Biochem. J. (1976) **158**, 391–400
Printed in Great Britain

Synthesis of δ-Aminolaevulinate Synthase by Isolated Liver Polyribosomes

By MALCOLM J. WHITING
Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5001, *Australia*

(*Received* 17 *February* 1976)

1. Postmitochondrial supernatants were prepared from the livers of chick embryos and were incubated under conditions that supported protein synthesis. δ-Aminolaevulinate synthase (EC 2.3.1.37) was synthesized by supernatants from livers treated with the porphyrinogenic drugs 2-allyl-2-isopropylacetamide and/or 3,5-diethoxycarbonyl-1,4-dihydrocollidine, but synthesis by supernatants from normal livers could not be detected. Synthesis of enzyme released from polyribosomes was measured by immunoprecipitation with specific antibody to the mitochondrial enzyme, and the specificity of the reaction was established by electrophoresis of dissociated immunoprecipitates on sodium dodecyl sulphate/polyacrylamide gels. 2. The relative synthesis of δ-aminolaevulinate synthase *in vitro* was comparable with that previously measured *in vivo*, and was correlated with the enzyme activity of the liver. 3. Enzyme synthesis *in vitro* occurred predominantly on free rather than membrane-bound polyribosomes. 4. The mol.wt. of the product synthesized *in vitro* was 70000±7000 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. However, pulse-labelling of the enzyme *in vivo* confirmed its mol.wt. to be 49000±5000 when isolated from the mitochondrion. A small amount of immunoprecipitable enzyme of mol.wt. 70000 was detected in the cytosol *in vivo*. In chick embryo liver, δ-aminolaevulinate synthase therefore appears to be synthesized on cytoplasmic polyribosomes as a polypeptide of mol.wt. 70000, which *in vivo* is rapidly incorporated into the mitochondrion, and is then extracted as a lower-molecular-weight form. 5. Haemin added to the postmitochondrial supernatant-containing incubation mixture at concentrations up to 10μM had no effect on general protein synthesis or the synthesis of δ-aminolaevulinate synthase. On the other hand, haemin treatment of induced chick embryo livers *in vivo* for 3h markedly decreased the relative synthesis of δ-aminolaevulinate synthase *in vitro*. These results suggest that haemin represses the synthesis of δ-aminolaevulinate synthase by decreasing the amount of mRNA for the enzyme available for translation.

The low basal activity of the first enzyme of the haem-biosynthetic pathway, δ-aminolaevulinate synthase [succinyl (3-carboxypropionyl)-CoA–glycine *C*-succinyltransferase (decarboxylating), EC 2.3.1.37] can be increased rapidly in liver mitochondria by many 'foreign' chemicals and steroids (Granick, 1966; Granick & Sassa, 1971). In chick embryo liver, up to 600-fold increases in enzyme activity can be observed 18h after treatment with two of the most potent porphyrinogenic chemicals, 2-allyl-2-isopropylacetamide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (Whiting & Granick, 1976*a*). These drug-induced increases in enzyme activity can be prevented by haemin (Granick *et al.*, 1975; Hayashi *et al.*, 1968).

Immunological studies have since shown that changes in enzyme activity are paralleled by changes in the amount of enzyme antigen present in liver mitochondria (Whiting & Granick, 1976*b*). In addition, synthesis of enzyme *de novo* occurs after drug induction such that the synthesis of δ-aminolaevulinate synthase can increase from less than 0.005% to account for over 1% of the synthesis of intracellular liver proteins (Whiting & Granick, 1976*b*). Haemin prevents the induced increase in enzyme synthesis.

Unlike most enzymes that are inducible by hormonal, nutritional or other stimuli, δ-aminolaevulinate synthase functions in the mitochondrion and not in the cytosol. The enzyme has been localized in the mitochondrial matrix or loosely attached to the inner mitochondrial membrane, where the substrate succinyl (3-carboxypropionyl)-CoA is available (McKay *et al.*, 1969; Zuyderhoudt *et al.*, 1969). Mitochondrial δ-aminolaevulinate synthase has been difficult to purify, although near homogeneous enzyme from chick embryo liver has now been obtained and characterized (Whiting & Granick, 1976*a*). A cytosol form of enzyme, amounting to up to 30% of the total hepatic activity, does accumulate in rat (Hayashi *et al.*, 1969), guinea pig (Whiting & Elliott, 1972) and cock liver (Ohashi & Kikuchi, 1972), but not chick embryo liver, after drug induction. Although of higher molecular weight, it is thought to

be a precursor of the mitochondrial form, because antibodies prepared against the mitochondrial enzyme can totally precipitate the cytosol form (Whiting & Elliott, 1972). Other mitochondrial and cytosol isoenzymes are immunologically distinct proteins that do not show cross-reactions (Ballard & Hanson, 1969; Kitto & Kaplan, 1966; Nisselbaum & Bodansky, 1966).

The appearance of a cytoplasmic form of δ-aminolaevulinate synthase, and the prevention of enzyme induction by cycloheximide, but not by chloramphenicol (Granick, 1966), suggest that, like most mitochondrial proteins, the enzyme is synthesized on cytoplasmic polyribosomes. In the present paper, it is shown directly that cytoplasmic polyribosomes do synthesize δ-aminolaevulinate synthase, and the effect of the proposed translational repressor haemin on the synthesis of δ-aminolaevulinate synthase has been investigated.

Experimental

Treatment of chick embryos

Unless noted otherwise, White Leghorn chick embryos (18 days old) were treated with 2-allyl-2-isopropylacetamide (2mg) and/or 3,5-diethoxycarbonyl-1,4-dihydrocollidine (4mg) for periods of 12h, as previously described (Whiting & Granick, 1976*a*).

Preparation of specific antibody

The preparation of a specific antibody to highly purified δ-aminolaevulinate synthase from chick-embryo liver mitochondria and its characterization by Ouchterlony immunodiffusion and quantitative immunoprecipitation studies has previously been described (Whiting & Granick, 1976*b*).

