

Role of haem in the induction of cytochrome *P*-450 by phenobarbitone

Studies in chick embryos *in ovo* and in cultured chick embryo hepatocytes

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The role of haem synthesis during induction of hepatic cytochrome *P*-450 haemoproteins was studied in chick embryos *in ovo* and in chick embryo hepatocytes cultured under chemically defined conditions. 1. Phenobarbitone caused a prompt increase in the activity of 5-aminolaevulinate synthase, the rate-limiting enzyme of haem biosynthesis, and in the concentration of cytochrome *P*-450. This induction response occurred without measurable initial destruction of the haem moiety of cytochrome *P*-450. 2. When intracellular haem availability was enhanced by exogenous haem or 5-aminolaevulinate, phenobarbitone-mediated induction of cytochrome *P*-450 was not affected in spite of the well known repression of 5-aminolaevulinate synthase by haem. These data are consistent with the concept that haem does not regulate the synthesis of cytochrome *P*-450 haemoproteins. 3. Acetate inhibited haem biosynthesis at the level of 5-aminolaevulinate formation. When intracellular haem availability was diminished by treatment with acetate, phenobarbitone-mediated induction was decreased. 4. This inhibitory effect of acetate on cytochrome *P*-450 induction was reversed by exogenous haem or its precursor 5-aminolaevulinate. These data suggest that inhibition of haem biosynthesis does not decrease synthesis of apo-cytochrome *P*-450. Moreover, they indicate that exogenous haem can be incorporated into newly formed apo-cytochrome *P*-450.

Cytochrome *P*-450 is the collective term for a group of haemoproteins that function as terminal oxidases in the metabolism of a wide variety of exogenous and endogenous substances. In the liver some of these substrates, e.g. phenobarbitone, differentially induce one or several cytochrome *P*-450 haemoproteins and thereby enhance microsomal mixed-function oxidase activity (for review, see Estabrook & Lindenlaub, 1979). Induction of cytochrome *P*-450 requires synthesis *de novo* of apoproteins in rough endoplasmic reticulum (Bar-Nun *et al.*, 1980) and of haem in mitochondria (Jones & Jones, 1969), but the regulation of these two processes during induction remains poorly understood (for review, see De Matteis & Aldridge, 1978). Co-ordination of haem and apoprotein synthesis has been demonstrated in the formation of other haemoproteins, such as haemoglobin (Grayzel *et al.*, 1966). Increased activity of ALA synthase (EC 2.3.1.37), the first and rate-limiting enzyme of haem biosynthesis, accompanies the induction of

cytochrome *P*-450 haemoproteins by phenobarbital (Marver, 1969).

Experimental inhibition of haem biosynthesis decreases drug-mediated induction of cytochrome *P*-450 (for review, see Tephly, 1978). For instance administration of acetate to rats decreased drug-mediated induction of hepatic ALA synthase and lowered induction of cytochrome *P*-450 (Piper & Tephly, 1974; Tephly, 1978). By contrast, experimental enhancement of intracellular haem availability, produced by injection of haem or the haem precursor ALA into rats did not raise hepatic cytochrome *P*-450 concentration (Song *et al.*, 1971; De Matteis & Gibbs, 1972; Druyan & Kelly, 1972). In fact, haem administration prevented drug-mediated induction of cytochrome *P*-450 (Marver, 1969; Bock *et al.*, 1971; Dehlinger & Schimke, 1972; Rajamanickam *et al.*, 1972, 1975; Bhat *et al.*, 1977). That this is not due to a principal inaccessibility of the apo-cytochrome *P*-450 haem site for exogenous haem is inferred from the recent observation that exogenous haem (Bhat *et al.*, 1977; Correia *et al.*, 1979; Farrell & Correia, 1980) and ALA (Levin & Kuntzman, 1969) are incorporated into cytochrome *P*-450. Moreover, haem added *in*

Abbreviations used: ALA, 5-aminolaevulinate; ALA synthase, 5-aminolaevulinate synthase.

in vitro to liver homogenates (Correia & Meyer, 1975) or mitochondria-rough-endoplasmic-reticulum complexes (Meier *et al.*, 1978) of rats treated with CoCl_2 and phenobarbitone increased cytochrome *P*-450 concentration. Hence, it is difficult to understand how endogenous and exogenous haem affect the synthesis and assembly of cytochrome *P*-450, particularly in the light of the fact that in the liver haem controls its own synthesis by negative feedback repression of ALA synthase (Burnham & Lascelles, 1963; Granick & Sassa, 1971).

With regard to the induction of ALA synthase by phenobarbitone, two mechanisms have been considered. Phenobarbitone may induce apocytochrome *P*-450 synthesis, which through its haem-binding capacity diverts intracellular haem from the pool regulating ALA synthase, thereby inducing this enzyme (De Matteis & Gibbs, 1972; Correia & Meyer, 1975; Rajamanickam *et al.*, 1975; Meier *et al.*, 1978). Alternatively, on the basis of studies in chick embryos, it recently has been proposed that primary destruction of cytochrome *P*-450 haem by drugs such as phenobarbitone may be a prerequisite of hepatic ALA synthase induction (Lim *et al.*, 1980).

