. The animals were killed at 2 and 6h after injection of allylisopropylacetamide. The results represent an average of two experiments and livers from two animals were pooled in each experiment. The absolute units are the same as given in Table 1.

Treatment	Time after injection (h) ...	δ-Aminolaevulinate synthetase		Catalase		Cytochrome *P*-450	
		2	6	2	6	2	6
Control		100	100 (17.5)	100	100 (320.5)	100	100 (0.95)
Allylisopropylacetamide (150mg/kg)		220	435	86	75	82	75
Allylisopropylacetamide (300mg/kg)		265	620	86	69	80	62
Allylisopropylacetamide (150mg/kg)+SKF-525A		110	145	92	95	96	98
Allylisopropylacetamide (300mg/kg)+SKF-525A		123	152	102	98	100	99
SKF-525A		125	162	107	95	102	108

synthetase and the concentration of cytochrome *P*-450 were determined at 2 and 6h after administration of allylisopropylacetamide. The results in Table 3 indicate that SKF-525A prevents the allylisopropylacetamide-mediated breakdown of cytochrome *P*-450 as well as the induction of δ-aminolaevulinate synthetase and depression of catalase activity. SKF-525A does not significantly inhibit the uptake of [2-^{14}C]-allylisopropylacetamide by the whole liver or the liver microsomes under these conditions (Table 4).

Discussion

It is worth while to discuss these results in the framework of Granick's (1966) model for the induction of δ-aminolaevulinate synthetase in mammalian liver by drugs. The model envisages haem, the end product of the pathway, as a co-repressor for the enzyme. The drugs have been visualized as inhibiting haem-binding to the apo-repressor, thereby preventing the formation of a functional repressor and thus leading to a derepression of the enzyme. The diverse chemical nature of the drugs that induce this enzyme has called for subtler modifications of this model. Burnham (1969) has proposed that the drugs as such may not compete with haem but a common metabolite X might be involved, the accumulation of which is facilitated by the inducers. The finding by Granick & Kappas (1967) and Kappas & Granick (1968) that some of the steroid metabolites are potent inducers

Table 4. *Effect of SKF-525A on the uptake of* [2-^{14}C]*allylisopropylacetamide*

SKF-525A was given intraperitoneally at a concentration of 50mg/kg, 30min before the injection of [2-^{14}C]-allylisopropylacetamide. Unlabelled allylisopropylacetamide was injected intraperitoneally together with [2-^{14}C]allylisopropylacetamide at two doses (150 or 300mg/kg). Radioactivity was adjusted to 2.5μCi/animal in each case. The animals were killed 2h after injection of allylisopropylacetamide. Two livers were pooled and processed to measure total incorporation into the liver and the microsomal fraction.

Treatment	Total uptake (μg of allylisopropylacetamide/g of liver)	Uptake in microsomes (μg of allylisopropylacetamide/g of liver)	% of total
Allylisopropylacetamide (150mg/kg)	79.7	1.7	2.1
Allylisopropylacetamide (300mg/kg)	134.2	2.4	1.8
Allylisopropylacetamide (150mg/kg)+SKF-525A	75.1	2.2	2.9
Allylisopropylacetamide (300mg/kg)+SKF-525A	158.8	2.4	1.5

of δ-aminolaevulinate synthetase has opened up the possibility that X could be a steroid metabolite. Drugs can have an influence on the concentrations of steroid metabolites since the two form common substrates for the microsomal mixed-function oxidase system as well as for glucuronidation and other conjugation steps.

On the basis of this model for the induction of δ-aminolaevulinate synthetase, a decrease in co-repressor concentrations would naturally be expected to facilitate enzyme induction. The two haem proteins that show decreased concentrations during treatment with allylisopropylacetamide are catalase and cytochrome *P*-450. The results presented here indicate the relationship between overproduction of haem and inhibition of catalase synthesis. In other words, the induction of δ-aminolaevulinate synthetase and the consequent overproduction of haem cause inhibition of catalase synthesis, rather than the depressed catalase activity being responsible for the induction of δ-aminolaevulinate synthetase. However, the decrease in concentration of cytochrome *P*-450 has the following distinct features: (*a*) cytochrome *P*-450 is the major microsomal haem protein and the haem moiety undergoes a rapid turnover, controlling the labile haem pool; (*b*) the decrease in the amount of cytochrome *P*-450 due to administration of allylisopropylacetamide is rapid and induction of δ-aminolaevulinate synthetase and haem overproduction are not necessary for this phenomenon to occur; (*c*) the decrease in cytochrome *P*-450 due to administration of allylisopropylacetamide involves the degradation of the haem moiety and hence a depletion of the haem pool.