Preparation of postmitochondrial supernatants from liver

Normal or drug-treated livers were homogenized as described by Ballard *et al.* (1974) in 4vol. of a solution, pH7.4, containing 0.2M-sucrose, 0.1M-KCl, 0.005M-$MgCl_2$, 0.02M-Tris/HCl, 0.001M-dithiothreitol and 0.5mg of heparin/ml (solution A). After centrifugation at 20000*g* for 10min, the resultant postmitochondrial supernatant was collected and used for protein-synthesis experiments.

Separation of free and membrane-bound polyribosomes

The method used was essentially as described by Bloemendal *et al.* (1974). Postmitochondrial supernatant (3ml) was layered over a discontinuous gradient consisting of 2.5ml of 1.5M-sucrose in solution A above 2.5ml of 2M-sucrose in solution A. Centrifugation was carried out at 105000*g* for 3h in a Ti50 rotor in a Beckman L2-65B ultracentrifuge. The pellet of free polyribosomes was resuspended in solution A in 2vol. with respect to the original liver weight. The cloudy layer of membrane-bound polyribosomes at the lower gradient boundary was diluted to 10ml with solution A and re-centrifuged at 105000*g* for 1h at 4°C. Again, the pellet was resuspended in solution A in 2vol. with respect to the original liver weight.

Measurement of protein synthesis in vitro

Postmitochondrial supernatant (0.5ml) was added to an incubation mixture, final volume 2.5ml, containing salts, amino acids and an energy-generating system at the concentrations specified by Ballard *et al.* (1974) and 25μCi of [4,5-^{3}H]leucine (sp. radioactivity 55Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.). In experiments with free and bound polyribosomes, the postmitochondrial supernatant was replaced with 0.25ml of polyribosomes and 0.5ml of a cytosol fraction, obtained after centrifuging postmitochondrial supernatant at 105000*g* for 60min at 4°C. After incubation at 30°C for 30min, solutions were rapidly cooled on ice and two 50μl samples taken for the determination of radioactivity incorporated into total protein, by using the method of Ballard *et al.* (1974). Then 0.25ml of 5% (v/v) Triton X-405 (Sigma Chemical Co., St. Louis, MO, U.S.A.) was added before centrifugation at 105000*g* for 60min. The clear supernatant, containing radioactive protein released from polyribosomes, was used for the measurement of radioactivity in soluble protein and radioactivity in immunoprecipitable δ-aminolaevulinate synthase.

Determination of radioactivity in δ-aminolaevulinate synthase

The supernatant from the protein-synthesis incubations contained no enzyme activity detectable by colorimetric assay. Therefore 50 units (defined below) of partially purified enzyme from chick-embryo liver mitochondria (sp. activity 600 units/mg of protein) were added as carrier protein to 1.0ml of incubation mixture, and δ-aminolaevulinate synthase was precipitated by the addition of a 30% excess of specific antibody. Duplicate mixtures were incubated for 60min at 25°C and for 16–20h at 4°C. The antigen–antibody precipitates were then collected and washed with 4×1ml of an ice-cold solution containing 10mM-sodium phosphate, pH7.6, 0.5% Triton X-405 and 0.9% (w/v) NaCl. They were dissolved in 100μl of NCS solubilizer (Amersham/Searle Corp., Arlington Heights, IL, U.S.A.) for radioactivity counting or in 50μl of a solution containing 2% (w/v) sodium dodecyl sulphate, 2% (w/v) dithiothreitol, 25% (v/v)

glycerol and 0.005% Bromophenol Blue in 0.01M-sodium phosphate buffer, pH 7.0, and heated for 2min at 100°C. Samples (30μl) of dissociated proteins were subjected to electrophoresis on sodium dodecyl sulphate/polyacrylamide gels (80mm × 0.5mm diam.). Gels were run, sliced and counted for radioactivity as previously described (Whiting & Granick, 1976*b*), by using a Packard liquid-scintillation spectrometer (model 3390) with an efficiency for ^3H of 53%.

Labelling studies in vivo

Normal and induced 18-day chick embryos were injected intravenously with 100μCi of [^3H]leucine as described by Whiting & Granick (1976*b*). After a labelling period of 45min, livers were removed and homogenized in 6vol. of buffer containing 0.25M-sucrose, 0.005M-Tris/HCl, 0.1mM-EDTA, 0.01mM-pyridoxal 5′-phosphate, pH 7.4, and the homogenate was centrifuged at 15000*g* for 10min. The supernatant was re-centrifuged at 105000*g* for 60min to obtain a cytosol fraction, and the pellet was resuspended in sucrose/buffer and the mitochondria were recovered by differential centrifugation (Whiting & Granick, 1976*b*). Soluble mitochondrial proteins were extracted by sonication, and after assay for δ-aminolaevulinate synthase activity, enzyme was isolated by quantitative immunoprecipitation (Whiting & Granick, 1976*b*). Duplicate samples (1.0ml) of the cytosol fraction were treated in the same way as for mitochondrial extracts, with 50 units of carrier enzyme being added to form an immunoprecipitate.

Assay of δ-aminolaevulinate synthase

Enzyme activity was measured as previously described (Whiting & Granick, 1976*a*). A unit of enzyme is defined as the amount required to catalyse the formation of 1nmol of δ-aminolaevulinate in 30min under the standard assay conditions.