We therefore have re-investigated the role of haem synthesis during induction of hepatic cytochrome *P*-450 in cultured chick embryo hepatocytes and in chick embryos *in ovo*. Induction of cytochrome *P*-450 and ALA synthase by phenobarbitone was studied under the following conditions: (1) inhibition of haem synthesis by acetate; (2) enhanced intracellular haem concentrations by administration of exogenous haem or the haem precursor ALA; (3) combined effects of acetate and ALA or haem.

In the present paper haem refers to iron protoporphyrin IX, irrespective of the oxidation state of the iron.

A preliminary account of part of this work was presented at the European Meeting on Cytochrome *P*-450, Sweden, 1980, and was published in the proceedings of this congress (Giger & Meyer, 1980).

Materials and methods

Materials

Chick embryos (Shaver hybrids) were obtained from Wolff Poultry Farm, Volketswil, Switzerland. Williams E medium without glucose and pyruvate, Ca^{2+} -free Hanks balanced salt solution (Hanks solution) and sodium phenobarbitone were supplied by the Hospital Pharmacy, University Hospital, Zurich, Switzerland. Collagenase type I (160 i.u./mg), GTP-dependent succinyl thiokinase [succinyl-CoA synthetase (GDP-forming); EC 6.2.1.4] from porcine heart, 5-aminolaevulinic chloride and 3,3',5-tri-iodothyronine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sodium

acetate 3-hydrate and 7-ethoxycoumarin were obtained from Riedel-DeHaen Co., Hannover, Germany, and Boehringer Co., Mannheim, Germany, respectively. Allylisopropylacetamide was generously provided by Hoffmann-La Roche, Basle, Switzerland. Haemin was obtained from Porphyrin Products, Logan, UT, U.S.A. [^{14}C]Succinate, with a specific radioactivity of 116 Ci/mol, was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals or biochemicals were obtained from Merck, Darmstadt, Germany, or Fluka, Buchs, Switzerland.

Preparation of cultured chick embryo hepatocytes

Hepatocytes were prepared from 15-day chick embryos under sterile conditions by the method of Althaus *et al.* (1979) with the following modifications. The livers were perfused *in situ* through the heart (flow rate 16 ml/min) with 0.9% NaCl, containing 2 mM-EDTA until most of the hepatic blood had disappeared and subsequently with 9 ml of 0.05% collagenase in Hanks solution. Livers were removed into collagenase/Hanks solution and 10–20 livers were incubated for 20 min at 37°C, then minced and again incubated for 30–35 min. Contaminating erythrocytes were removed (Sassa & Kappas, 1977). The isolated liver cells were diluted to about 1.5×10^6 cells/ml of Williams E medium without glucose and pyruvate, but containing 3,3',5-tri-iodothyronine (1 $\mu\text{g}/\text{ml}$ of medium). Of this cell suspension, 10 ml was plated in 10 cm Flacon culture dishes and incubated at 37°C in a humidified incubator in a CO_2/air (1:19) atmosphere for 44 h with one medium change after 24 h.

With this isolation procedure a yield of about 10^8 cells/g wet wt. of liver was obtained. Over 90% of these cells could be classified as hepatocytes. After plating, hepatocytes formed a practically confluent monolayer in about 12 h. Insulin or serum was not necessary for cell attachment.

Additions to the medium were dissolved in water and the pH was adjusted to 7.4 before filtration (Millipore, 0.22 μm); haemin was dissolved in 0.25% sodium carbonate and the pH was adjusted to 8.0 with HCl. Control cultures were exposed to the appropriate solvents. Phenobarbitone was added for the last 16 h of culture; the final concentration was 0.4 mM. ALA (10 μM) and haem (3 μM) were administered twice, 2 h before and 4 h after the phenobarbitone addition. Acetate was present from the beginning of the culture.

Studies in chick embryos *in ovo*

The same time sequence was followed for injections into chick embryos *in ovo* as for additions to the culture medium. All chemicals were injected into

the fluid surrounding the embryo starting with the administration of acetate on day 15 of the embryonic development. On day 16, 5 mg of phenobarbitone was injected concomitantly with acetate. ALA (750 µg) or haem (280 µg) were injected 2 h before and 4 h after the phenobarbitone administration.

Preparation of homogenates and microsomes

Monolayers of three culture dishes were usually maintained under identical culture conditions. One dish per experimental group was used for determination of ALA synthase activity, two dishes for preparation of microsomes. Tissue fractionation was performed at 4°C as follows. One culture dish was washed twice with 0.9% NaCl, the cells scraped off with a rubber policeman and suspended in a final volume of 0.5 ml of 0.9% NaCl containing 10 mM-Tris, 0.1 mM-pyridoxal phosphate and 0.5 mM-EDTA, pH 7.4 (ALA synthase buffer). Homogenates were prepared with a Potter-Elvehjem homogenizer (glass-Teflon homogenizer; clearance 0.08–0.14 mm, 120 rev./min, 11 complete strokes), sonicated for 5 s with a Kontes cell disruptor (130 Watts/cm²; type 881440; Kontes, Vineland, NY, U.S.A.) and stored at –20°C at a protein concentration of 10 mg/ml until used for the determination of ALA synthase activity.

