Biochem. J. (1972) **129**, 1095–1099
Printed in Great Britain

The Effect of Exogenous δ-Aminolaevulinate on Rat Liver Haem and Cytochromes

By ROBERT DRUYAN and ALDON KELLY
Department of Medicine, The University of Chicago Pritzker School of Medicine, and Argonne Cancer Research Hospital, Chicago, Ill. 60637, U.S.A.

(*Received* 19 *May* 1972)

The activity of δ-aminolaevulinate synthetase is generally regarded as rate-limiting for hepatic haem biosynthesis. It has been suggested that cytochrome synthesis may also be regulated by changes in δ-aminolaevulinate synthetase activity. This hypothesis was studied by injecting product, δ-aminolaevulinate, into adult rats over a 4–240h period. The concentrations of hepatic mitochondrial cytochromes *a*, *b*, *c* and c_1 were unchanged by treatment with δ-aminolaevulinate, allylisopropylacetamide or phenobarbital. In control animals, total microsomal haem content equalled the sum of cytochromes b_5 plus *P*-450. After δ-aminolaevulinate administration the total amount of microsomal haem, measured as the pyridine haemochromogen, exceeded these components, indicating the formation of a 'free' haem pool. Haem synthesis does not appear rate-limiting for hepatic cytochrome synthesis in the adult rat.

The regulation of δ-aminolaevulinate synthetase activity has received detailed study since its induction was reported by Granick & Urata (1963). δ-Aminolaevulinate synthetase is induced by many chemicals (Granick, 1966) and its activity is inhibited by haem (Scholnick *et al.*, 1969). Although δ-aminolaevulinate synthetase activity appears to be rate-limiting for haem biosynthesis (Granick, 1966), its role in the regulation of overall haemoprotein synthesis remains unclear.

Several experimental models have been used to explore possible relationships between δ-aminolaevulinate synthetase activity and synthesis of specific haemoproteins. δ-Aminolaevulinate synthetase activity has been bypassed by adding the product, δ-aminolaevulinate. In tissue cultures of chick blastoderm, added δ-aminolaevulinate promotes haemoglobin synthesis (Levere & Granick, 1965). In developing *Polyphemus* moths, an increase in cytochrome *c* concentration follows δ-aminolaevulinate injection (Soslau *et al.*, 1971).

The effect of allylisopropylacetamide, a potent inducer of δ-aminolaevulinate synthetase (Granick, 1966), has also been examined on the activity and concentration of haemoproteins. The activity of L-tryptophan 2,3-dioxygenase increased after allylisopropylacetamide injection (Marver *et al.*, 1966*b*). L-Tryptophan 2,3-dioxygenase requires haem as a prosthetic group for enzymic activity. From studies of allylisopropylacetamide-treated rats, it has been suggested that δ-aminolaevulinate synthetase activity is involved in the regulation of mitochondrial biogenesis (Beattie & Stuchell, 1970; Beattie, 1971). After allylisopropylacetamide induction of δ-aminolaevulinate synthetase, several changes were observed in hepatic mitochondria: the concentrations of rat liver cytochromes $a+a_3$, *b* and $c+c_1$ increased by 30–50%; the incorporation of [^{14}C]glycine into haem was enhanced; and both *in vivo* and *in vitro*, incorporation of labelled leucine into protein was increased.

Because allylisopropylacetamide has diverse chemical and morphological sequelae beyond its effect on δ-aminolaevulinate synthetase, we have studied the effect of injected δ-aminolaevulinate on hepatic haemoproteins to evaluate the possible role of δ-aminolaevulinate synthetase in their regulation.

Materials and Methods

L-Tryptophan and δ-aminolaevulinate hydrochloride were purchased from Schwarz/Mann (Orangeburg, N.Y., U.S.A.). On the basis of its colour yield after condensation with acetylacetone (Urata & Granick, 1963), δ-aminolaevulinate was 99% pure. Allylisopropylacetamide was a gift from Hoffmann–LaRoche (Nutley, N.J., U.S.A.); NADH and crystalline bovine haemoglobin were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); sodium deoxycholate was from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.).