Results

Synthesis of δ-aminolaevulinate synthase by liver postmitochondrial supernatants

Under appropriate conditions, liver postmitochondrial supernatants have been shown to incorporate amino acids into proteins (Richardson *et al.*, 1971), and the synthesis of specific enzymes by liver polyribosomes in cell-free extracts has been reported (Beck *et al.*, 1972; Ballard *et al.*, 1974). The synthesis of hepatic δ-aminolaevulinate synthase is known to be increased over 500-fold *in vivo* after treatment of 18-day chick embryos with the drugs 2-allyl-2-isopropylacetamide (2mg) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (4mg) for 12h (Whiting & Granick, 1976*b*). To test whether the synthesis of δ-aminolaevulinate synthase could be measured *in vitro*, postmitochondrial supernatants from the livers of normal and drug-treated chick embryos were incubated in the presence of [^3H]leucine under conditions that allowed protein synthesis. The labelled soluble proteins released from the polyribosomes were mixed with a specific antibody directed against chick-embryo liver mitochondrial δ-aminolaevulinate synthase, and the radioactivity in the immunoprecipitates was determined. The results are shown in Table 1.

Postmitochondrial supernatants from solvent-treated control livers synthesized an amount of total protein similar to that of the postmitochondrial supernatants from induced livers, but there was a large difference in the amount of radioactivity in the immunoprecipitates formed with non-radioactive carrier enzyme. A relatively small amount was obtained from incubation of normal liver postmitochondrial supernatant, whereas immunoprecipitates from drug-treated liver supernatant contained radioactivity representing 2.2% of the total protein labelled, or 6.7% of the labelled protein released from the polyribosomes. Although no enzyme activity was detectable after incubation of either supernatant, the

Table 1. *Synthesis of δ-aminolaevulinate synthase by postmitochondrial supernatants from chick-embryo liver*

Postmitochondrial supernatants (0.5ml) from normal and drug-treated chick-embryo livers were incubated at 30°C for 30min, with appropriate salts, amino acids, an energy supply and [^3H]leucine, as described in the Experimental section. Synthesis of enzyme released from polyribosomes was measured by immunoprecipitation with specific antibody. Radioactivity is expressed as 10^{-3} × d.p.m./2.5ml of incubation mixture.

Treatment	Enzyme activity (units/g of liver)	Radioactivity incorporated into: Total protein (*a*)	Released protein (*b*)	Immuno-precipitate (*c*)	Relative synthesis (*c*/*a*)
Solvent	~5	2300	720	1.1	0.04
2-Allyl-2-isopropylacetamide (2mg) + 3,5-diethoxycarbonyl-1,4-dihydrocollidine (4mg) for 12h	2000	1780	590	39.8	2.23

results indicate that the synthesis of δ-aminolaevulinate synthase, as measured by specific immunoprecipitation, occurred during the incubation of drug-treated liver postmitochondrial supernatants.

The time-course of synthesis of immunoprecipitable δ-aminolaevulinate synthase and total and soluble protein is shown in Fig. 1. After a lag of 5 min, which allowed temperature equilibration to 30°C, proteins were synthesized at a linear rate for 25min. Other workers have found liver polyribosomes to have dissociated after this time (Beck *et al.*, 1972). Up to 45% of protein synthesized was released from the polyribosomes, in agreement with Ballard *et al.* (1974). The radioactivity measured after immunoprecipitation of released protein also increased in a near-linear fashion with time, similar to the release of labelled protein from the polyribosomes. As the time for a ribosome to traverse half of an average-size mRNA molecule is around 3 min (Palmiter & Schimke, 1973), the results suggest that initiation of protein and enzyme synthesis is occurring under the incubation conditions. Support for this contention was provided by the finding that aurintricarboxylic acid (1 mM) and NaF (20 mM), compounds that have been proposed to inhibit protein synthesis by preventing initiation (Stewart *et al.*, 1971; Hoerz & McCarty, 1971), inhibited by 90% both enzyme synthesis and general protein synthesis by the liver supernatant. Cycloheximide (20 μg/ml) also inhibited enzyme synthesis, whereas chloramphenicol (20 μg/ml) did not.

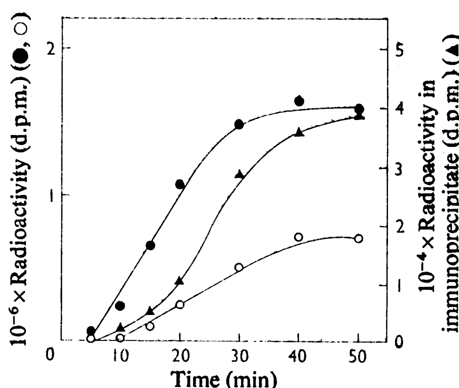

Fig. 1. *Time-course of the synthesis of protein and immunoprecipitable δ-aminolaevulinate synthase by drug-treated chick-embryo liver postmitochondrial supernatants*

Liver postmitochondrial supernatants were prepared and incubated to allow protein synthesis, and the incorporation of [³H]leucine into protein fractions was determined as described in the Experimental section. Radioactivity is expressed as the total incorporation per 2.5 ml of reaction mixture used for protein synthesis *in vitro*: ●, total protein; ○, released protein; ▲, immunoprecipitable protein.

Other experiments using liver postmitochondrial supernatants from chick embryos treated with 2-allyl-2-isopropylacetamide and/or 3,5-diethoxycarbonyl-1,4-dihydrocollidine for 12h showed a correlation between the enzyme activity of the liver homogenate and the synthesis of δ-aminolaevulinate synthase by the postmitochondrial supernatant prepared from the homogenate. This point is illustrated in Fig. 2, where the results of 16 different experiments are presented. In five separate experiments the apparent relative synthesis of δ-aminolaevulinate synthase by normal liver postmitochondrial supernatants was 0.04, 0.16, 0.17, 0.18 and 0.25%, compared with values of approx. 0.7% for 2-allyl-2-isopropylacetamide-treated, 1.4% for 3,5-diethoxycarbonyl-1,4-dihydrocollidine-treated and 2.0% for 2-allyl-2-isopropylacetamide + 3,5-diethoxycarbonyl-1,4-dihydrocollidine-treated chick-embryo liver supernatants. However, the small amount of radioactivity present in immunoprecipitates from incubation mixtures containing normal liver supernatant was due to non-specific trapping of labelled proteins, since sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of these precipitates revealed no significant peaks of radioactivity corresponding to δ-aminolaevulinate synthase (see Fig. 3, and under 'Comparison between δ-aminolaevulinate synthase synthesized *in vitro* and *in vivo*'). Thus there was no detectable synthesis of δ-aminolaevulinate synthase by normal liver postmitochondrial supernatants, a finding consistent with previous pulse-labelling studies *in vivo*, which have shown that the relative synthesis of the mitochondrial enzyme in normal liver is less than 0.005% and difficult to measure accurately (Whiting & Granick, 1976*b*). Mixing experiments using normal and drug-treated liver postmitochondrial supernatants indicated that no inhibitor of δ-aminolaevulinate synthase synthesis was present in normal liver cytosol. Also, addition of both drugs directly to the system *in vitro* had no effect on enzyme synthesis.