For preparation of microsomes, monolayers were washed with 0.25 M-sucrose, removed, suspended in a final volume of 10 ml of 0.25 M-sucrose, homogenized and sonicated as described above. The homogenate was centrifuged at 10 000 g for 12 min and the resulting supernatant was centrifuged at

105 000 g for 90 min to sediment the microsomal pellet. Microsomes were suspended in 0.1 M-sodium phosphate buffer, pH 7.4, to a protein concentration of 2–4 mg/ml and stored at –20°C until used for the determination of cytochrome *P*-450 concentration and 7-ethoxycoumarin de-ethylase activity. Livers from experiments *in ovo* were perfused *in situ* through the heart with ice-cold 0.9% NaCl. A 10% homogenate (w/v) in ALA synthase buffer was prepared from small pieces of two to three pooled livers and sonicated, as described above. The remaining liver tissue was taken up in 10 ml of 0.25 M-sucrose and the same fractionation procedure, but without sonication, was applied as for the preparation of microsomes from culture. The resulting microsomal pellet was washed and re-centrifuged at 105 000 g in 0.1 M-sodium phosphate buffer containing 30% glycerol and 0.5 mM-EDTA, pH 7.4.

Analytical assays

Microsomal cytochrome *P*-450 concentration was measured in microcuvettes as described previously by Althaus *et al.* (1979) and 7-ethoxycoumarin de-ethylase activity was determined by the method of Poland & Greenlee (1978). ALA synthase activity was measured by the incorporation of [1,4-¹⁴C]succinate into ALA and isolation of ALA by ion-exchange chromatography by the method of Strand *et al.* (1972), but with the following modifications. (a) The reaction mixture contained 0.1 M-potassium fluoride (Yoda *et al.*, 1975). (b) The succinate, arsenite and malonate concentration was 4 mM, 10 mM and 100 mM respectively. (c) GTP was

Table 1. Phenobarbitone-mediated changes in cytochrome *P*-450 concentration, 7-ethoxycoumarin de-ethylase and ALA synthase activity in chick embryos *in ovo* and in cultured chick embryo hepatocytes

In ovo, chick embryos were treated with 5 mg of phenobarbitone dissolved in 0.1 ml of water 16 h before killing on day 17. The controls were injected with solvent. In hepatocyte culture, hepatocytes from 15-day chick embryos were cultured for 44 h in Williams E medium. Monolayers were exposed to 0.4 mM-phenobarbitone for the last 16 h of culture. Hepatic cytochrome *P*-450 concentration and 7-ethoxycoumarin de-ethylase activity were measured in microsomes and ALA synthase activity was determined in homogenates, as described in the Materials and methods section. Results are means ± S.E.M. The numbers of experiments are indicated in parentheses. **P* < 0.001, compared with control values (Student's *t* test).

	<i>In ovo</i>		In hepatocyte culture	
	Control	Phenobarbitone-treated	Control	Phenobarbitone-treated
Cytochrome <i>P</i> -450 concentration (pmol of cytochrome <i>P</i> -450/mg of protein) (% of control)	195 ± 13 (9)	465 ± 37 (10)* 237 ± 9 (10)	112 ± 5 (9)	213 ± 8 (13)* 196 ± 3 (12)
7-Ethoxycoumarin de-ethylase activity (pmol of hydroxycoumarin/mg of protein per min) (% of control)	774 ± 77 (8)	1782 ± 191 (9)* 205 ± 9 (9)	328 ± 12 (6)	725 ± 33 (4)* 224 ± 4 (4)
ALA synthase activity (pmol of ALA/mg of protein per 30 min) (% of control)	153 ± 11 (6)	1327 ± 157 (8)* 817 ± 92 (8)	293 ± 25 (8)	661 ± 56 (9)* 229 ± 8 (9)

used instead of ATP as cofactor of the succinyl thiokinase from porcine heart. (d) The third ion-exchange column was equilibrated with 1M-sodium acetate instead of 0.05 M solution. The final incubation volume was 0.5ml, including 0.05 ml of homogenate. For all enzyme assays product formation was linear with respect to time and protein concentration over the concentration range used. Protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

Phenobarbitone added to the culture medium markedly induced cytochrome *P*-450 and enhanced 7-ethoxycoumarin de-ethylase activity; the magnitude of the phenobarbitone-mediated induction response was comparable with the response in chick embryos *in ovo* (Table 1). Control ALA synthase activity was higher in hepatocyte culture than *in ovo* and was promptly induced after exposure to phenobarbitone in both systems. After phenobarbitone addition there was no initial decrease in hepatic cytochrome *P*-450 concentration, in contrast with the well known destructive effect of allylisopropylacetamide on cytochrome *P*-450 in chick embryos *in ovo* (Table 2). The addition of 7–66 mM-acetate to hepatocyte cultures diminished the phenobarbitone-mediated induction of cytochrome *P*-450 (Fig. 1). The inhibitory effect of acetate was dose-dependent. By contrast, induction of ALA synthase by phenobarbitone in the same monolayers was markedly enhanced in the presence of 44 and 66 mM-acetate (Fig. 1). Thus phenobarbitone-mediated induction of ALA synthase and cytochrome *P*-450 were clearly dissociated after acetate treatment. Because these results in cultured hepatocytes were contrary to the findings reported in rats *in vivo* (Piper & Tephly, 1974), we studied the effects of

