

## Haem Control in Experimental Porphyria

### THE EFFECT OF HAEMIN ON THE INDUCTION OF $\delta$ -AMINOLAEVULINATE SYNTHASE IN ISOLATED CHICK-EMBRYO LIVER CELLS

Gopesh SRIVASTAVA, John D. BROOKER, Brian K. MAY and William H. ELLIOTT  
*Department of Biochemistry, The University of Adelaide, Adelaide, South Australia 5001, Australia*

(Received 1 October 1979)

2-Allyl-2-isopropylacetamide-mediated induction of hepatic porphyria was studied in isolated chick-embryo liver cells. Increased  $\delta$ -aminolaevulinate synthase activity occurred within 1 h of induction and continued to increase for 8 h. Protoporphyrins synthesized during this time accumulated to a concentration 10-fold greater than that in the control. Removal of 2-allyl-2-isopropylacetamide from the cells by washing at 3 h immediately inhibited further increases in  $\delta$ -aminolaevulinate synthase synthesis. However substitution of 2-allyl-2-isopropylacetamide at 3 h by deferoxamine methane-sulphonate, an inhibitor of haem synthesis, allowed continued  $\delta$ -aminolaevulinate synthase induction at an unaltered rate, even though this agent did not, by itself, induce enzyme synthesis. Exogenously added haemin was shown completely to inhibit 2-allyl-2-isopropylacetamide-mediated  $\delta$ -aminolaevulinate synthase induction at concentrations as low as 20 nM, a value that is less than the reported physiological one. The duration of inhibition was dependent on the concentration of added haemin and was followed by a period of  $\delta$ -aminolaevulinate synthase synthesis at a rate similar to that of the control. These data are consistent with the hypothesis that  $\delta$ -aminolaevulinate synthase synthesis is regulated by the concentration of intracellular haem and that induction is initiated by 2-allyl-2-isopropylacetamide-mediated destruction of haem. Induction of  $\delta$ -aminolaevulinate synthase was shown to be dependent on both RNA and protein synthesis, and a study of the comparative effects of cordycepin, cycloheximide and haem has shown that, at haemin concentrations up to 50 nM, the inhibition of  $\delta$ -aminolaevulinate synthase synthesis followed kinetics similar to the effect of cordycepin, with no synergism between cordycepin and 50 nM-haemin. However, at a haemin concentration of 2  $\mu$ M, the inhibition of  $\delta$ -aminolaevulinate synthase synthesis followed similar kinetics to the effect of cycloheximide. These data demonstrate the control of  $\delta$ -aminolaevulinate synthase synthesis by low concentrations of haemin and suggests that the primary effect of haemin is at the level of transcription.

The biosynthesis of hepatic haemoproteins is coupled to the intracellular concentration of haem, which is controlled by the activity of the haem biosynthetic pathway. This pathway is tightly regulated, and one of the major rate-controlling enzymes is  $\delta$ -aminolaevulinate synthase [succinyl-CoA-glycine C-succinyltransferase (decarboxylating) EC 2.3.1.37]. The intracellular concentration of this enzyme is normally low (Granick & Urata, 1963), but a wide variety of xenobiotics and steroids

are known to increase markedly its activity in mammalian (De Matteis, 1971) and avian liver cells (Granick, 1963; Granick *et al.*, 1975; Sassa & Kappas, 1977; Morgan *et al.*, 1977; Tomita *et al.*, 1974), and to cause the synthesis of high concentrations of porphyrins (Granick, 1966). This experimental porphyria in its biochemistry resembles human acute intermittent porphyria (Granick, 1966), and a number of chemical inducers are known to precipitate attacks of the disease in susceptible individuals (Granick & Urata, 1963).

Immunological studies have shown that, in experimentally induced porphyria, the synthesis of  $\delta$ -

Abbreviation used: Bt<sub>2</sub>cAMP, *N*<sup>6</sup>*O*<sup>2</sup>-dibutyryladenosine 3':5'-cyclic monophosphate.

aminolaevulinate synthase occurs *de novo* (Whiting & Granick, 1976) and haem, the final product of the pathway, inhibits enzyme induction (Granick, 1966; Tomita *et al.*, 1974; Tyrrell & Marks, 1972; Sassa & Kappas, 1977). Although there is general agreement that haem plays an important role in the physiological control of  $\delta$ -aminolaevulinate synthase activity (Granick, 1966; Tomita *et al.*, 1974; Tyrrell & Marks, 1972; Ohashi & Kikuchi, 1972), there is no such consensus on the mechanism of its action, and many different schemes have been suggested. Granick (1966) originally proposed that haem acts as a co-repressor for  $\delta$ -aminolaevulinate synthase transcription; chemical inducers were regarded as haem analogues that compete for the apo-(repressor protein), thus preventing haem from repressing  $\delta$ -aminolaevulinate synthase mRNA transcription. However, later studies by Sassa & Granick (1970) and Tyrrell & Marks (1972) suggested that, contrary to this transcriptional theory, haem exerted its effect at a post-transcriptional stage. The finding (Granick *et al.*, 1975; Sassa & Granick, 1970) that there was apparently no concentration-dependent competition between haem and porphyria-inducing chemicals further argued against the original concept of Granick (1966). Ohashi & Kikuchi (1972) and Hayashi *et al.* (1972) have proposed that, in adult chickens, haem exerts its effect by controlling transport of cytoplasmically synthesized  $\delta$ -aminolaevulinate synthase into the mitochondria. However, haem does not affect  $\delta$ -aminolaevulinate synthase transport in embryonic chick liver (Tomita *et al.*, 1974; Tyrrell & Marks, 1972; Sassa & Granick, 1970).