Comparison between δ-aminolaevulinate synthase synthesized in vitro and in vivo

The antibody–antigen precipitates obtained from reaction mixtures for protein synthesis *in vitro* were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to verify the specificity of the immunoprecipitation reaction in the presence of the other labelled proteins and a 1000-fold excess of free [³H]leucine.

As shown in Fig. 3, over 70% of the radioactivity present in immunoprecipitates from incubation mixtures directed by postmitochondrial supernatants from drug-treated livers was present in one major protein peak (slice 7). The mol.wt. of this protein was determined as 70000±7000 after calibration of the gel system with protein standards by the method of

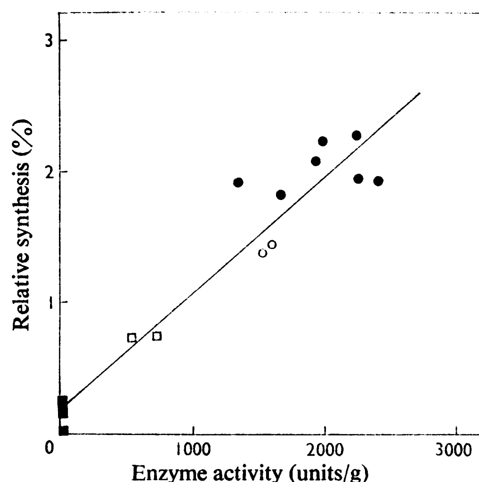

Fig. 2. *Correlation between enzyme activity in liver homogenates and enzyme synthesis in vitro by the postmitochondrial supernatants*

Groups of three to five chick embryos were treated with solvent (■), 2-allyl-2-isopropylacetamide (2mg) (□), 3,5-diethyoxycarbonyl-1,4-dihydrocollidine (4mg) (○), or 2-allyl-2-isopropylacetamide (2mg) plus 3,5-diethoxycarbonyl-1,4-dihydrocollidine (4mg) (●), for 12h. Livers were removed, homogenized and homogenates assayed for δ-aminolaevulinate synthase activity as described in the Experimental section. Postmitochondrial supernatants were prepared from the liver homogenates and incubated to allow protein synthesis. The relative synthesis of δ-aminolaevulinate synthase is expressed as (d.p.m. incorporated into immunoprecipitable protein × 100) divided by (d.p.m. incorporated into total protein).

Weber & Osborn (1969). Some radioactivity of unknown significance was always present in a second peak of lower molecular weight (slice 13). As discussed above, immunoprecipitates from incubation mixtures containing normal postmitochondrial supernatant showed no significant radioactivity in any peaks (Fig. 3), indicating a high degree of specificity in the immunoprecipitation technique.

The mol.wt. of purified chick-embryo liver mitochondrial δ-aminolaevulinate synthase by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis is 49000 (Whiting & Granick, 1976*a*). This value was also obtained in previous studies where enzyme labelled *in vivo* was isolated from mitochondrial extracts by immunoprecipitation. To confirm in the present work that the antibody preparation specifically precipitated mitochondrial δ-aminolaevulinate synthase, chick embryos were drug treated, then pulse-labelled with [^{3}H]leucine, and radioactive δ-aminolaevulinate was isolated from the mitochondria as described in the Experimental section. As shown in Fig. 3, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis proved that mitochondrial δ-aminolaevulinate synthase of mol.wt. 49000 was specifically precipitated by the antibody used in this work.

Overall, the results suggest that δ-aminolaevulinate synthase is synthesized as a precursor of mol.wt. 70000 before incorporation into the mitochondrion. Previous work has shown that rat liver cytosolic δ-aminolaevulinate synthase is of larger size than the mitochondrial enzyme (Whiting & Elliott, 1972). However, δ-aminolaevulinate synthase activity has not been detected in the cytosol of chick embryo liver. Because of the greater sensitivity of radioimmunochemical methods, the cytosol fractions from normal and induced chick-embryo livers, which had been pulse-labelled *in vivo*, were immunoprecipitated, along with the mitochondrial fractions. The results are shown in Table 2.

In agreement with previous work (Whiting & Granick, 1976*b*), a large difference was seen in the amount of radioactivity in the immunoprecipitates from the mitochondrial extracts (column *c*), indicating a large difference in the relative synthesis of enzyme *in vivo* in normal and drug-treated livers. In addition, a smaller difference was measured in the amount of immunoprecipitable radioactivity from cytosol fractions (column *e*). When examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 4), the radioactivity profiles of the dissociated immunoprecipitates were at background value, except for a single peak from the drug-treated cytosol. The mobility of this peak was identical with that of δ-aminolaevulinate synthase synthesized by isolated liver polyribosomes *in vitro*. Thus, both *in vivo* and *in vitro*, δ-aminolaevulinate synthase is synthesized by cytoplasmic polyribosomes as a polypeptide of mol.wt. 70000.

From the results in Table 2, the proportion of immunoprecipitable radioactive δ-aminolaevulinate synthase in the cytosol *in vivo* can be estimated as less than 3% of the total per liver, implying that the newly synthesized enzyme is rapidly incorporated into the mitochondrion. However, preliminary experiments to allow incorporation of δ-aminolaevulinate synthase synthesized *in vitro* into isolated mitochondria have been unsuccessful.