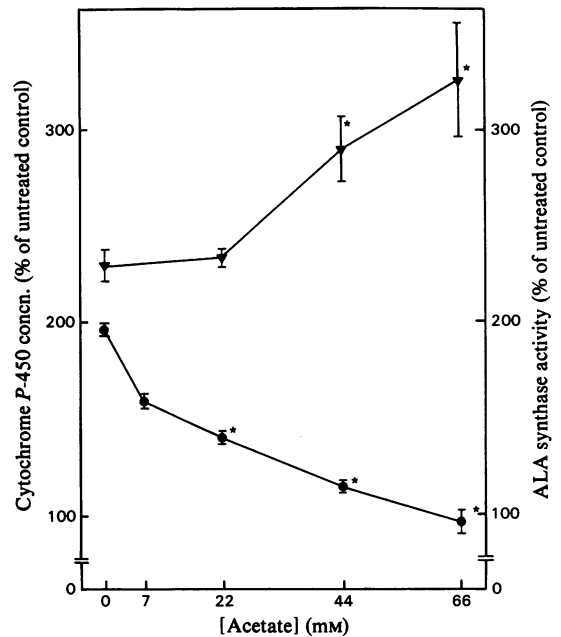


Fig. 1. Effect of acetate on phenobarbitone-mediated induction of cytochrome *P*-450 and ALA synthase in cultured chick embryo hepatocytes

Chick embryo hepatocyte cultures were exposed to various concentrations of acetate over the entire 44 h culture period and to 0.4 mM-phenobarbitone for the last 16 h of culture; controls were treated with solvent. Cytochrome *P*-450 concentration (●) and ALA synthase activity (▼) were measured in microsomes and in homogenates respectively. The results are expressed as percentages of the untreated controls. The absolute values of control and phenobarbitone-induced cytochrome *P*-450 concentration and ALA synthase activity are shown in Table 1. Each point represents the mean \pm S.E.M. for three to six experiments. * $P < 0.001$, compared with the phenobarbitone-treated group cultured in the absence of acetate (paired Student's *t* test).

Table 2. Early time course of phenobarbitone- and allylisopropylacetamide-mediated induction of cytochrome *P*-450 in chick embryos *in ovo*

Phenobarbitone (5 mg) or allylisopropylacetamide (5 mg) was injected into the fluid surrounding the 18-day chick embryo. Controls received 0.1 ml of water. After 1, 2, 3 and 4 h microsomes from two to three pooled chick embryo livers were prepared and cytochrome *P*-450 concentration was determined. Results are means \pm S.E.M. of three experiments for the control and phenobarbitone-treated group. Cytochrome *P*-450 values after allylisopropylacetamide are derived from a single experiment. * $P < 0.01$ compared with controls (paired Student's *t* test).

Time (h)	Cytochrome <i>P</i> -450 concn. (pmol/mg of protein)		
	Control	Phenobarbitone	Allylisopropylacetamide
1	241 \pm 15	254 \pm 27	141
2	243 \pm 2	275 \pm 17	180
3	238 \pm 6	267 \pm 37	184
4	254 \pm 12	320 \pm 12*	236