In the present study we have re-examined the effects of haem on  $\delta$ -aminolaevulinate synthase induction by using a newly developed system of isolated chick-embryo liver cells (Srivastava *et al.*, 1979). In this system we have shown that initiation of  $\delta$ -aminolaevulinate synthase induction requires the presence of a chemical inducer, in this case 2-allyl-2-isopropylacetamide, but that continued presence of this inducer is not necessary provided that haem synthesis is prevented. This is compatible with a scheme in which 2-allyl-2-isopropylacetamide causes a depletion of intracellular haem, and in which haem is the principal controlling agent in the induction of  $\delta$ -aminolaevulinate synthase. Furthermore, our data conflict with those of Tyrrell & Marks (1972) and show that, at a concentration of 20 nM, haemin will completely inhibit  $\delta$ -aminolaevulinate synthase induction and that this effect is primarily at the level of transcription.

## Experimental

### Materials

Eagle's basal medium was purchased from Com-

monwealth Serum Laboratories, Melbourne, Vic., Australia; deferoxamine methane sulphonate was a gift from Ciba-Geigy Australia Ltd., N.S.W., Australia; 2-allyl-2-isopropylacetamide was a gift from Hoffmann-La Roche, Nutley, NJ, U.S.A.; cycloheximide, cordycepin and haemin were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; chick embryos were obtained from the Parafield Poultry Farm, Adelaide, South Australia, Australia.

### Preparation of cell suspension

An isolated liver-cell suspension from 17-day chick embryos was prepared as described previously (Srivastava *et al.*, 1979), and the cells were incubated on a gyrotary shaker (150 cycles/min) in Eagle's basal medium containing 50  $\mu$ M-Bt<sub>2</sub>cAMP, 1  $\mu$ g of insulin/ml and 0.05  $\mu$ g of cortisol/ml.

### Cell-washing procedure

After incubation of the cells for the appropriate time, portions were removed and centrifuged at 175 g for 3 min. The cell pellet was quickly rinsed with 1 ml of fresh medium and then carefully resuspended in the original volume of medium containing either 2-allyl-2-isopropylacetamide, deferoxamine methanesulphonate or nothing, as appropriate. The cells were incubated at 37°C on a gyrotary shaker (150 cycles/min) for 10 min and then centrifuged at 175 g for 3 min. The washing procedure was repeated twice more to ensure complete removal of the inducing drug (2-allyl-2-isopropylacetamide).

### $\delta$ -Aminolaevulinate synthase assay

$\delta$ -Aminolaevulinate synthase activity from sonicated mitochondria was determined by the colorimetric method of Poland & Glover (1973), except that assay mixtures also contained 30 mM-sodium laevulinate. Total protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

### Protoporphyrin determination

Total intracellular protoporphyrin was measured by the spectrophotofluorometric assay of Granick *et al.* (1975).

## Results

Isolated chick-embryo liver cells were prepared as described previously (Srivastava *et al.*, 1979) and incubated in Eagle's basal medium supplemented with Bt<sub>2</sub>cAMP, insulin and cortisol. Addition of 2-allyl-2-isopropylacetamide to these cells caused a marked increase in  $\delta$ -aminolaevulinate synthase activity and the accumulation of protoporphyrin (Table 1). Increased concentrations of protoporphyrin occur because the activity of ferrochelatase, which catalyses the incorporation of Fe<sup>2+</sup> into haem,

Table 1. Inhibition of synthesis of  $\delta$ -aminolaevulinate synthase by exogenously added  $\delta$ -aminolaevulinate and protoporphyrin

Isolated chick-embryo liver cells were incubated in Eagle's basal medium supplemented with Bt<sub>2</sub>cAMP (50  $\mu$ M), insulin (1  $\mu$ g/ml) and cortisol (0.05  $\mu$ g/ml). Further additions were made at 0h. After 8h incubation the cells were sonicated, mitochondria isolated and the  $\delta$ -aminolaevulinate synthase activity determined. Protoporphyrin was determined spectrophotofluorometrically after 10h of incubation. Results are means  $\pm$  s.e.m. of four separate determinations. Abbreviations used: DES, deferoxamine methanesulphonate; AIA, allylisopropylacetamide.