Synthesis of δ-aminolaevulinate synthase by membrane-bound and free polyribosomes

The concept that membrane-bound polyribosomes are responsible for the synthesis of exportable proteins, whereas free polyribosomes synthesize intracellular proteins, has been widely discussed (see Campbell, 1970; Tata, 1971). Support for this idea has been obtained from studies on the polyribosomal site of synthesis of serum albumin (Takagi & Ogata, 1972), ferritin (Redman, 1969) and other proteins,

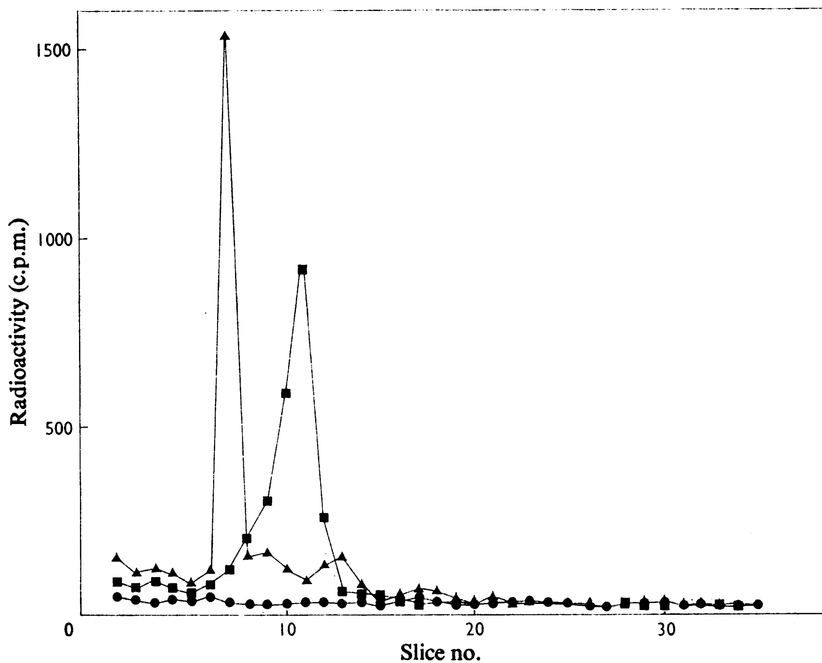

Fig. 3. *Comparison by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of mitochondrial δ-aminolaevulinate synthase with the enzyme synthesized by liver postmitochondrial supernatants*

The liver of a drug-treated chick embryo was labelled *in vivo* for 45 min. The mitochondria were isolated and extracted, and δ-aminolaevulinate synthase was immunoprecipitated as described in the Experimental section. Antibody–antigen precipitates were also prepared from protein-synthesis reaction mixtures *in vitro* containing postmitochondrial supernatants from normal and drug-treated chick-embryo livers. All immunoprecipitates were dissociated in sodium dodecyl sulphate, and portions subjected to polyacrylamide-gel electrophoresis to determine the radioactivity profiles: mitochondrial δ-aminolaevulinate synthase labelled *in vivo* (■); immunoprecipitates from the incubation of drug-treated (▲) and normal (●) postmitochondrial supernatants. Slice 1 is the top of the gel.

Table 2. *Immunoprecipitation of mitochondrial and cytosol fractions from normal and induced chick-embryo liver labelled in vivo*

Livers from normal and drug-treated chick embryos were labelled and fractionated as described in the Experimental section. One liver was used per experiment. Radioactivity incorporated is expressed as $10^{-3}\times$ d.p.m./liver.

		Radioactivity incorporated into:					
Treatment	Enzyme activity (units/g)	Cell protein (*a*)	Mitochondrial protein (*b*)	Immuno-precipitate from mitochondrial extract (*c*)	Cytosol protein (*d*)	Immuno-precipitate from cytosol (*e*)	Relative synthesis (%) (*c/a*)
Normal	~5	3550	820	0.69	670	0.88	0.019
2-Allyl-2-isopropylacetamide (2 mg)+ 3,5-diethoxycarbonyl-1,4-dihydrocollidine (4 mg) for 12 h	1610	3220	820	39.2	600	1.85	1.22

but in some cases both classes of cytoplasmic polyribosomes as isolated appear to participate in the synthesis of specific proteins.

The rapid induction of hepatic δ-aminolaevulinate synthase after drug treatment suggests that this protein must be imported by existing mitochondria. This

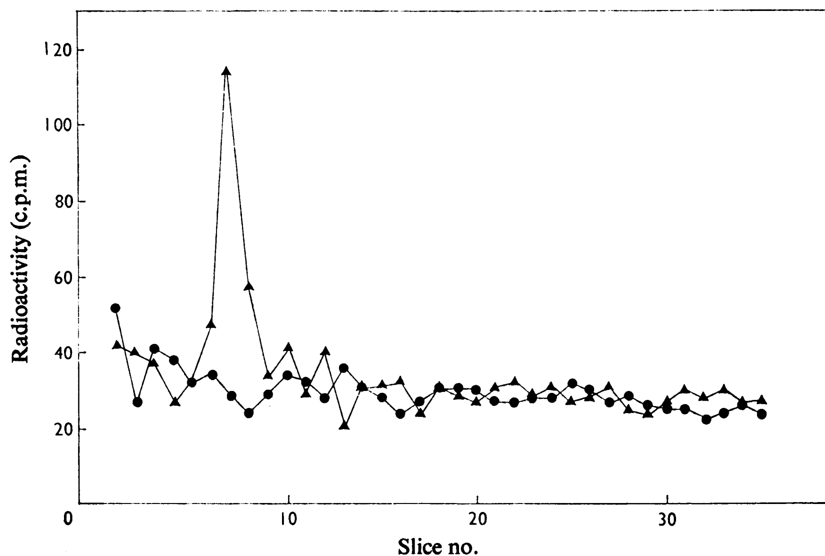

Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of dissociated immunoprecipitates obtained from liver cytosol fractions labelled in vivo*

Cytosol fractions were prepared from normal and drug-treated chick-embryo livers that had been pulse-labelled with [^{3}H]leucine as described in the Experimental section. To 1.0ml of cytosol fraction was added 50 units of partially purified enzyme as carrier, Triton X-405 to a concentration of 0.5% and a 25% excess of specific antibody to the mitochondrial enzyme. After immunological incubation, immunoprecipitates were washed, dissociated and 25 μl of dissociated protein solution was run on the gel. Slice 1 is the top of the gel. ●, Normal cytosol; ▲, drug-treated cytosol.

