STUDIES ON THE MECHANISM OF INDUCTION OF δ-AMINOLEVULINIC ACID SYNTHASE

A Thesis

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by

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SUMMARY

1. Isolated liver cells were prepared from 17-day old chick embryos and incubated in Eagle's basal medium. Induction of δ-aminolevulinate (ALA) synthase occurred upon addition of chemical inducers 2-allyl-2-isopropyl-acetamide (AIA) or 3,5-diethoxycarbonyl-1,4-dihydro-collidine (DDC) and was totally dependent on the presence of Bt_2cAMP (or cAMP). Total porphyrins synthesized over a 10 h period accumulated to a concentration 10-fold greater than that in the control which did not contain AIA.

2. Removal of the inducer AIA by washing the cells at 3 h and resuspending in fresh media lacking AIA resulted in an immediate fall in the rate of enzyme synthesis. However, substitution of AIA at 3 h by deferoxamine methanesulphonate (DES), an inhibitor of heme synthesis, allowed continued ALA-synthase synthesis at an unaltered rate, even though this agent did not, by itself induce enzyme synthesis. Similar results were obtained when DDC was used as the pre-inducing drug. These results suggest that once induction of ALA-synthase is established, the inducer is no longer required and induction continues unchecked provided DES is present. The probable effect of the latter is to inhibit heme synthesis. The results are in keeping with the idea that after drug removal heme accumulation switches off ALA-synthase induction.

3. Exogenously added hemin was shown to completely inhibit AIA-mediated ALA-synthase induction at concentrations as low as 20 nM, a value that is less than the reported physiological one. The duration of inhibition was dependant

on the concentration of added hemin and was followed by a period of ALA-synthase synthesis at a rate similar to that of the control which contained AIA but no hemin. These data are consistant with the hypothesis that ALA-synthase synthesis is regulated by the concentration of intracellular heme.

4. The hypothesis was tested that induction of the heme degradative enzyme heme oxygenase might result in sufficient heme depletion to cause ALA-synthase induction. Isolated chick embryo liver cells were incubated in the presence of exogenous hemin and DES; after 11 h, the hemin was removed by extensive washing and the incubation was then continued in the presence of DES alone for a further 15 h. Microsomal heme oxygenase activity increased 3-fold in the first 11 h and following cell washings, ALA-synthase levels increased 23-fold. Heme oxygenase induction was dependant on the initial concentrations of exogenous hemin, and ALA-synthase induction correlated both with the induction of heme oxygenase and inhibition of endogenous heme synthesis by DES. The results demonstrate that induction of ALA-synthase can be achieved in the absence of a chemical inducer and suggest that removal of intracellular heme is the mechanism. These data are compatable with the hypothesis that chemical induction of ALA-synthase is a consequence of heme depletion.

5. Induction of ALA-synthase was shown to be dependant on both RNA and protein synthesis (reported by others). A study

of the comparative effect of cordycepin, cycloheximide and hemin has shown that at hemin concentrations up to 50 nM, the inhibition of ALA-synthase induction resembled the effect of cordycepin. However, at a hemin concentration of 2 μM, the inhibition of ALA-synthase induction resembled the effect of cycloheximide. These results are discussed with regard to the regulation of hepatic heme synthesis and the acute hepatic porphyria diseases of man.

ACKNOWLEDGEMENTS

I wish to sincerely thank Professor W.H. Elliott, Dr. B.K. May and Dr. J.D. Brooker for supervision, encouragement and rewarding discussion throughout the course of this work. Iain Borthwick provided much appreciated advice and helpful discussion at all stages of this project. Assistance and support was always forthcoming from the other members of the group and is gratefully acknowledged. I also wish to thank Miss J. Thompson and Miss L. Cirocco for competent technical assistance. My thanks are due to Mrs J. Brooker for her skilled typing of this thesis. Financial support of a University of Adelaide Post-graduated Research award is also acknowledged.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written, except where due reference is made in the text.

GOPESH SRIVASTAVA.