Additions ( $\mu$ g/ml, except where shown otherwise)	$\delta$ -Aminolaevulinate synthase (nmol of $\delta$ -aminolaevulinate/h per mg of protein)	Protoporphyrin (pmol/10h per mg of protein)
None	0.10 $\pm$ 0.02	6.0 $\pm$ 0.80
DES (500)	0.10 $\pm$ 0.02	16.8 $\pm$ 0.86
AIA (500)	7.50 $\pm$ 0.87	115.1 $\pm$ 1.87
AIA (500) + DES (500)	9.40 $\pm$ 0.91	146.7 $\pm$ 2.03
AIA (500) + ALA (50 $\mu$ M)	2.73 $\pm$ 0.09	—
AIA (500) + ALA (50 $\mu$ M) + DES (500)	9.01 $\pm$ 0.92	—
AIA (500) + protoporphyrin (1 $\mu$ M)	1.84 $\pm$ 0.24	—
AIA (500) + protoporphyrin (1 $\mu$ M) + DES (500)	8.73 $\pm$ 0.83	—

becomes rate-limiting. However, haem synthesis does occur, but this can be inhibited by addition of an iron chelator, deferoxamine methanesulphonate (Granick *et al.*, 1975; Sassa & Granick, 1970). Addition of this agent to an 2-allyl-2-isopropylacetamide-induced chick-embryo-liver-cell system caused a synergistic increase in  $\delta$ -aminolaevulinate synthase activity (Table 1), even when 2-allyl-2-isopropylacetamide was used at its maximum effective concentration. Separate studies with these cells have shown that up to 80% of added 2-allyl-2-isopropylacetamide is metabolized within 10h as determined by paper chromatography. However, deferoxamine methanesulphonate alone did not induce  $\delta$ -aminolaevulinate synthase synthesis.

Addition of excess  $\delta$ -aminolaevulinate or protoporphyrin to 2-allyl-2-isopropylacetamide-induced cells resulted in marked inhibition of drug-induced  $\delta$ -aminolaevulinate synthase synthesis. This inhibition was completely prevented by deferoxamine methanesulphonate. Sassa & Granick (1970) have shown that deferoxamine methanesulphonate inhibits ferrochelatase activity, thereby blocking the conversion of protoporphyrin into haem. To confirm that this was the site of action of the chelator we studied protoporphyrin accumulation in chick-embryo liver cells after induction. Addition of 2-allyl-2-isopropylacetamide to the isolated cells increased protoporphyrin accumulation, which was further enhanced by the addition of deferoxamine methanesulphonate (Table 1). Deferoxamine methanesulphonate alone caused a 2-fold increase in protoporphyrin concentrations, but did not induce  $\delta$ -aminolaevulinate synthase synthesis. These results confirm the data of Sassa & Granick (1970) and suggest that induction of  $\delta$ -aminolaevulinate synthase activity is regulated by the intracellular

concentration of haem. Unsel & De Matteis (1978) have shown that 2-allyl-2-isopropylacetamide interacts with, and degrades, cytochrome *P*-450-associated haem, thereby decreasing the concentration of intracellular haem. However, it is not known whether this reaction plays a primary role in initiating  $\delta$ -aminolaevulinate synthase synthesis.

If induction of  $\delta$ -aminolaevulinate synthase synthesis is a direct consequence of haem depletion, then the continued presence of 2-allyl-2-isopropylacetamide should be unnecessary after this depletion, provided that regeneration of intracellular haem is prevented. To test this hypothesis we incubated isolated chick-embryo liver cells with 2-allyl-2-isopropylacetamide for up to 3h then washed away the 2-allyl-2-isopropylacetamide and continued the incubation with added deferoxamine methanesulphonate. Fig. 1(a) shows that, under these conditions, deferoxamine methanesulphonate alone promoted full enzyme induction for at least a further 5h. In the control after removal of 2-allyl-2-isopropylacetamide and in the absence of added deferoxamine methanesulphonate or 2-allyl-2-isopropylacetamide, enzyme induction ceased after 1h. Similar results were obtained by measuring protoporphyrin accumulation (Fig. 1b). There was no apparent synergism between deferoxamine methanesulphonate and 2-allyl-2-isopropylacetamide in this experiment.

The effect of increasing concentrations of haemin on  $\delta$ -aminolaevulinate synthase induction was examined. Enzyme activity was measured 8h after induction by 2-allyl-2-isopropylacetamide and deferoxamine methanesulphonate. The results in Fig. 2 show an unusual biphasic effect. Concentrations of haemin between 20 and 50nM progressively inhibited  $\delta$ -aminolaevulinate synthase synthesis to approx.