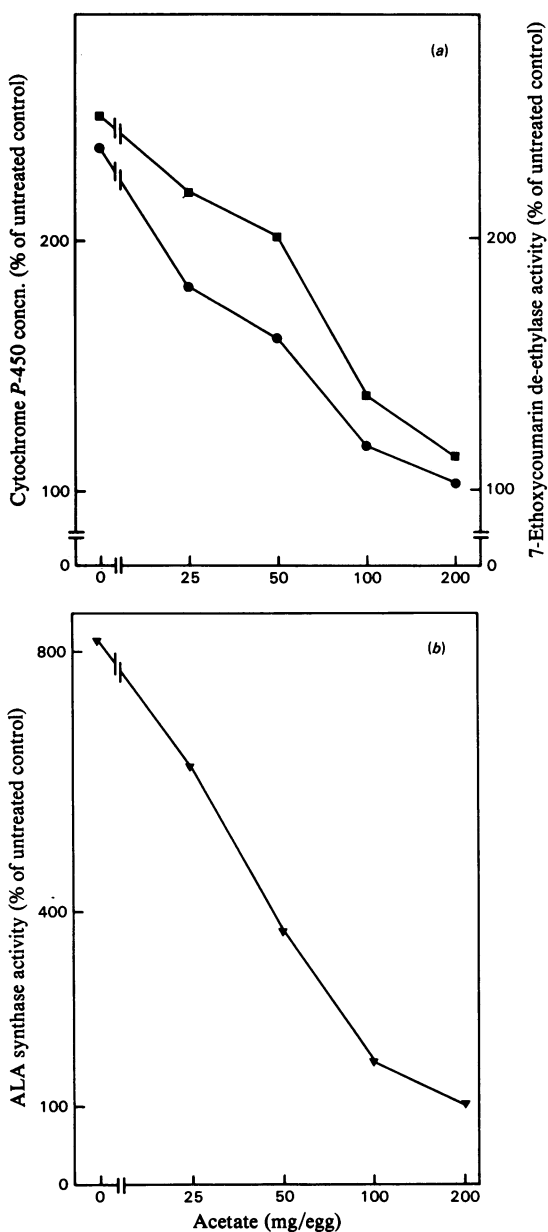


Fig. 2. Effect of acetate on phenobarbitone-mediated induction of cytochrome P-450, 7-ethoxycoumarin de-ethylase and ALA synthase in chick embryos *in ovo*. Chick embryos received two injections of acetate 44 and 16h before killing on day 17. The dose of acetate shown in the Figure represents the sum of the two injections. Simultaneously with the second dose of acetate, 5 mg of phenobarbitone was injected. Cytochrome P-450 concentration (●) and 7-ethoxycoumarin de-ethylase activity (■) were measured in microsomes (Fig. 2a) and ALA synthase activity (▼) was determined in homogenates (Fig. 2b). The results are expressed as percentages of the values in the untreated control. The absolute values of control and phenobarbitone-

acetate on cytochrome P-450 and ALA synthase induction by phenobarbitone *in ovo*. A total dose of 100mg of acetate per egg, comparable with the concentration used in the studies with rats, completely blocked the induction response of cytochrome P-450 by phenobarbitone. The effect of acetate on cytochrome P-450 induction was dose-dependent and was closely paralleled by diminished 7-ethoxycoumarin de-ethylase activity (Fig. 2a). However, in contrast with the findings in hepatocyte culture, induction of ALA synthase *in ovo* was impaired by acetate (Fig. 2b). Uninduced levels of ALA synthase and cytochrome P-450 were not altered by acetate in chick embryos *in ovo* and in cultured hepatocytes (results not shown).

Acetate may compete for CoA with succinate, a substrate of ALA synthase. Decreased availability

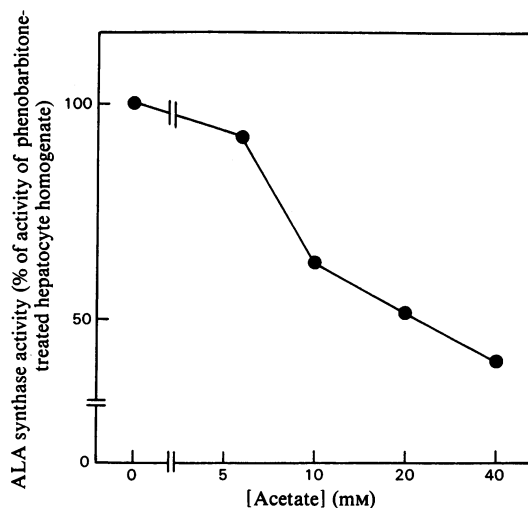


Fig. 3. Effect *in vitro* of acetate on ALA synthase activity in homogenate from cultured chick embryo hepatocytes treated with phenobarbitone

Homogenates from phenobarbitone-treated cultured chick embryo hepatocytes were prepared and ALA synthase activity was determined. Increasing amounts of acetate were added to the incubation mixture in the ALA synthase assay. Results are expressed as the percentages of ALA synthase activity in the absence of acetate. Each point represents the mean of two to four independent determinations. The mean value of ALA synthase activity in the absence of acetate in homogenate from phenobarbitone-treated cultured chick embryo hepatocytes was 780 pmol of ALA/mg of protein per 30 min.

induced cytochrome P-450 concentration and ALA synthase activity are shown in Table 1. Each point represents the mean of two to seven experiments.

of succinyl-CoA in hepatocyte culture would not be detected in the assay of ALA synthase in homogenates, where acetate is no longer present and an excess of succinate, CoA, GTP and succinyl thiokinase assure constant formation of succinyl-CoA. When acetate was added to the incubation mixture for assay of ALA synthase activity containing homogenate from phenobarbitone-treated cultured hepatocytes, a progressive inhibition of ALA formation was observed (Fig. 3). These data suggest that acetate in culture inhibits ALA production and haem formation, thereby derepressing ALA

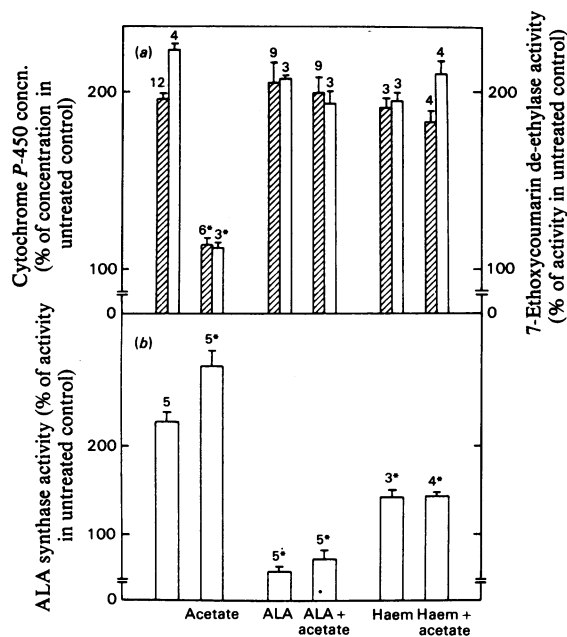


Fig. 4. Effect of haem, ALA and acetate on phenobarbitone-mediated induction of cytochrome P-450, 7-ethoxycoumarin de-ethylase and ALA synthase in cultured chick embryo hepatocytes