Male Sprague–Dawley rats (200–250g) were killed by cervical fracture. Washed mitochondria (Beattie, 1968) were prepared from two rat livers (16–21 g total wet wt.) pooled during homogenization. The post-mitochondrial supernatant was centrifuged for 10min at 12000*g* and a microsomal fraction was collected from the supernatant by centrifugation at 105000*g*

for 60min; the microsomal fraction was re-homogenized in 0.15M-KCl and recentrifuged. Analyses of mitochondria were performed immediately; microsomal pellets were stored at −15°C for 12–72h in 0.1M-sodium phosphate buffer, pH7.4.

Mitochondrial cytochrome concentrations were measured by difference spectroscopy (Williams, 1964). For each sample, derived from two livers, duplicate analyses at two protein concentrations were made; mean values are cited. A sample of mitochondria, equivalent to 75–150mg of protein, was used for haem extraction.

Cytochrome *P*-450 concentrations were measured on microsomal suspensions by using the difference spectrum generated by CO (Omura & Sato, 1964). To minimize light-scattering and microsomal sedimentation, and to permit assays at higher protein concentrations, cytochrome b_5 was measured with microsomal fractions solubilized in deoxycholate. Each 1ml cuvette contained: microsomal fraction, equivalent to 4–10mg of protein; sodium phosphate, pH7.4, 50μmol; sodium deoxycholate, 1% (w/v). Cytochrome b_5 was reduced by 0.2μmol of fresh NADH. Calculations were made as described by Omura & Sato (1964).

Haems were measured after the formation of pyridine haemochromogens. Mitochondrial and microsomal pellets were delipidated with acetone, $CHCl_3$–methanol (2:1, v/v) and acetone in subdued light. Haems were extracted with 3×10vol. of acetone–HCl (2.5ml of 36% HCl per 100ml of acetone). The pooled extracts were flash evaporated. Haems were dissolved in alkaline pyridine (pyridine–1M-NaOH–water, 5:1:5, by vol.). The pH was adjusted to 13 with 1M-NaOH, if necessary. The concentrations of mitochondrial haem *a* and protohaem were determined as described by Reiske (1967). For microsomal samples, in which only protohaem was detected, the method of Porra & Jones (1963) was used.

L-Tryptophan 2,3-dioxygenase activity was assayed as described by Knox *et al.* (1970). Total activity was determined after preincubation with methaemoglobin. To measure holoenzyme activity, methaemoglobin was omitted from the preincubation step. Apoenzyme activity was calculated from total activity minus holoenzyme activity.

Protein concentrations were determined by the biuret reaction (Gornall *et al.*, 1949) with crystalline bovine serum albumin (Pentex, Kankakee, Ill., U.S.A.) as a standard. Proteins were first precipitated with 10% (w/v) trichloroacetic acid, and precipitates were acetone-washed. A Cary model 14 spectrophotometer, equipped with a dual-range slide wire, was used to record difference spectra. Tissue fractionation and haem extraction, except for flash evaporation, was done at 4°C.

Results

The dosages and schedules for rats injected with δ-aminolaevulinate, allylisopropylacetamide or phenobarbital are summarized in Table 1. Rats treated with δ-aminolaevulinate hourly (group 1E) were killed 1h after the fourth injection; other rats were killed 16h after the last injection. All injections were made intraperitoneally except for rats in group 2A, where a single dose of allylisopropylacetamide was given subcutaneously. Rats treated with allylisopropylacetamide or phenobarbital were given only water before death, whereas other rats were fed *ad libitum*.

After allylisopropylacetamide treatment, a 20% increase in liver weight was observed, in agreement with earlier reports (Marver *et al.*, 1966*a*). Livers from δ-aminolaevulinate-treated rats were not similarly affected. Mitochondrial cytochrome concentrations, measured on four control samples(eight livers) were: cytochrome *a*, 198±24; cytochrome *b*, 272±24; cytochrome *c*, 233±24; cytochrome c_1, 157±17nmol/g of mitochondrial protein. No significant differences in cytochrome concentrations were observed when control values were compared with those obtained from rats treated with δ-aminolaevulinate, allylisopropylacetamide or phenobarbital. Concentrations of mitochondrial haems, extracted from control mitochondrial samples, were:

Table 1. *Dosage and schedule of injections of δ-aminolaevulinate, allylisopropylacetamide or phenobarbital*

Group	Drug	Dosage (mg/kg)	Schedule
1A	δ-Aminolaevulinate	15	One injection
1B	δ-Aminolaevulinate	15	Daily for 2 days
1C	δ-Aminolaevulinate	15	Daily for 3 days
1D	δ-Aminolaevulinate	10	Twice daily for 10 days
1E	δ-Aminolaevulinate	15	Hourly for 4 injections
2A	Allylisopropylacetamide	400	One injection
2B	Allylisopropylacetamide	150	Daily for 2 days
3	Phenobarbital	125	One injection

haem *a*, 269±67; protohaem, 311±91 nmol/g of protein. Although no apparent difference was observed between haem concentrations in control and treated rats, the large standard deviations among control values preclude a meaningful statistical comparison. In separate experiments, the conditions used for haem extraction were varied, but better precision was not achieved.