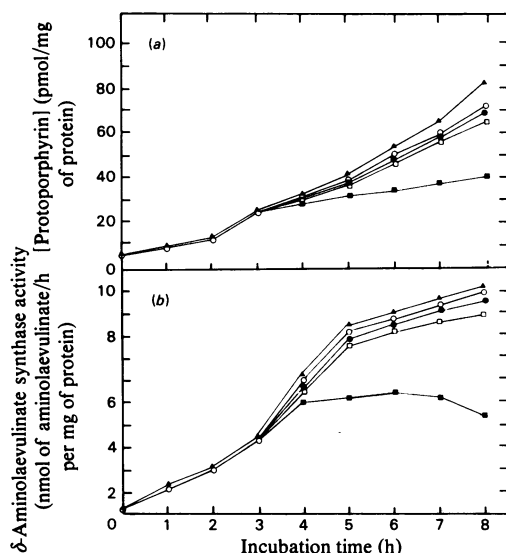


Fig. 1. Time course of  $\delta$ -aminolaevulinate synthase induction in the presence and absence of deferoxamine methanesulphonate

Isolated chick-embryo liver cells were incubated in regular media (see Table 1) supplemented with 2-allyl-2-isopropylacetamide (500  $\mu$ g/ml) for 3 h, washed with fresh media as described in the Experimental section and reincubated in fresh media containing the following additions: 2-allyl-2-isopropylacetamide (500  $\mu$ g/ml) and deferoxamine methanesulphonate (500  $\mu$ g/ml) ( $\circ$ ); 2-allyl-2-isopropylacetamide (500  $\mu$ g/ml) ( $\bullet$ ); deferoxamine methanesulphonate (500  $\mu$ g/ml) ( $\square$ ); no additions ( $\blacksquare$ ); and control without washing ( $\blacktriangle$ ). Intracellular protoporphyrin (a) and mitochondrial  $\delta$ -aminolaevulinate synthase activity (b) were determined after various incubation times. Values are the means for four separate experiments.

50%, but concentrations greater than 200 nM were required for any additional inhibition. Complete inhibition of enzyme synthesis was observed at a haemin concentration greater than 1  $\mu$ M. Separate experiments showed  $\delta$ -aminolaevulinate synthase activity measured in sonicated liver mitochondrial extracts of 2-allyl-2-isopropylacetamide-induced chick embryos was not inhibited by haem concentrations of up to 2  $\mu$ M. Furthermore, an  $\delta$ -aminolaevulinate synthase assay was not affected by addition of postmitochondrial supernatant extract from 2-allyl-2-isopropylacetamide-induced cells pre-treated with 2  $\mu$ M-haemin, demonstrating that the enzyme was not inhibited by a haem metabolite. This suggests therefore that haem is not a feedback inhibitor of  $\delta$ -aminolaevulinate synthase activity in our system, a conclusion in agreement with that of

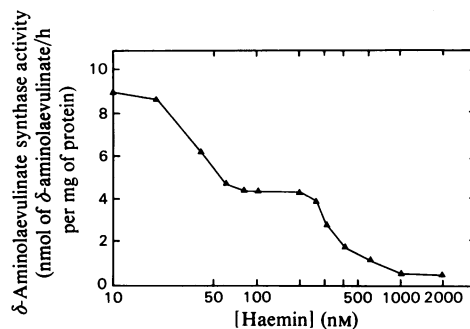


Fig. 2. Effect of exogenous haemin on  $\delta$ -aminolaevulinate synthase activity

Isolated chick-embryo liver cells were incubated in regular media supplemented at 0 h with 2-allyl-2-isopropylacetamide (500  $\mu$ g/ml), deferoxamine methanesulphonate (500  $\mu$ g/ml) and various concentrations of haemin dissolved in 0.2 M-KOH/ethanol (1:1, v/v). After 8 h incubation, mitochondrial  $\delta$ -aminolaevulinate synthase activity was determined. Values are the means of six separate determinations.

Whiting & Granick (1976) and Wolfson *et al.* (1979).

To determine the time course of haem inhibition, the effect of various concentrations of haemin on  $\delta$ -aminolaevulinate synthase synthesis was determined over an 8 h incubation period. The results (Fig. 3a) show that, even at the lowest concentration (20 nM), haemin caused a complete inhibition of  $\delta$ -aminolaevulinate synthase induction for 2 h, after which time reversal of the inhibition occurred. The period of complete inhibition of enzyme synthesis increased with increasing haemin concentrations up to 50 nM. Between 50 and 200 nM haemin, the effect on  $\delta$ -aminolaevulinate synthase synthesis was unchanged, compatible with the plateau observed in Fig. 2. At a haemin concentration of 2  $\mu$ M, enzyme synthesis was completely inhibited over the 8 h incubation period (Fig. 3b). However, concentrations of haemin up to 5  $\mu$ M did not affect general protein synthesis as determined by incorporation of L-[4,5- $^3$ H]leucine into trichloroacetic acid-insoluble protein (results not shown).