Table 3. *Synthesis of δ-aminolaevulinate synthase by free and membrane-bound polyribosomes*

Free and membrane-bound polyribosomes were prepared from the liver postmitochondrial supernatants of drug-treated chick embryos as described in the Experimental section, and incubated with cytosol fraction (0.5ml) for the measurement of protein synthesis. Radioactivity is expressed as 10^{-3} × d.p.m. incorporated by the polyribosomes present in 0.5ml of postmitochondrial supernatant. Results are the average of duplicate separations.

	Radioactivity incorporated into:				
Additions	Total protein (*a*)	Soluble protein (*b*)	Immuno-precipitate (*c*)	δ-Aminolaevulinate synthase (*d*)	Relative synthesis (%) (*d*/*a*)
Normal postmitochondrial supernatant	660	320	1.07	—	—
Drug-treated post-mitochondrial supernatant	1080	620	17.3	16.2	1.50
Free polyribosomes	560	410	12.1	11.0	1.96
Bound polyribosomes	350	200	3.5	2.4	0.69

process would require transport across the mitochondrial membranes, and may be mechanistically similar to the process of protein export across the cell membrane. Membrane-bound and free polyribosomes were therefore isolated and examined for their ability to synthesize δ-aminolaevulinate synthase.

As shown in Table 3, when drug-treated liver postmitochondrial supernatant was separated into free and membrane-bound polyribosomes, the relative synthesis of δ-aminolaevulinate synthase was increased in the free polyribosome fraction. Some synthesis by the membrane-bound polyribosomes was still apparent, but when corrected for radioactivity contributed by non-specific trapping of labelled proteins, as estimated from immunoprecipitation of an incubation mixture containing normal postmitochondrial supernatant, the synthesis of enzyme relative to total protein synthesis was threefold higher in the free polyribosome fraction. Electrophoresis of the immunoprecipitates confirmed that only half of

Table 4. *Effect of haemin on the synthesis of δ-aminolaevulinate synthase in vitro*

Chick embryos were treated with 2-allyl-2-isopropylacetamide (2mg) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (4mg) for a total of 12 or 15h. Haemin was either injected intravenously (0.4ml of a neutral 1mM solution) to groups of two or three embryos at 3h before killing, or was added *in vitro* to protein-synthesis incubation mixtures as indicated. Livers were removed, postmitochondrial supernatants prepared and the synthesis of δ-aminolaevulinate synthase and protein was measured *in vitro* as described in the Experimental section. Radioactivity is expressed as $10^{-3}\times$d.p.m. incorporated/2.5ml incubation mixture.

Treatment	Enzyme activity (units/g of liver)	Radioactivity incorporated into: Total protein (*a*)	Immunoprecipitate (*b*)	Relative synthesis (%) (*b*/*a*)
Expt. 1				
(a) 2-Allyl-2-isopropylacetamide+3,5-diethoxy-carbonyl-1,4-dihydrocollidine for 15h	2460	1090	15.6	1.43
(b) As in (a), with 10μM-haemin added *in vitro*	2460	1090	15.2	1.40
(c) As in (a), with haemin administered *in vivo*	1660	1180	5.3	0.45
(d) Duplicate of (c)	1770	1060	5.7	0.54
Expt. 2				
(a) 2-Allyl-2-isopropylacetamide+3,5-diethoxy-carbonyl-1,4-dihydrocollidine for 12h	1590	1750	24.6	1.40
(b) As in (a), with 10μM-haemin added *in vitro*	1590	1720	21.2	1.23
(c) As in (a), with haemin administered *in vivo*	1110	1680	7.0	0.43

the radioactivity in the immunoprecipitate from proteins synthesized by membrane-bound polyribosomes was due to δ-aminolaevulinate synthase. This small amount of synthesis could be the result of contamination with free polyribosomes. Thus the results indicate that the synthesis of δ-aminolaevulinate synthase is carried out preferentially, and perhaps even exclusively, on free polyribosomes.

Effect of haemin on the synthesis of δ-aminolaevulinate synthase in vitro

Low concentrations of haemin (less than 1μM) are known to prevent drug-induced increases in the synthesis of δ-aminolaevulinate synthase in chick-embryo liver cells (Granick *et al.*, 1975). Since haemin has been proposed to act at the post-transcriptional level (Sassa & Granick, 1970; Tyrrell & Marks, 1972), its effect on the synthesis of δ-aminolaevulinate synthase *in vitro* was investigated. When added directly to the incubation mixture at concentrations up to 10μM, no significant effect was observed on general protein synthesis, or on the synthesis of δ-aminolaevulinate synthase (Table 4). However, when chick embryos were treated intravenously with haemin for 3h *in vivo* and then a postmitochondrial supernatant was prepared and incubated to allow protein synthesis, the synthesis of δ-aminolaevulinate synthase was decreased by over 70%, with no change in total protein synthesis. In addition, the enzyme activity in the liver was decreased by approx. 30%.