ABBREVIATIONS

The abbreviations used in this thesis are acceptable to the *Biochemical Journal*, or are defined in the text.

CHAPTER ONE

GENERAL INTRODUCTION

The field of heme, hemoproteins, heme metabolism and their relation to the disease porphyria has been reviewed extensively by Granick and Sassa (1971), Marver and Schmid (1972), Tschudy (1974). More recently knowledge in this field has been critically assessed by De Matteis and Aldridge (1978), Granick and Beale (1978) and Elder (1981). Therefore, only a brief introduction will be given with emphasis on areas relevant to the work in this thesis.

1.1 THE BIOSYNTHESIS AND DEGRADATION OF HEME

The biosynthetic and degradative pathways of heme are shown in Fig. 1.1. The former is most active in two cell types; erythropoietic cells, in which heme is required for the synthesis of hemoglobin, and liver parenchymal cells which synthesize hemoproteins such as catalase, tryptophan pyrrolase and cytochrome P-450. Secondary to developing erythrocytes, the liver is the major site of heme synthesis. The former accounts for about 80%, and the latter for about 20% of heme which turns over daily.

The activity of the heme biosynthetic pathway in hepatocytes is tightly regulated and normally low. But heme seldom acts by itself in cellular metabolism; rather, it functions in the form of hemoproteins. Thus the synthesis of heme and its relevant apo-protein must be co-ordinated, since neither accumulates in cells (Marver *et al.*, 1966; Gonzales-Cadavid *et al.*, 1972; Druyan *et al.*, 1973; Bellzard *et al.*, 1973 and Gross and Robinowitz, 1972).

FIGURE 1.1

THE BIOSYNTHESIS AND DEGRADATION OF HEME

Numerical key to enzymes:

1: ALA-synthase

2: ALA dehydratase

3: PBG deaminase

4: Uroporphyrinogen III cosynthase

5: Uroporphyrinogen decarboxylase

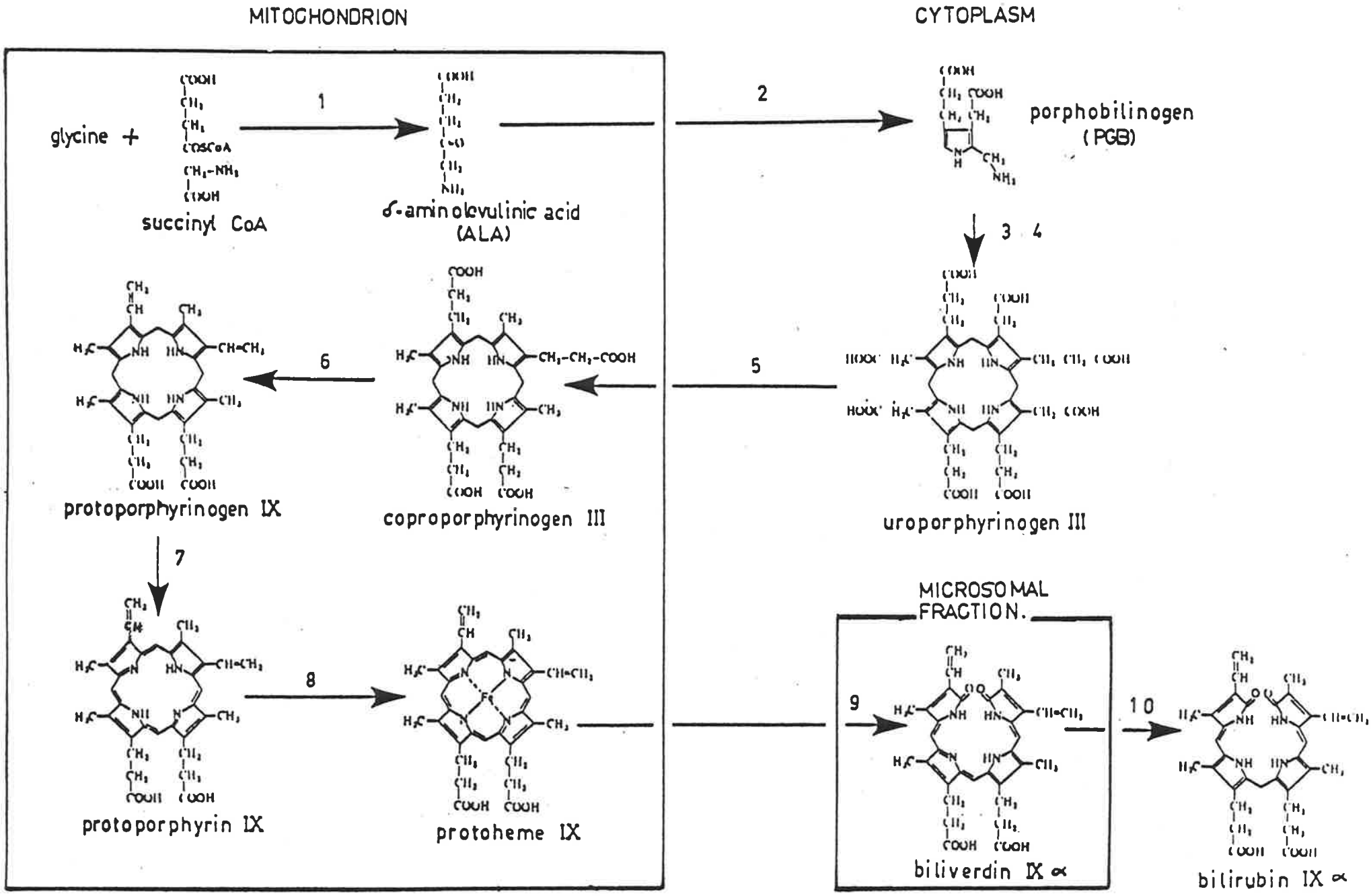
6: Coproporphyrinogen oxidase

7: Protoporphyrinogen oxidase

8: Heme synthetase

9: Heme oxygenase

10: Biliverdin reductase


MITOCHONDRION
CYTOPLASM
glycine +
succinyl CoA
1
δ-aminolevulinic acid (ALA)
2
porphobilinogen (PGB)
3 4
uroporphyrinogen III
5
coproporphyrinogen III
6
protoporphyrinogen IX
7
protoporphyrin IX
8
protoheme IX
MICROSOMAL FRACTION.
9
biliverdin IX α
10
bilirubin IX α

The first and major rate limiting enzyme of the heme biosynthetic pathway in animals and bacteria is δ-amino-levulinic acid (ALA) synthase [succinyl-CoA glycine C-succinyl transferase (decarboxylating) EC 2.3.1.3.7] (Enzyme 1, Fig. 1.1). This fact is best illustrated by the demonstration that addition of exogenous ALA will increase the synthesis of porphyrins and heme (Granick, 1966; Doss, 1969; De Matteis and Gibbs, 1972; Druyan and Kelly, 1972), while addition of the precursors of ALA will not. A comparison of the activities of the various enzymes of pathway is also compatible with ALA-synthase normally being the rate limiting step (De Matteis, 1975). Evidence suggests that the enzyme (and possibly its mRNA) from mammals and avian livers has a relatively short half life and is normally present at low concentrations.

In mammalian and avian cells, ALA-synthase is localized in the mitochondria. The small amount found in other sub-cellular fractions, such as the cytoplasm, is probably due to enzyme released from mitochondria which have been damaged or disrupted during tissue fractionation (Patton and Beattie, 1973). By comparing the distribution of ALA-synthase in sub-mitochondrial fractions from liver, with that of marker enzymes, it appears that ALA-synthase is free in the matrix, or loosely bound to the inner mitochondrial membrane (Patton and Beattie, 1973; McKay *et al.*, 1969; Barnes *et al.*, 1971; Zuyderhoudt *et al.*, 1969). However, in the livers of animals (rats, adult chickens etc.), treated with porphyrinogenic drugs (Section 1.3), where ALA-synthase level is much higher than normal, a significant proportion of the total activity

is present in the cytoplasm (Yamauchi *et al.*, 1980), much more than can be accounted for by mitochondrial damage. After ALA-synthase the next most rate limiting enzyme is heme synthase, also known as ferrochelatase. It catalyzes the incorporation of Fe^{2+} into protoporphyrin IX to form heme.