Addition of various concentrations of haemin 2 h after 2-allyl-2-isopropylacetamide induction (Fig. 3b) shows the same effect as Fig. 3(a). There was an initial complete inhibition of  $\delta$ -aminolaevulinate synthase synthesis followed by a haemin-concentration-dependent recovery. Haemin at a concentration of 2  $\mu$ M was completely inhibitory over the whole incubation period. These results suggest that the isolated chick-embryo liver cells are extremely

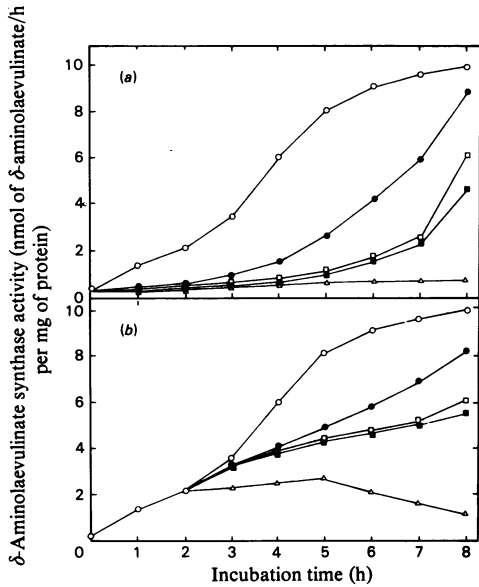


Fig. 3. Time course of  $\delta$ -aminolaevulinate synthase induction in the presence of various concentrations of haemin

Isolated chick-embryo liver cells were incubated in regular media supplemented with 2-allyl-2-isopropylacetamide (500  $\mu$ g/ml) and deferoxamine methanesulphonate (500  $\mu$ g/ml). Various concentrations of haemin were added at 0h (a) or 2h (b) and mitochondrial  $\delta$ -aminolaevulinate synthase activity was determined after various incubation times. No addition (O), 20nM-haemin (●), 50nM-haemin (□), 200nM-haemin (■), 2  $\mu$ M-haemin ( $\Delta$ ). Values are the means for four separate experiments.

sensitive to low concentrations of haemin, and that significant  $\delta$ -aminolaevulinate synthase induction is observed only after 2-allyl-2-isopropylacetamide-mediated removal of endogenous haem.

Therefore, when the cells were induced with 2-allyl-2-isopropylacetamide for 2h followed by washing and replacement of 2-allyl-2-isopropylacetamide with deferoxamine methanesulphonate to maintain induction (see Fig. 1), these cells should have been extremely sensitive to haem. Fig. 4 shows the effect of 50nM-haemin on  $\delta$ -aminolaevulinate synthase synthesis after cells were preinduced with 2-allyl-2-isopropylacetamide for 2h and the drug then removed by washing. The control, in which no additions were made subsequent to washing, presumably reflects translation of pre-existing  $\delta$ -aminolaevulinate synthase mRNA, and addition of 50nM-haemin did not affect this. However, in cells preincubated with deferoxamine methanesulphonate plus haemin or 2-allyl-2-isopropylacetamide plus haemin, some additional increase in enzyme syn-

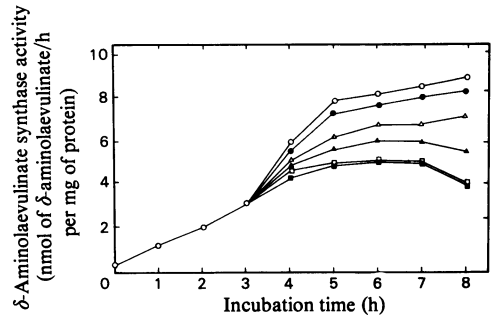


Fig. 4. Effect of deferoxamine methanesulphonate on  $\delta$ -aminolaevulinate synthase induction after the removal of 2-allyl-2-isopropylacetamide by washing

Isolated chick-embryo liver cells were incubated in regular media supplemented with 2-allyl-2-isopropylacetamide (500  $\mu$ g/ml) for 3h, washed with fresh media as described in the Experimental section and reincubated in fresh media containing the following additions: 2-allyl-2-isopropylacetamide (500  $\mu$ g/ml) (O); deferoxamine methanesulphonate (500  $\mu$ g/ml) (●); 2-allyl-2-isopropylacetamide (500  $\mu$ g/ml) and haemin (50nM) ( $\Delta$ ); deferoxamine methanesulphonate (500  $\mu$ g/ml) and haemin (50nM) ( $\blacktriangle$ ); no addition (□); and haemin (50nM) (■). Mitochondrial  $\delta$ -aminolaevulinate synthase activity was determined after various incubation times. Values are the means for four separate experiments.

thesis did occur. This was unexpected, since, as shown in Fig. 3, 50nM-haemin inhibits  $\delta$ -aminolaevulinate synthase synthesis completely for 5h in the presence of 2-allyl-2-isopropylacetamide, and therefore the curves in Fig. 4 would be expected to be coincident with the control in which induction is inhibited. At present there is no explanation for this decreased effect, although diminished haemin uptake by these washed cells is a possibility.