These results show that a haemin does not directly affect the translation of endogenous mRNA for δ-aminolaevulinate synthase *in vitro*, but when administered *in vivo* to chick embryos for 3h, haemin can decrease δ-aminolaevulinate synthase activity and the capacity of liver postmitochondrial supernatants to synthesize the enzyme. In mixing experiments, liver supernatants from haemin-treated chick embryos did not inhibit the synthesis of δ-aminolaevulinate synthase as carried out by liver supernatants from drug-treated chick embryos. Thus haemin appears to act *in vivo* by decreasing the amount of mRNA for the enzyme, rather than by causing the accumulation of a specific translational inhibitor of enzyme synthesis.

Discussion

The present work has demonstrated that postmitochondrial supernatants prepared from drug-treated, but not normal, chick-embryo livers are able to synthesize a polypeptide which has been identified as δ-aminolaevulinate synthase. The identification was based on the ability of a specific antibody against purified mitochondrial δ-aminolaevulinate synthase to precipitate the newly synthesized enzyme, and the fact that, when measured *in vitro*, the synthesis of the polypeptide is strongly correlated with the activity of δ-aminolaevulinate synthase in the liver. This latter finding has also been reported from studies of mitochondrial δ-aminolaevulinate synthase synthesis *in vivo* (Whiting & Granick, 1976*b*). In addition, the δ-aminolaevulinate synthase-repressor haemin, when administered *in vivo*, was able to inhibit specifically the synthesis of the immunoprecipitable polypeptide

by postmitochondrial supernatants. These results then strongly support the identification of the polypeptide as δ-aminolaevulinate synthase, although peptide-'mapping' studies will be required to conclusively prove a relationship to the mitochondrial enzyme because of the larger molecular weight of the polypeptide synthesized *in vitro*.

Previous pulse-labelling experiments *in vivo* have shown that the drugs 2-allyl-2-isopropylacetamide and 3,5-diethoxycarbonyl-1,4-dihydrocollidine dramatically increase the synthesis of mitochondrial δ-aminolaevulinate synthase by up to 500-fold in chick-embryo liver (Whiting & Granick, 1976*b*). At 12h after drug treatment, 1.0% of the total radioactivity in intracellular liver proteins was isolated in δ-aminolaevulinate synthase from the mitochondria. By using the same drug treatment, the synthesis of δ-aminolaevulinate synthase measured *in vitro* in the present study was estimated to be around 2.0% of total protein synthesis when corrected for nonspecific trapping of labelled protein in the immunoprecipitates.

The apparent higher relative enzyme synthesis by isolated liver polyribosomes compared with intact animals could be due to several factors. First, the molecular weight of the δ-aminolaevulinate synthase polypeptide that was synthesized *in vitro* was 1.4 times that of the polypeptide labelled *in vivo* and isolated from the mitochondria. Also, the synthesis of δ-aminolaevulinate synthase *in vivo* was probably underestimated, since no correction was made for losses of mitochondria during cell fractionation, or incomplete extraction of enzyme from the mitochondria by sonication. In addition, if only the immunoprecipitable radioactivity migrating as a single peak on a sodium dodecyl sulphate/polyacrylamide gel is included in the calculations of relative synthesis, as in the studies *in vivo*, the value for δ-aminolaevulinate synthase synthesis *in vitro* is decreased to around 1.4%. Thus when these factors are taken into account, there is reasonable agreement between the relative synthesis of δ-aminolaevulinate synthase occurring either in the intact liver or by incubation of isolated liver polyribosomes.

The finding that the δ-aminolaevulinate synthase polypeptide synthesized in the absence of mitochondria was shown to have a mol.wt. of 70000 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, as compared with a mol.wt. of 49000 for the enzyme subunit from mitochondria, is consistent with previous results, which have indicated that cytosolic δ-aminolaevulinate synthase is larger than the enzyme extracted from mitochondria. Whiting & Elliott (1972) have shown that although rat liver δ-aminolaevulinate synthase from the cytosol was of mol.wt. 178000 by Sephadex chromatography, and much larger than the native enzyme from mitochondria (mol.wt. 78000), an antibody against the mitochondrial enzyme was able to immunoprecipitate the cytosol enzyme completely. The cytosol enzyme was therefore regarded as a precursor of the mitochondrial form, and a structural modification was postulated to occur during the process of transport or incorporation of enzyme into the mitochondrion. The present results provide further support for this model, and demonstrate that a proteolytic cleavage of the δ-aminolaevulinate synthase subunit polypeptide may take place during the transfer process. However, the possibility cannot be excluded that the decrease in the molecular weight of the enzyme occurs after disruption of the mitochondrion, and is totally unrelated to the mechanism of incorporation of the enzyme into the mitochondrion.

More detailed information on the molecular structure and properties of the cytosol enzyme would be of great interest for comparison with the purified mitochondrial enzyme, and could help in conclusively proving a precursor/product relationship between the two proteins. Unfortunately, the activity of δ-aminolaevulinate synthase in the cytosol fraction from drug-treated chick-embryo liver is undetectable by colorimetric assay (Whiting & Granick, 1976*a*), suggesting that newly synthesized enzyme is rapidly and efficiently incorporated into the mitochondria. However, a very small amount of immunoprecipitable enzyme of mol.wt. 70000 was detected in the cytosol fraction of pulse-labelled chick-embryo livers in the present work. This finding confirms that the enzyme is synthesized extramitochondrially as a polypeptide of mol.wt. 70000 *in vivo* as well as *in vitro*.

More than 90% of mitochondrial proteins are made on cytoplasmic ribosomes (Hawley & Greenawalt, 1970), yet almost nothing is known about how proteins are transported and integrated into the mitochondria (Schatz & Mason, 1974). Since the amount of δ-aminolaevulinate synthase present in mitochondria can be increased up to 600-fold by drugs in 18h (Whiting & Granick, 1976*a*), this enzyme is uniquely suitable for a study of protein uptake by mitochondria. The rapid induction and short half-life of around 1h, plus the intramitochondrial localization of being loosely attached to, or inside, the inner membrane (McKay *et al.*, 1969; Zuyderhoudt *et al.*, 1969), imply that this enzyme must be imported across the inner and outer membranes by existing mitochondria.