The effect of deoxycholate on the measurement of cytochrome b_5 was studied in preliminary experiments. Eight different microsomal samples, each containing 2–4 mg of protein/ml, were compared by two assays: identical samples were assayed (1) in suspension in 0.05 M-sodium phosphate buffer, pH 7.4, and (2) in solubilized form in 0.05 M-sodium phosphate buffer containing 1% sodium deoxycholate. The measurements were essentially identical, since differences were 0.1–3.5%. The activity of NADH–cytochrome b_5 reductase was not limiting for these measurements, since the addition of 0.8 mg of this enzyme did not increase the spectral yield generated by adding NADH alone. [NADH–cytochrome b_5 reductase was purified through gel-filtration and ammonium sulphate precipitation steps (Strittmater, 1967). Activity, measured with $K_3Fe(CN)_6$ as a substrate, was 17.5 μmol/min per mg of protein.]

Neither δ-aminolaevulinate nor allylisopropylacetamide injections altered the concentrations of cytochrome b_5 or *P*-450 in microsomal fractions (Table 2). Consistent with previous reports, we found that a single phenobarbital injection increased cytochrome b_5 concentrations by 30% (Omura *et al.*, 1969), and resulted in a doubling of cytochrome *P*-450 concentrations (Orrenius & Ernster, 1964).

Table 2. *Effect of injections of δ-aminolaevulinate, allylisopropylacetamide or phenobarbital on concentrations of microsomal cytochrome b_5 or P-450*

Cytochrome concentrations are expressed as nmol/g of microsomal protein. Dosages and schedules for rats receiving δ-aminolaevulinate, allylisopropylacetamide and phenobarbital are shown in Table 1. Numbers of experiments are given in parentheses.

Sample		Cytochrome b_5	Cytochrome *P*-450
Control	(4)	383±35	543± 66
1A	(3)	418±26	581± 58
1B	(3)	400±35	599±160
1C	(3)	365±34	513± 12
1D	(1)	403	497
1E	(1)	392	592
2A	(1)	447	485
2B	(1)	409	465
3	(1)	482	1111

Total microsomal haem was measured as the alkaline pyridine haemochromogen on microsomal samples containing 50–125 mg of protein. For control rats, the sum of cytochrome b_5 plus *P*-450 corresponded closely to the total microsomal haem concentration (Fig. 1). The highest total haem concentration was found in phenobarbital-treated rats. The increase could be accounted for by the increased microsomal cytochrome concentration. Rats treated with allylisopropylacetamide (not shown here) did not differ from the control animals. With δ-aminolaevulinate injections, however, total microsomal haem increased above the sum of cytochromes b_5 plus *P*-450. For two groups, treated with δ-aminolaevulinate for 2 and 3 days, this increment was statistically significant ($P<0.05$). (Differences between total haem and total cytochrome concentrations were compared among treated and control rats by using a single-tailed Student's *t* test.) Similar changes were observed among rats receiving δ-aminolaevulinate for 10 days, or hourly.

L-Tryptophan 2,3-dioxygenase activity (Fig. 2) remained constant after daily δ-aminolaevulinate injections or after phenobarbital; the ratio of holoenzyme to total activity was 0.1–0.3. After hourly

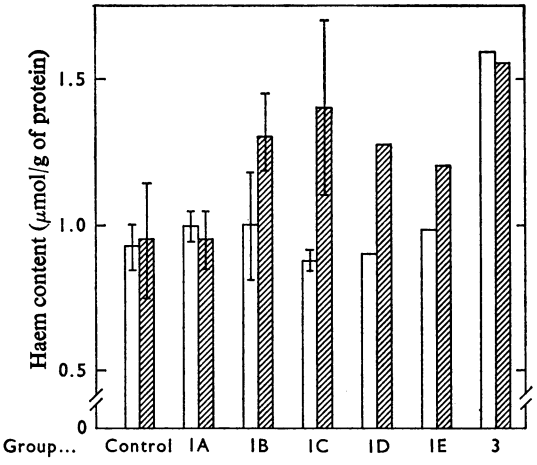

Fig. 1. *Microsomal concentrations of cytochromes and total haem in control and injected rats*