To determine whether the haem effect was transcriptional or post-transcriptional, we examined the effect of cordycepin (12  $\mu$ g/ml) and cycloheximide (10  $\mu$ g/ml) on  $\delta$ -aminolaevulinate synthase induction. Cordycepin inhibited by 98% the incorporation of [5,6- $^3$ H]uridine into total cellular RNA over a 10h period, and, when added at zero time, completely prevented 2-allyl-2-isopropylacetamide-induced  $\delta$ -aminolaevulinate synthase synthesis (results not shown). Cycloheximide inhibited by 96% the incorporation of L-[4,5- $^3$ H]leucine into total cellular protein over 10h and also completely inhibited  $\delta$ -aminolaevulinate synthase induction when added at zero time (results not shown). Therefore both RNA and protein synthesis are required for induction of  $\delta$ -aminolaevulinate synthase activity. When induction was allowed to proceed for 2h before addition of the inhibitors, the

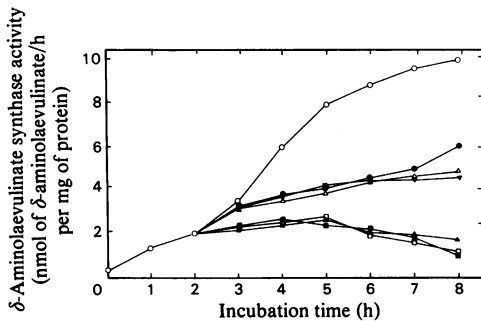


Fig. 5. Comparison of the effects of haemin, cycloheximide and cordycepin on the induction of  $\delta$ -aminolaevulinate synthase

Isolated chick-embryo liver cells were incubated in regular media supplemented with 2-allyl-2-isopropylacetamide (500  $\mu$ g/ml) and deferoxamine methanesulphonate (500  $\mu$ g/ml) for 2 h. The following additions were then made: no addition (○); 50 nM-haemin (●); cordycepin (12.5  $\mu$ g/ml) (△); cordycepin plus 50 nM-haemin (▼); cycloheximide (10  $\mu$ g/ml) (▲); 2  $\mu$ M-haemin (□); and cordycepin plus 2  $\mu$ M-haemin (■). Mitochondrial  $\delta$ -aminolaevulinate synthase activity was measured after various incubation times. Values are the means for four separate experiments.

results shown in Fig. 5 were obtained. In the presence of cordycepin, enzyme activity increased slightly for a further 1–2 h, then remained at that value for an additional 5 h. After addition of cycloheximide, further increases in  $\delta$ -aminolaevulinate synthase activity were inhibited, and activity subsequently declined with a  $t_{1/2}$  of 4–5 h. These results were compared with the effects of various concentrations of added haemin. After 2 h of  $\delta$ -aminolaevulinate synthase induction, either 50 nM- or 2  $\mu$ M-haemin was added to the cells. The results show that, in the presence of 50 nM-haemin, the resulting change in enzyme activity was identical with that caused by cordycepin. However, the response to 2  $\mu$ M-haemin was similar to that of cycloheximide. Furthermore, although 50 nM-haemin was shown to completely inhibit  $\delta$ -aminolaevulinate synthase induction (Fig. 2a), there was no synergistic effect with cordycepin (Fig. 5). However, addition of 2  $\mu$ M-haemin to cordycepin-treated cells caused an immediate inhibition of  $\delta$ -aminolaevulinate synthase induction, a response analogous to that of cycloheximide. These results show that haemin may have at least two concentration-dependent effects within the cell: at low concentrations (less than 200 nM) to inhibit the transcription of  $\delta$ -aminolaevulinate synthase mRNA; at high concentrations (greater than 1  $\mu$ M) to inhibit translation of  $\delta$ -aminolaevulinate synthase mRNA.