At the same time some of the results obtained in the present investigation show that degradation of cytochrome *P*-450 is not always followed by induction of δ-aminolaevulinate synthetase. Conditions such as treatment with allylisopropylacetamide together with cycloheximide and glucose interfere with δ-aminolaevulinate synthetase induction although degradation of cytochrome *P*-450 is taking place. These results can, however, be explained on the basis that cycloheximide and glucose interfere with the protein-synthetic machinery. A similar result is observed under conditions of continuous treatment with allylisopropylacetamide as well as phenobarbital pretreatment followed by treatment with allylisopropylacetamide. In this case an explanation on the basis that allylisopropylacetamide is responsible for induction of δ-aminolaevulinate synthetase and its metabolite for degradation of cytochrome *P*-450 would tend to dissociate the two events. However, the results obtained with SKF-525A indicate that such an explanation is not feasible. SKF-525A, which inhibits metabolism of allylisopropylacetamide, prevents cytochrome *P*-450 breakdown as well as induction of δ-aminolaevulinate synthetase. An alternative explanation can be offered on the basis that, in the process of δ-aminolaevulinate synthetase induction, metabolite X might not only prevent haem-binding to the apo-repressor but its own binding to the apo-repressor might be essential and lead to the formation of a positive inducer. Thus it is possible that under conditions of continuous treatment with allylisopropylacetamide, and phenobarbital pretreatment followed by allylisopropylacetamide, the availability of metabolite X might have been interfered with. This would explain the lack of an inductive response of δ-aminolaevulinate synthetase even though cytochrome *P*-450 is breaking down.

The possibility still exists that degradation of cytochrome *P*-450 is quite unrelated to the process of induction of δ-aminolaevulinate synthetase; but

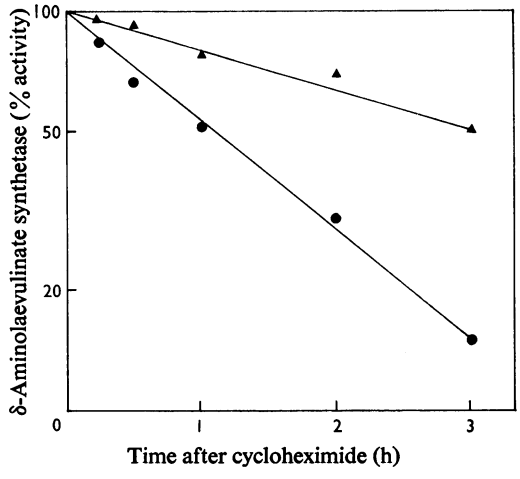

Fig. 4. *Effect of phenobarbital on the half-life of δ-aminolaevulinate synthetase*

The details are given in the Experimental section. Each point represents an average of two experiments where three livers have been pooled in each experiment. ▲, Phenobarbital treatment; ●, allylisopropylacetamide treatment.

the following considerations, in addition to those stated above, tend to implicate a role for this haem protein in the control of δ-aminolaevulinate synthetase. (*a*) Although Granick (1966) originally proposed a transcriptional control of δ-aminolaevulinate synthetase with the chick-embryo liver system, the model can also be applied as one operating at the translational level. Sassa & Granick (1970) have indicated that both allylisopropylacetamide and haem act at the translational level. In such a situation, the microsomal haem pool could exert a profound influence at a site where protein synthesis is taking place. (*b*) The rapid degradation of cytochrome *P*-450 is observed only with drugs such as allylisopropylacetamide and diethoxycarbonyl-1,4-dihydrocollidine, which are potent inducers of δ-aminolaevulinate synthetase. Compounds like phenobarbital and hexachlorobenzene, which are moderate or weak inducers of δ-aminolaevulinate synthetase in the whole animal, do not lead to a decrease in the concentration of cytochrome *P*-450.

This also leads to the question whether weak or moderate inducers of δ-aminolaevulinate synthetase act by a mechanism different from those of the potent inducers. The results presented in Fig. 4 indicate that under conditions of administration of phenobarbital, the half-life of δ-aminolaevulinate synthetase is increased as compared with that obtained with allylisopropylacetamide. It is a possibility that drugs like phenobarbital and hexachlorobenzene might lead to elevated activity of δ-aminolaevulinate synthetase by decreasing the rate of enzyme degradation.

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