Cytochrome concentrations (open bars) are the sum of cytochromes b_5 and *P*-450. Total haem (stippled bars) was measured independently. Standard deviations are shown as vertical lines. Allylisopropylacetamide-treated rats, not shown here, did not differ from the controls. The increment of 'free' haem in rats treated with δ-aminolaevulinate for 2 or 3 days (groups 1B and 1C respectively) was statistically significant.

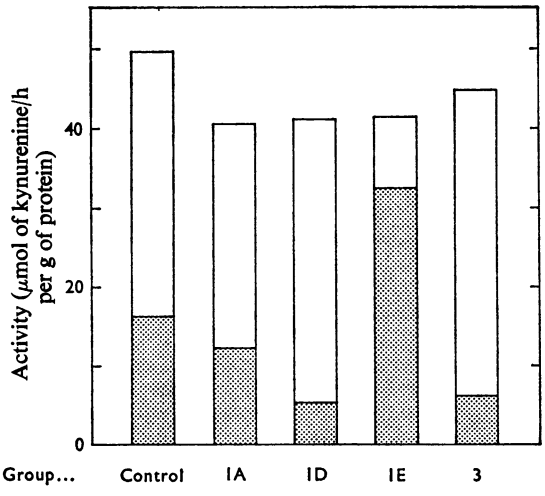

Fig. 2. *L-Tryptophan 2,3-dioxygenase activity in liver cytosol from control, δ-aminolaevulinate-treated and phenobarbital-treated rats*

Enzyme activity is expressed as μmol of kynurenine formed/h per g of cytosol protein. The stippled bar represents holoenzyme activity, assayed without methaemoglobin during preincubation. The open bar represents apoenzyme activity, calculated from total activity (assayed with methaemoglobin) minus holoenzyme activity.

δ-aminolaevulinate injections, this proportion changed, and the activity of the holoenzyme form was 80% of the total activity.

Discussion

Two assumptions underlie the use of injected δ-aminolaevulinate to bypass δ-aminolaevulinate synthetase in this study: (1) that δ-aminolaevulinate is incorporated into hepatic cytochromes; (2) that the endogenous pool of δ-aminolaevulinate is small relative to the injected dose of δ-aminolaevulinate. In earlier studies, incorporation of δ-aminolaevulinate into six hepatic cytochromes was described (Aschenbrenner *et al.*, 1970; Druyan *et al.*, 1969; Levin & Kunzman, 1969). Cytochrome labelling is detected within 15min of intraperitoneal δ-amino[^{3}H]laevulinate injection, and incorporation stops within 6h (Druyan *et al.*, 1971). δ-Aminolaevulinate concentrations in liver are low, but precise values are unavailable. δ-Aminolaevulinate measurements (Urata & Granick, 1963) made on a perchloric acid supernatant solution from 5g of rat liver yielded an $E_{553}<0.01$. This reading corresponds to a δ-aminolaevulinate concentration of less than 4nmol/100g wet wt. of tissue. Presumably the lability of δ-aminolaevulinate reflects its rapid conversion into porphobilinogen and porphyrins.

After δ-aminolaevulinate injection we found that the concentrations of six hepatic concentrations remained constant when δ-aminolaevulinate was given over a 4–240h period. For five of these cytochromes, turnover rates for the haem moieties range between 2 and 6 days (Aschenbrenner *et al.*, 1970; Druyan *et al.*, 1969; Levin & Kunzman, 1969) (cytochrome c_1 has not been studied in this regard). Hence, the intervals selected for δ-aminolaevulinate injection and subsequent killing were suitable to detect changes in cytochrome concentrations. With rapid turnover (i.e., a 3h half-life) the effect of precursor injection on product concentration will be transient, owing to rapid degradation. The short half-life of L-tryptophan 2,3-dioxygenase probably explains why the altered partition between apo- and holo-enzyme forms was observed only among rats killed 1h after the last δ-aminolaevulinate injection.