## Discussion

The above results describe the induction of hepatic porphyria in isolated chick-embryo liver cells and the effect of haem in controlling the synthesis of  $\delta$ -aminolaevulinate synthase. Our data show that deferoxamine methanesulphonate, an iron chelator and inhibitor of ferrochelatase, has a synergistic effect with 2-allyl-2-isopropylacetamide in promoting synthesis of  $\delta$ -aminolaevulinate synthase and protoporphyrin accumulation, and suggests, therefore, that the intracellular concentration of haem is a major regulator of the haem-biosynthetic pathway. These results confirm the observations of Sassa & Granick (1970) that iron chelators such as deferoxamine methanesulphonate prevent the formation of haem and thereby promote 2-allyl-2-isopropylacetamide-induced porphyria. The fact that deferoxamine methanesulphonate itself is not an inducer implies that the normal pool of intracellular haem has a very low rate of turnover or utilization, such that it does not significantly diminish in amount even after haem biosynthesis has been inhibited for several hours. We have also shown for the first time that after the initial induction event by 2-allyl-2-isopropylacetamide, continued  $\delta$ -aminolaevulinate synthase synthesis and protoporphyrin accumulation can be maintained by deferoxamine methanesulphonate alone. This confirms the report (Granick *et al.*, 1975; Sassa & Granick, 1970) that haem and 2-allyl-2-isopropylacetamide do not compete for a common site on a repressor protein and suggests that the primary effect of 2-allyl-2-isopropylacetamide in induction is to cause a rapid turnover and degradation of intracellular haem (Unsel'd & De Matteis, 1978; Ortiz de Montellano *et al.*, 1978, 1979), thereby removing the negative control on  $\delta$ -aminolaevulinate synthase synthesis. Subsequent replacement of 2-allyl-2-isopropylacetamide by deferoxamine methanesulphonate completely inhibits the formation of endogenous haem from protoporphyrin and therefore maintains  $\delta$ -aminolaevulinate synthase synthesis at the maximum induced rate.

The effect of increasing concentrations of haemin on  $\delta$ -aminolaevulinate synthase induction shows that synthesis of this enzyme is extremely sensitive to haem. Concentrations as low as 20 nM-haemin completely inhibited 2-allyl-2-isopropylacetamide-induced  $\delta$ -aminolaevulinate synthase synthesis for a period of 2 h, followed by a recovery period in which the rate of enzyme synthesis approached the control values. Increasing concentrations of haemin extended the inhibitory period, but did not affect the subsequent recovery of induction. Above 1  $\mu$ M, haemin was completely inhibitory throughout the course of the experiment. These data directly demonstrate that 2-allyl-2-isopropylacetamide-in-

duced  $\delta$ -aminolaevalinate synthase synthesis is regulated by very low concentrations of haem. Furthermore, haem control is reversible, as evidenced by recovery of induction after 2-allyl-2-isopropylacetamide-mediated degradation of the added haemin.

These results are of particular importance with regard to the role of haem in  $\delta$ -aminolaevalinate synthase induction, since haemin concentrations as high as  $50\mu\text{M}$  have previously been used to define the site of action of haem (Tomita *et al.*, 1974; Tyrrell & Marks, 1972). Our results show that these concentrations are up to several orders of magnitude too high and that such amounts may be toxic; they also show clearly that all previous studies on haem inhibition of induction that did not include the time course cannot be interpreted with any confidence. Furthermore, Sassa & Granick (1970) have estimated the intracellular concentration of haem in chick embryo liver cells to be of the order of 50–100 nM, a value that is shown in our experiments to be sufficient to completely inhibit  $\delta$ -aminolaevalinate synthase synthesis and to prevent induction of hepatic porphyria.

The biphasic shape of the haemin-inhibition curve is surprising in that there is a plateau between 50 nM and 200 nM haemin, whereas a continuously increasing inhibition might be expected. This could be due to the secondary effects of 2-allyl-2-isopropylacetamide-mediated haem destruction and a haemin-concentration-dependent induction of haem oxygenase at higher substrate concentrations (Bissell & Hammahes, 1976). As a consequence of these two effects, the actual concentration of haemin necessary for inhibition of  $\delta$ -aminolaevalinate synthase induction may be even less than that observed in our experiments.

The 2-allyl-2-isopropylacetamide-mediated induction of hepatic porphyria in isolated chick-embryo liver cells has been shown to be dependent on both RNA and protein synthesis. Our data show that, after 2 h of induction, addition of cordycepin had little effect on the increase in  $\delta$ -aminolaevalinate synthase activity for approx. 1 h, after which time enzyme activity was constant for the remaining period of the assay. This indicates continued translation of pre-existing  $\delta$ -aminolaevalinate synthase mRNA, which achieves a steady-state equilibrium between enzyme synthesis and turnover. Addition of cycloheximide to 2-allyl-2-isopropylacetamide-induced cells immediately inhibited the increase in  $\delta$ -aminolaevalinate synthase activity, which then declined with a  $t_{1/2}$  of 4–5 h. This value for  $\delta$ -aminolaevalinate synthase turnover is in agreement with the data of Tyrrell & Marks (1972) and Sassa & Granick (1970). In comparison with the kinetics of the above two responses, the data of Fig. 4 show that, at a concentration of haemin (50 nM) sufficient completely to inhibit induction of  $\delta$ -amino-

laevalinate synthase synthesis by 2-allyl-2-isopropylacetamide, the change in enzyme activity closely paralleled the cordycepin curve. 50 nM-Haemin did not have any synergistic effect with cordycepin, suggesting that this concentration of haemin inhibits  $\delta$ -aminolaevalinate synthase synthesis at the level of transcription. Addition of  $2\mu\text{M}$ -haemin to cordycepin-treated cells showed a synergistic effect, and the response was similar to that of cycloheximide. These data indicate that, at low concentrations, haem is a very effective regulator of  $\delta$ -aminolaevalinate synthase synthesis and acts at the level of transcription.