In contrast to heme biosynthesis in animal and avian species the status of ALA-synthase in plants is uncertain. ALA-synthase has not been detected in extracts of any g reening plant tissue, including algae. Rather, ALA in these tissues appears to arise by way of a completely different route, not involving glycine or succinyl CoA, but utilising α-ketoglutarate and glutamate. Beale *et al.* (1975) have discussed hypothetical pathways for the synthesis of ALA in plants. One possible intermediate is γ,δ-dioxovalerate.

1.2 THE PORPHYRIA DISEASES OF MAN

Under normal conditions the intermediates of the heme biosynthetic pathway (the porphyrins and their precursors ALA and porphoblinogen) seldom accumulate and appear in the urine only in very small amounts. There are conditions, however, where control of the heme biosynthetic pathway is disturbed and far more porphyrins and earlier precursors are synthesized than become incorporated into heme. These porphyrins accumulate in large amounts and are excreted. These conditions are known as porphyrias, and on the basis of the tissue localization of the disorders, have been

classified as either erythropoietic or hepatic forms. The erythropoietic porphyrias consist of two distinct varieties, congenital erythropoietic porphyria and erythropoietic protoporphyria, which differ in the type and amount of porphyrin over-produced.

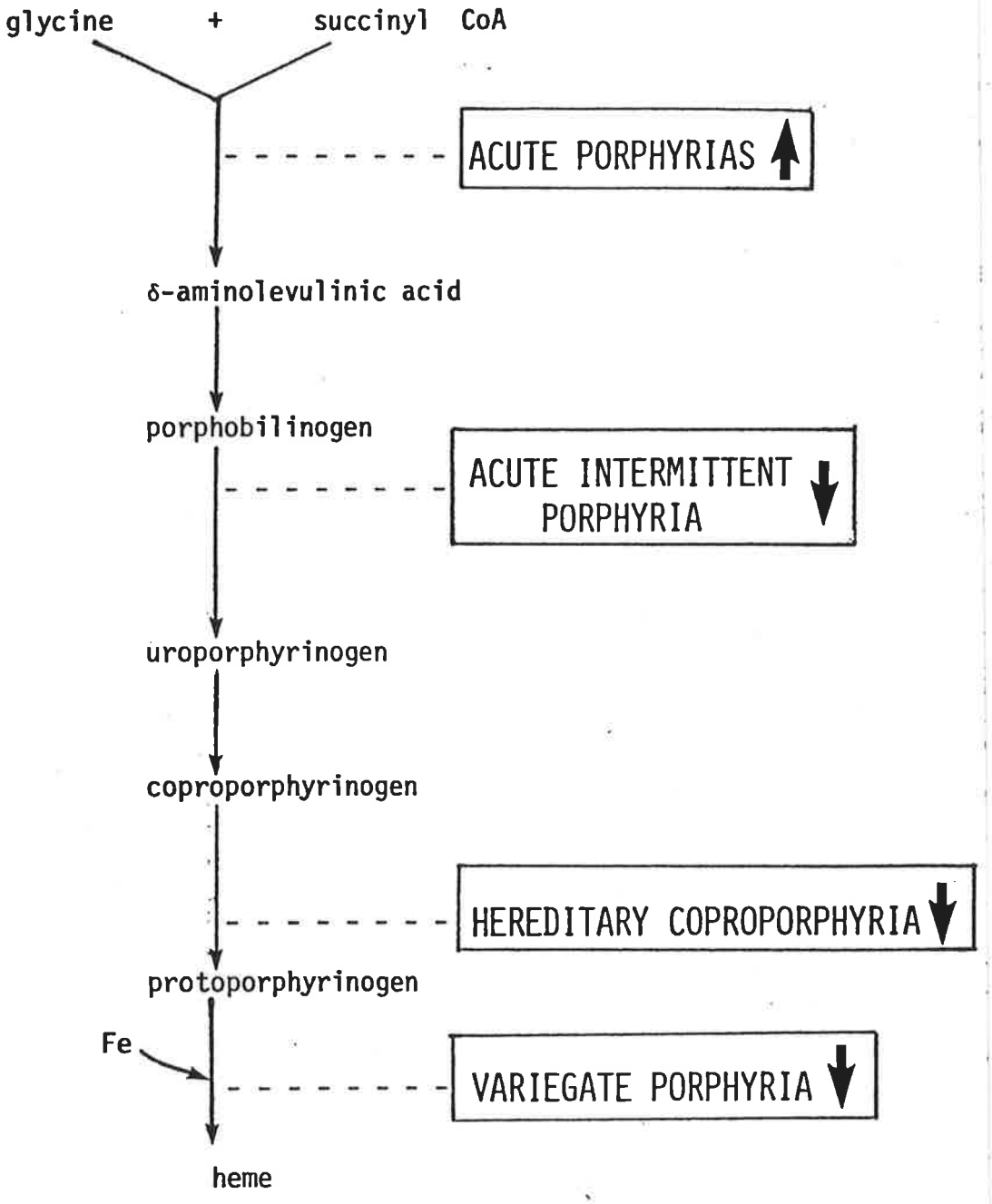
In attacks of the hepatic acute porphyrias (acute intermittent porphyria, variegate porphyria and hereditary coproporphyria), the common feature is elevated levels of ALA-synthase combined with a decreased level of at least one other enzyme of the pathway (Fig. 1.2). These diseases have the following characteristics (a) They are inherited as autosomal dominant defects. (b) The disease becomes clinically manifest as acute neurologic disorders and is usually only observed after puberty. (c) The disease is usually latent but neurologic symptoms may be precipitated by low concentrations of various drugs including anticonvulsants, barbiturates, hypotensive agents, steroids and tranquillizers.