Monolayers were exposed to phenobarbitone (0.4 mM) for the last 16 h of incubation. Where indicated, haem (3 μ M) or ALA (10 μ M) was administered 2 h before and 4 h after the addition of phenobarbitone; acetate was present from the beginning of culture. Cytochrome P-450 concentration (hatched bars) and 7-ethoxycoumarin de-ethylase activity (open bars) were measured in microsomes (a) and ALA synthase activity was determined in homogenates (b). The results are expressed as percentages of those in untreated controls. The absolute values for control and phenobarbitone-induced cultures are shown in Table 1. Each bar represents the mean \pm S.E.M. for the numbers of experiments indicated above each bar. * $P < 0.01$, compared with the phenobarbitone-treated group in the absence of acetate, ALA and haem (paired Student's *t* test).

synthase. *In ovo*, acetate does not enhance ALA synthase induction (Fig. 2b) as will be discussed below.

Haem or its precursor ALA, added to the culture medium or injected into the fluid surrounding the chick embryo, markedly impaired the phenobarbitone-mediated induction of ALA synthase (Figs. 4–6). In cultured hepatocytes, phenobarbitone alone caused an increase in ALA synthase to 229% of controls. Treatment with haem decreased this induction response to 144%. The inhibitory effect of haem was even more pronounced in chick embryos *in ovo* (Fig. 5). Addition of ALA

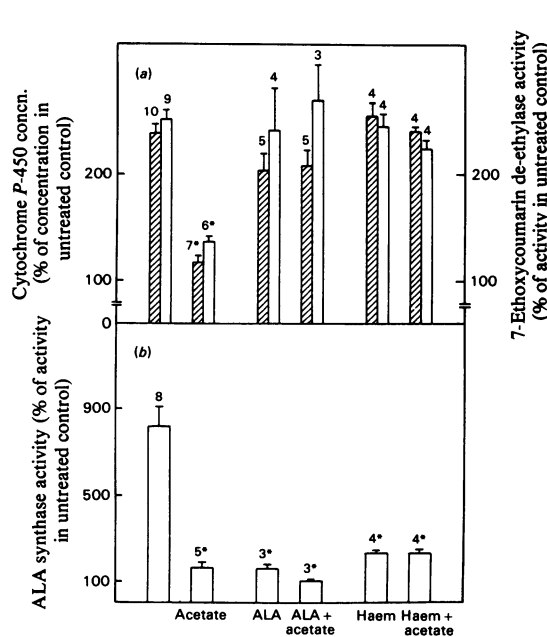


Fig. 5. Effect of haem, ALA and acetate on phenobarbitone-mediated induction of cytochrome P-450, 7-ethoxycoumarin de-ethylase and ALA synthase in chick embryos *in ovo*.

Eggs were injected with phenobarbitone (5 mg) on day 16 of embryonic development 16 h before killing. Controls were injected with solvent. Acetate (50 mg) was administered twice, 44 h and 16 h before killing. Haem (280 μ g) or ALA (750 μ g) was injected 2 h before and 4 h after the administration of phenobarbitone, cytochrome P-450 concentration (hatched bars) and 7-ethoxycoumarin de-ethylase activity (open bars) were measured in microsomes (a) and ALA synthase activity was determined in homogenates (b). The results are expressed as a percentage of untreated controls. The absolute values for control and phenobarbitone-induced groups are shown in Table 1. Each bar represents the mean \pm S.E.M. for the numbers of experiments indicated above each bar. * $P < 0.01$, compared with the phenobarbitone-treated group in the absence of acetate, ALA and haem (paired Student's *t* test).

to the culture medium or injection of ALA *in ovo* completely abolished the induction response. In spite of the impaired increase in ALA synthase activity, both *in ovo* and in culture, phenobarbitone-mediated induction of cytochrome *P*-450 was not or only minimally affected by haem and ALA. Moreover, ALA and haem did neither enhance nor diminish the increase in 7-ethoxycoumarin de-ethylase activity. These effects of haem were observed *in ovo* over a 20h induction period (Fig. 6).