Only concentrations of haemin above  $1\mu\text{M}$  have post-transcriptional effects. These results conflict with those of Tyrrell & Marks (1972) and Sassa & Granick (1970), who showed no apparent transcriptional effect of exogenous haemin on the induction of  $\delta$ -aminolaevalinate synthase activity in cultured chick-embryo liver cells. A possible reason for this conflict may be indicated by our finding that, after washing of 2-allyl-2-isopropylacetamide-pre-treated cells, haemin caused only a partial inhibition of  $\delta$ -aminolaevalinate synthase synthesis compared with complete inhibition in unwashed cells. A similar effect was observed when haemin was replaced with its precursor,  $\delta$ -aminolaevalinate. Possibly the prolonged cell culture used by the above authors or the extensive washing of chick-embryo liver cells in our experiments may have decreased the ability of these cells to absorb exogenous haemin. However, it should be emphasized that our washed cells were still capable of responding to endogenously synthesized haem, as is demonstrated by Fig. 1, in which, after washing,  $\delta$ -aminolaevalinate synthase synthesis ceases in the absence of deferoxamine methanesulphonate or 2-allyl-2-isopropylacetamide.

From experiments reported in the present paper we conclude, therefore, that in chick-embryo liver cells, induction of  $\delta$ -aminolaevalinate synthase synthesis and hepatic porphyria is primarily regulated by the intracellular concentration of haem. The effect of haem on the synthesis of  $\delta$ -aminolaevalinate synthase is at the level of transcription, and translational effects seen at higher concentrations may be related to haem toxicity. Clearly, however, conclusions based solely on the results of inhibitor experiments must be regarded as tentative, and confirmation by direct measurement of  $\delta$ -aminolaevalinate synthase mRNA is required.

We are grateful for the technical assistance of Miss J. Thompson. This work was supported by the National Health and Medical Research Council of Australia.

## References

- Bissell, D. M. & Hammahes, L. E. (1976) *Arch. Biochem. Biophys.* **176**, 103–112

- De Matteis, F. (1971) *Biochem. J.* **124**, 769-779
- Granick, S. (1963) *J. Biol. Chem.* **238**, 2247-2249
- Granick, S. (1966) *J. Biol. Chem.* **241**, 1359-1375
- Granick, S. & Urata, G. (1963) *J. Biol. Chem.* **238**, 821-827
- Granick, S., Sinclair, P., Sassa, S. & Grieninger, G. (1975) *J. Biol. Chem.* **250**, 9215-9225
- Hayashi, N., Kurashima, Y. & Kikuchi, G. (1972) *Arch. Biochim. Biophys.* **148**, 10-21
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Morgan, R. O., Stephens, J. K., Fischer, P. W. F. & Marks, S. (1977) *Biochem. Pharmacol.* **26**, 265-275
- Ohashi, A. & Kikuchi, G. (1972) *Arch. Biochem. Biophys.* **153**, 34-46
- Ortiz de Montellano, P. R., Mico, B. A. & Yost, G. S. (1978) *Biochem. Biophys. Res. Commun.* **83**, 132-137
- Ortiz de Montellano, P. R., Kunze, K. L., Yost, G. S. & Mico, B. A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 746-749
- Poland, A. & Glover, E. (1973) *Science* **179**, 476-477
- Sassa, S. & Granick, S. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 517-522
- Sassa, S. & Kappas, A. (1977) *J. Biol. Chem.* **252**, 2428-2436
- Srivastava, G., May, B. K. & Elliott, W. H. (1979) *Biochem. Biophys. Res. Commun.* **90**, 42-49
- Tomita, Y., Ohashi, A. & Kikuchi, G. (1974) *J. Biochem. (Tokyo)* **75**, 1007-1015
- Tyrrell, D. L. J. & Marks, G. S. (1972) *Biochem. Pharmacol.* **21**, 2077-2093
- Unsold, A. & De Matteis, F. (1978) *Int. J. Biochem.* **9**, 865-869
- Whiting, M. J. & Granick, S. (1976) *J. Biol. Chem.* **251**, 1347-1353
- Wolfson, S. J., Bartczak, A. & Bloomer, J. R. (1979) *J. Biol. Chem.* **254**, 3543-3546