Although it has been demonstrated that the mitochondrial protein, cytochrome *c*, is synthesized mainly on membrane-bound polyribosomes, and it has been proposed that the endoplasmic reticulum plays an important role in mitochondrial biogenesis (Gonzalez-Cadavid & De Cordova, 1974), δ-aminolaevulinate synthase has been shown to be synthesized mainly on free polyribosomes. Thus the mechanism of transport of this enzyme across the mitochondrial membranes may differ from that for cytochrome *c*,

and is probably not the same as that involved in the export of proteins such as albumin across the liver-cell membrane. A model of vectorial translation of protein by cytoplasmic ribosomes attached to the outer mitochondrial membrane, as proposed in yeast by Kellems *et al.* (1975), also seems unlikely. On the other hand, the incorporation does not appear to be a simple uptake process, since the addition of mitochondria to incubation mixtures actively synthesizing δ-aminolaevulinate synthase did not result in measurable transfer of immunoprecipitable enzyme from the soluble fraction (M. J. Whiting, unpublished work).

From studies with chick-embryo liver cells in tissue culture, Sassa & Granick (1970) and Tyrrell & Marks (1972) have proposed that 3,5-diethoxycarbonyl-1,4-dihydrocollidine and 2-allyl-2-isopropylacetamide increase the amount of mRNA for δ-aminolaevulinate synthase, whereas haemin represses enzyme synthesis in a manner similar to cycloheximide, and therefore acts at the translational level. The present results further support the first postulate, that the action of inducing chemicals is to increase the amount of mRNA for the enzyme in the cytoplasm. However, haemin itself did not directly affect the translation of endogenous mRNA for δ-aminolaevulinate synthase *in vitro*. On the other hand, when administered *in vivo* for 3h, the ability of liver post-mitochondrial supernatants to synthesize δ-aminolaevulinate synthase was greatly decreased. These results are best interpreted as indicating that *in vivo*, haemin decreases the amount of mRNA for the enzyme available for translation, a proposal not easily reconciled with the results of Sassa & Granick (1970) and Tyrrell & Marks (1972). A direct assay for the mRNA for δ-aminolaevulinate synthase is needed to clarify the exact site of action of haem in the feedback repression of the synthesis of δ-aminolaevulinate synthase.

I thank Professor S. Granick of the Rockefeller University, New York, for the antibody which was prepared in his laboratory, and Professor W. H. Elliott for helpful suggestions. This work was supported by a Queen Elizabeth II Post-doctoral Fellowship.

References

Ballard, F. J. & Hanson, R. W. (1969) *J. Biol. Chem.* **244**, 5625–5630

Ballard, F. J., Hopgood, M. F., Reshef, L., Tilghman, S. & Hanson, R. W. (1974) *Biochem. J.* **144**, 199–207

Beck, J. P., Beck, G., Wong, K. Y. & Tomkins, G. M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3615–3619

Bloemendal, H., Benedetti, E. C. & Bont, W. S. (1974) *Methods Enzymol.* **30**, 313–327

Campbell, P. N. (1970) *FEBS Lett.* **7**, 1–7

Gonzalez-Cadavid, N. F. & De Cordova, C. S. (1974) *Biochem. J.* **140**, 157–167

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

Granick, S. & Sassa, S. (1971) in *Metabolic Pathways* (Vogel, H. J., ed.), vol. 5, pp. 77–141, Academic Press, New York

Granick, S., Sinclair, P., Sassa, S. & Grieninger, G. (1975) *J. Biol. Chem.* **250**, 9215–9225

Hawley, E. S. & Greenawalt, J. W. (1970) *J. Biol. Chem.* **245**, 3574–3583

Hayashi, N., Yoda, B. & Kikuchi, G. (1968) *J. Biochem. (Tokyo)* **63**, 446–452

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

Hoerz, W. & McCarty, K. S. (1971) *Biochim. Biophys. Acta* **228**, 526–535

Kellems, R. E., Allison, V. F. & Butow, R. A. (1975) *J. Cell Biol.* **65**, 1–14

Kitto, G. B. & Kaplan, N. O. (1966) *Biochemistry* **5**, 3966–3980

McKay, R., Druyan, R., Getz, G. S. & Rabinowitz, M. (1969) *Biochem. J.* **114**, 455–461

Nisselbaum, J. S. & Bodansky, O. (1966) *J. Biol. Chem.* **241**, 2661–2664

Ohashi, A. & Kikuchi, G. (1972) *Arch. Biochem. Biophys.* **153**, 34–46

Palmiter, R. D. & Schimke, R. T. (1973) *J. Biol. Chem.* **248**, 1502–1512

Redman, C. M. (1969) *J. Biol. Chem.* **244**, 4308–4315

Richardson, A., McGown, E., Henderson, L. M. & Swan, P. B. (1971) *Biochim. Biophys. Acta* **254**, 468–477

Sassa, S. & Granick, S. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 517–522

Schatz, G. & Mason, T. L. (1974) *Annu. Rev. Biochem.* **43**, 51–87

Stewart, M. L., Grollman, A. P. & Husang, M. T. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 97–101

Takagi, M. & Ogata, K. (1972) *Biochem. Biophys. Res. Commun.* **42**, 125–131

Tata, J. R. (1971) *Sub-Cell. Biochem.* **1**, 83–89

Tyrrell, D. L. J. & Marks, G. S. (1972) *Biochem. Pharmacol.* **21**, 2077–2093

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Whiting, M. J. & Elliott, W. H. (1972) *J. Biol. Chem.* **247**, 6818–6826

Whiting, M. J. & Granick, S. (1976*a*) *J. Biol. Chem.* **251**, 1340–1346

Whiting, M. J. & Granick, S. (1976*b*) *J. Biol. Chem.* **251**, 1347–1353

Zuyderhoudt, F. M., Borst, P. & Huijing, F. (1969) *Biochim. Biophys. Acta* **178**, 408–411