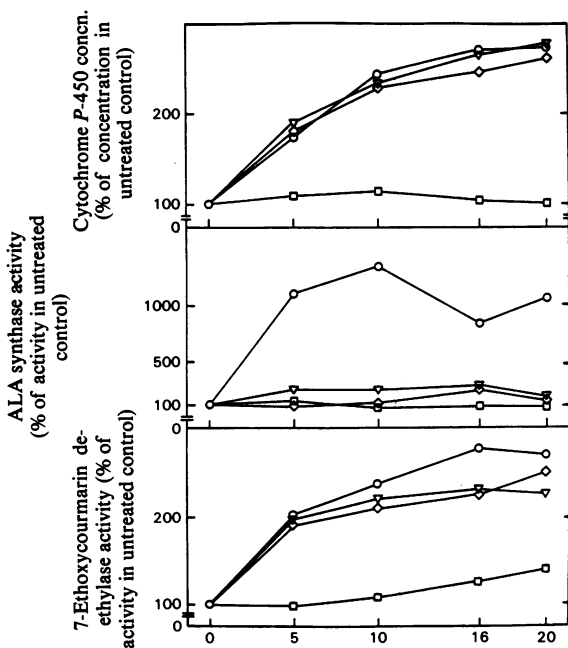


Fig. 6. Time course of the effect of haem and acetate on phenobarbitone-mediated induction of cytochrome *P*-450, ALA synthase and 7-ethoxycoumarin de-ethylase in chick embryos *in ovo*

All chick embryos were treated with phenobarbitone (5 mg) on day 16 of embryonic development (○), except the control group. Acetate (50 mg) was injected twice 24 h before and simultaneously with phenobarbitone (□); haem (280 μg) was administered 2 h before and 4 h after phenobarbitone treatment (▽) and one group of chick embryos was exposed to both acetate and haem (◇). Homogenates and microsomes from two to three pooled livers per group were prepared and cytochrome *P*-450 concentration, ALA synthase and 7-ethoxycoumarin de-ethylase activity were determined. The results are expressed as percentages of untreated controls, which received only the solvents. Mean values ± S.E.M. of the untreated controls for all time points were: 249 ± 7 pmol of cytochrome *P*-450/mg of protein, 227 ± 15 pmol of ALA/mg of protein per 30 min, 805 ± 11 pmol of hydroxycoumarin/mg of protein per min.

Both haem and ALA almost completely reversed the inhibitory effect of acetate on cytochrome *P*-450 induction *in ovo* and in culture (Figs. 4–6). In culture, the enhanced phenobarbitone-mediated induction of ALA synthase caused by acetate was markedly diminished by the addition of exogenous haem or ALA (Fig. 4). The effects on cytochrome *P*-450 induction *in ovo* and in culture were again paralleled by changes in 7-ethoxycoumarin de-ethylase activity. These effects of haem and ALA were not due to shifts in the time course of the induction (Fig. 6). The inhibitory effect of acetate on induction of cytochrome *P*-450 was prevented by haem and ALA already at early time points and persisted for the whole observation period. If haem or ALA was present only for the last 12 h of culture, there was still a partial reversal of the effect of acetate on cytochrome *P*-450 induction. In chick embryos *in ovo* not exposed to phenobarbitone, treatment with haem or ALA and in combination with acetate did not significantly affect basal concentrations of cytochrome *P*-450 and activities of ALA synthase (results not shown).

Discussion

The present data suggest that during induction of microsomal cytochrome *P*-450 by phenobarbitone, apo-(cytochrome *P*-450) synthesis proceeds independently of haem biosynthesis. This concept is derived from the following observations in chick embryos *in ovo* and in cultured chick embryo hepatocytes. (1) Exogenous haem and haem formed from exogenous ALA do not affect the phenobarbitone-mediated induction of cytochrome *P*-450. (2) Acetate inhibits the phenobarbitone-mediated enhancement of haem biosynthesis at the level of ALA formation. (3) Exogenous haem and ALA reverse the inhibitory effect of acetate on cytochrome *P*-450 induction.

To investigate the role of haem availability for cytochrome *P*-450 synthesis, intracellular haem concentration was increased by the administration of exogenous haem or its precursor ALA. Neither control nor phenobarbitone-induced cytochrome *P*-450 concentrations were increased by enhanced haem availability in chick embryos *in ovo* or in cultured chick embryo hepatocytes. These data are in accordance with studies in rats *in vivo* (De Matteis & Gibbs, 1972; Druyan & Kelly, 1972; Bhat *et al.*, 1977) and do not support a rate-limiting role of haem synthesis for basal or induced cytochrome *P*-450 synthesis nor the presence in the hepatocyte of sizeable amounts of reconstitutable free apocytochrome (Correia & Meyer, 1975; Negishi & Kreibich, 1978).

Cultured adult rat hepatocytes appear to be different in this regard. A rapid initial decrease in cytochrome *P*-450 concentration is partially preven-

ted by the addition of ALA to the medium (Guzelian & Bissell, 1976; Decad *et al.*, 1977; Paine & Hockin, 1981). The decrease in cytochrome *P*-450 may reflect preferential destruction of the haem moiety of this haemoprotein, because partial reversal of this decrease by exogenous ALA suggests that haem formed from ALA is incorporated into remaining free apocytochrome *P*-450.