As mentioned earlier, an increase in ALA-synthase levels measured in liver biopsy and autopsy samples has been reported for most forms of acute hepatic porphyria, and this is probably the main reason for increased production of porphyrins and their precursors. But whether the primary lesion in these conditions is with a gene directly controlling ALA-synthase or, more likely, a defect in one or more of the following enzymes of the pathway is not proven, although there is considerable evidence to support the latter view.

As well as being studied from a medical viewpoint,

FIGURE 1.2

CHANGES IN ENZYME LEVELS IN THE ACUTE PORPHYRIAS



porphyria is of historical interest. Following their investigation into the possible reasons for the reported insanity of King George III, Macalpine and Hunter (1969) stated that "a picture unfolded which revealed the purple thread of porphyria running through the royal houses from the Tudors to Hanoverians, and from the Hanoverians to the present day". Thus they have suggested that George III, and Queen of Scots, James VI and George IV suffered from porphyria.

1.3 INDUCTION OF ALA-SYNTHASE BY DRUGS IN NORMAL LIVER

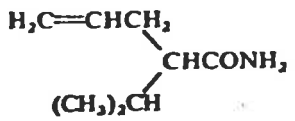
Whilst the acute hepatic porphyrias are usually latent, a number of drugs are known to precipitate overt attacks (Granick and Urata, 1963). Similar drugs at higher concentrations have also been observed to cause elevation of ALA-synthase levels in normal mammalian livers (De Matteis, 1971) and in avian liver cells (Granick, 1966; Sassa and Kappas, 1977; Morgan *et al.*, 1977 and Tomita *et al.*, 1974). Elevated levels of porphyrins also occur (Granick, 1966). The biochemical features of this experimental porphyria thus resemble those of human disease, the administration of chemical inducers triggering the response in both systems. These experimental systems have therefore provided a basis for further study of the mechanism by which drugs cause increased ALA-synthase levels (and therefore presumably acute attacks of the disease if the two are