These results now suggest that the rate of haem synthesis is not a significant determinant in the regulation of hepatic cytochrome concentration under physiological conditions. In contrast, with foetal (Levere & Granick, 1965) or differentiating tissues (Soslau *et al.*, 1971), haem synthesis, and specifically the activity of δ-aminolaevulinate synthetase, appears to have a regulatory role in haemoprotein synthesis. Although not extensively examined, we were unable to confirm the effects of allylisopropylacetamide on mitochondrial cytochrome concentrations (Beattie & Stuchell, 1970; Beattie, 1971). Barnes *et al.* (1971) also found no increase in hepatic cytochromes after allylisopropylacetamide treatment.

The existence of a free, rapidly renewed haem pool has been inferred from several independent experimental approaches. One component of early labelled bilirubin is excreted within minutes of injection of the labelled precursor, and probably derives from haem, rather than from haemoprotein degradation (Levitt *et al.*, 1968). It has been shown that injected δ-aminolaevulinate partially inhibits the allylisopropylacetamide-mediated increase in hepatic δ-aminolaevulinate synthetase activity (Hayashi *et al.*, 1972). This inhibition represents either co-repression of enzyme synthesis by haem (Granick, 1966) or feedback inhibition of δ-aminolaevulinate synthetase activity by haem (Scholnick *et al.*, 1969). In either case, the effector is probably free haem, generated in response to the injected δ-aminolaevulinate. Cycloheximide does not inhibit incorporation of labelled δ-aminolaevulinate into all haems and haemoproteins and this observation is also consistent with a free haem pool. During 95% inhibition of protein synthesis by cycloheximide, labelling of microsomal haems (Levitt *et al.*, 1968; Garner & McLean, 1969) and cytochrome b_5 (Druyan *et al.*, 1971; Garner &

McLean, 1969) is unchanged. Haem exchange has been observed between methaemoglobin and cytochrome b_5 (Druyan *et al.*, 1971) and the incorporation of δ-amino[^{3}H]laevulinate into cytochrome b_5 in the absence of protein synthesis probably reflects exchange between cytochrome b_5 and a rapidly renewed haem pool. In view of these results, the interpretation of microsomal haemoprotein turnover results, based on haem labelling, appears equivocal. Demonstration of haem exchange, together with a 'free' microsomal haem pool, renders it unlikely that a clear interpretation can be ascribed to disappearance of radioactive haem from microsomal haemoproteins *in vivo*.

A close agreement between total microsomal haem and the sum of cytochromes b_5 and *P*-450 has been previously reported. This relationship obtains for untreated (Omura & Sato, 1964; Waterfield *et al.*, 1969), allylisopropylacetamide-treated (De Matteis, 1971; Waterfield *et al.*, 1969), and 3,5-dicarbethoxy-1,4-dihydrocollidine-treated (Waterfield *et al.*, 1969) animals. In rabbits, an increment of microsomal haem was found after phenobarbital (Waterfield *et al.*, 1969); in rats, no increment was observed by De Matteis (1971) or by us. Our analytical results confirm these reports. After δ-aminolaevulinate injections, an increment of excess of microsomal haem was observed, providing direct evidence for a free haem pool. Although our results on mitochondrial haem concentrations lack sufficient precision to permit useful comparisons with the corresponding mitochondrial cytochromes, on *a priori* grounds it seems reasonable to speculate that a haem pool exists in this organelle as well, since ferrochelatase, the final enzyme of haem synthesis, is localized to the inner mitochondrial membrane, where it is firmly bound (Jones & Jones, 1969; McKay *et al.*, 1969).

The changes observed in the partition of L-tryptophan 2,3-dioxygenase activity between its apoenzyme and holoenzyme forms provide a functional counterpart to our analytical results, and indicate that the incremental haem, generated in response to δ-aminolaevulinate, is not sequestered to microsomal fractions. In rats receiving δ-aminolaevulinate hourly, the holoenzyme fraction represented 80% of total L-tryptophan 2,3-dioxygenase activity, whereas in the other animals, the holoenzyme fraction ranged between 10 and 30% of the total L-tryptophan 2,3-dioxygenase activity. These results support the view of Marver *et al.* (1966*b*) that there is an interrelationship between haem synthesis and L-tryptophan 2,3-dioxygenase activity.

The Argonne Cancer Research Hospital is operated by the University of Chicago for the United States Atomic Energy Commission.

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