In chick embryos *in ovo* and in cultured chick embryo hepatocytes exogenous haem and ALA partially or completely prevented the induction of ALA synthase (Fig. 4) as previously described by Granick (1966), Tyrrell & Marks (1972), Strand *et al.* (1972), Tomita *et al.* (1974), Granick *et al.* (1975) and Srivastava *et al.* (1980). ALA apparently has to be transformed into haem to repress the induction of ALA synthase (Granick *et al.*, 1975; Srivastava *et al.*, 1980), and this haem from exogenous ALA is incorporated into haemoproteins (Levin & Kuntzman, 1969; Druyan & Kelly, 1972). Our observation that the marked repression of ALA synthase by exogenous haem did not interfere with cytochrome *P*-450 induction indicates that exogenous haem and haem generated from exogenous ALA was incorporated into newly formed apocytochrome *P*-450, overriding the block of ALA production.

Decreased availability of haem for cytochrome *P*-450 synthesis was experimentally produced by inhibition of haem formation. Acetate inhibited the phenobarbitone-mediated increase of haem biosynthesis and markedly lowered or prevented induction of cytochrome *P*-450 (Figs. 1–3). Haem or ALA administration to acetate-treated chick embryos and hepatocyte cultures reversed the effect of acetate and fully restored the cytochrome *P*-450 induction response (Figs. 4–6). These results strongly suggest that inhibition of haem formation by acetate did not impair apocytochrome *P*-450 synthesis and that exogenous haem was incorporated into newly formed apo-cytochrome *P*-450. The increase in cytochrome *P*-450 concentration was paralleled by enhanced 7-ethoxycoumarin de-ethylase activity, a cytochrome *P*-450-dependent mixed-function oxidase activity, indicating functional reconstitution. In previous attempts to incorporate exogenous haem into cytochrome *P*-450 by this and other laboratories, haem was incorporated into cytochrome *P*-450 only if prosthetic haem had previously been removed or destroyed by allylisopropylacetamide (Bhat *et al.*, 1977; Correia *et al.*, 1979; Farrell & Correia, 1980) or CoCl_2 (Correia & Meyer, 1975; Meier *et al.*, 1978; Sinclair *et al.*, 1980). On the other hand, unlike the findings in the present study in chick embryos, haem administration to rats without concomitant inhibition of haem synthesis or destruction of prosthetic haem decreased concentration and prevented phenobarbitone-mediated induction of

cytochrome *P*-450 (Marver *et al.*, 1968; Marver, 1969; Rajamanickam *et al.*, 1972; Dehlinger & Schimke, 1972; Bhat *et al.*, 1977). Moreover, the inhibitory effect of haem was not restricted to cytochrome *P*-450, but apparently included decreased protein and phospholipid synthesis (Marver, 1969). It is possible, therefore, that under the conditions used in rats exogenous haem exerted generalized toxicity (Maines & Kappas, 1975).

Acetate inhibits haem biosynthesis probably at the level of ALA formation. ALA reversed the effects of acetate on phenobarbitone-mediated induction of cytochrome *P*-450. Moreover, *in vitro*, acetate inhibited ALA formation (Fig. 3) and this effect was partially reversed by increased concentrations of succinate (Piper & Tephly, 1974). Thus the most likely mechanism appears to be competition with succinate for CoA. The same competition may operate in culture and prevent ALA formation. The ensuing haem depletion could lead to increased induction of ALA synthase (Fig. 1). This concept is further underlined by the observation that enhanced ALA synthase induction by acetate was completely counteracted by exogenous ALA (Fig. 4). *In ovo*, there was no such enhanced phenobarbitone-mediated induction by acetate, but a dose-dependent decrease in ALA synthase activity was observed (Fig. 2*b*), as previously described in rats by Piper & Tephly (1974). This suggests additional and presently unknown effects of acetate in intact animals on ALA synthase induction.

Phenobarbitone produced a prompt and sustained increase in ALA synthase activity and cytochrome *P*-450 concentration (Fig. 6) without measurable initial destruction of the haem moiety of cytochrome *P*-450 (Table 2). Initial destruction of the haem moiety of cytochrome *P*-450 therefore appears unlikely to be a prerequisite for induction of hepatic ALA synthase by phenobarbitone (Lim *et al.*, 1980). Our data rather support the concept that phenobarbitone depletes an intracellular haem pool by inducing apo-cytochrome *P*-450. Newly formed apo-cytochrome *P*-450 through its haem-binding capacity may divert haem from the pool regulating ALA synthase and thereby induce ALA synthase. Data from experiments in rats also favour this mechanism as ALA synthase is induced by phenobarbitone without an initial decrease in cytochrome *P*-450 (Marver, 1969; Meyer & Marver, 1971; Satyanarayana & Padmanaban, 1973). In contrast, allylisopropylacetamide induces ALA synthase by a different mechanism. Allylisopropylacetamide has the unique property of rapidly decreasing cytochrome *P*-450 concentrations (Table 2; De Matteis, 1971; Meyer & Marver, 1971; Levin *et al.*, 1973; Krupa *et al.*, 1974) by covalently binding to the prosthetic haem (Ortiz de Montellano *et al.*, 1979) causing destruction of haem. This haem

depletion potentiates the induction of ALA synthase, as previously suggested (De Matteis, 1971, 1973; Meyer & Marver, 1971).

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