FIGURE 1.3

FOUR CLASSES OF COMPOUNDS WHICH INDUCE HEPATIC PORPHYRIA IN EXPERIMENTAL ANIMALS

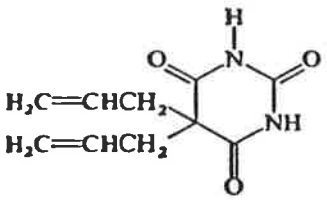
1. Derivatives of branched aliphatic acetamides,

   e.g. 2-allyl-2-isopropylacetamide (A1A).



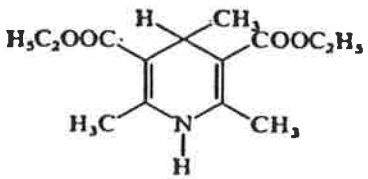
2. Barbiturates with at least one unsaturated side chain,

   e.g. 5,5-diallylbarbituric acid.



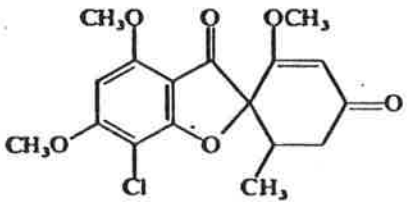
3. Compounds chemically related to collidine,

   e.g. 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC).



4. Compounds chemically related to griseofulvin,

   e.g. griseofulvin.



causally related).

Granick (1966) classified porphyrinogenic drugs into 4 main groups (Fig. 1.3) although in general there is no one structural feature common to all known inducing drugs. The only property that they appear to share is a high degree of lipid solubility (Wetterberg, 1976).

The drug mediated induction of ALA-synthase in the animal models has been demonstrated to involve *de novo* enzyme synthesis (Whiting and Granick, 1976). It should be noted however, that Simpson and Beattie (1980) have suggested that an increase in the activity of the enzyme in rat liver mitochondria involves activation of pre-existing enzyme rather than synthesis. At present it is difficult to reconcile this viewpoint with available evidence (Whiting and Granick, 1976) to the contrary.

Induction of ALA-synthase is prevented by exogenous heme (Granick, 1966; Tyrrell and Marks, 1972 and Whiting and Granick, 1976). But although there is general agreement that heme plays an important role in the physiological control of ALA-synthase (Granick, 1966; Ohashi and Kikuchi, 1972; Tyrrell and Marks, 1972; Tomita *et al.*, 1974), there is no concensus as to the mechanism by which drugs override this control and induce enzyme synthesis. A number of theories have been proposed and these are discussed in detail later in this chapter. Also, there is no general agreement on the mechanism of heme repression, and several different possibilities have been proposed; these are also discussed later in this chapter.

1.4 INDUCTION OF ALA-SYNTHASE AND ENZYMES OF SMOOTH ENDOPLASMIC RETICULUM

It is known that there is a very wide range of different drugs, chemicals and even steroids which cause increased hepatic heme biosynthesis; but what is the physiological rationale for this? In fact increased heme biosynthesis is only one aspect of a broad adaptive response of the body against foreign chemicals. Many of these chemicals also cause increased synthesis of smooth endoplasmic reticulum and changes in the levels of various enzymes involved in reactions which convert nonpolar compounds to polar, and therefore more excretable forms. This response is often referred to as induction of the microsomal mixed function oxidase system (Fig. 1.4). The most pronounced change in this response is an increased level of the hemoprotein cytochrome P-450. Evidence (Correia and Meyer, 1975; Rajamanickam *et al.*, 1975) indicates increased cytochrome P-450 is due to an increased rate of synthesis of the protein. As cytochrome P-450 is a hemoprotein, increased cytochrome P-450 would require increased heme synthesis (Estrabrook *et al.*, 1970).

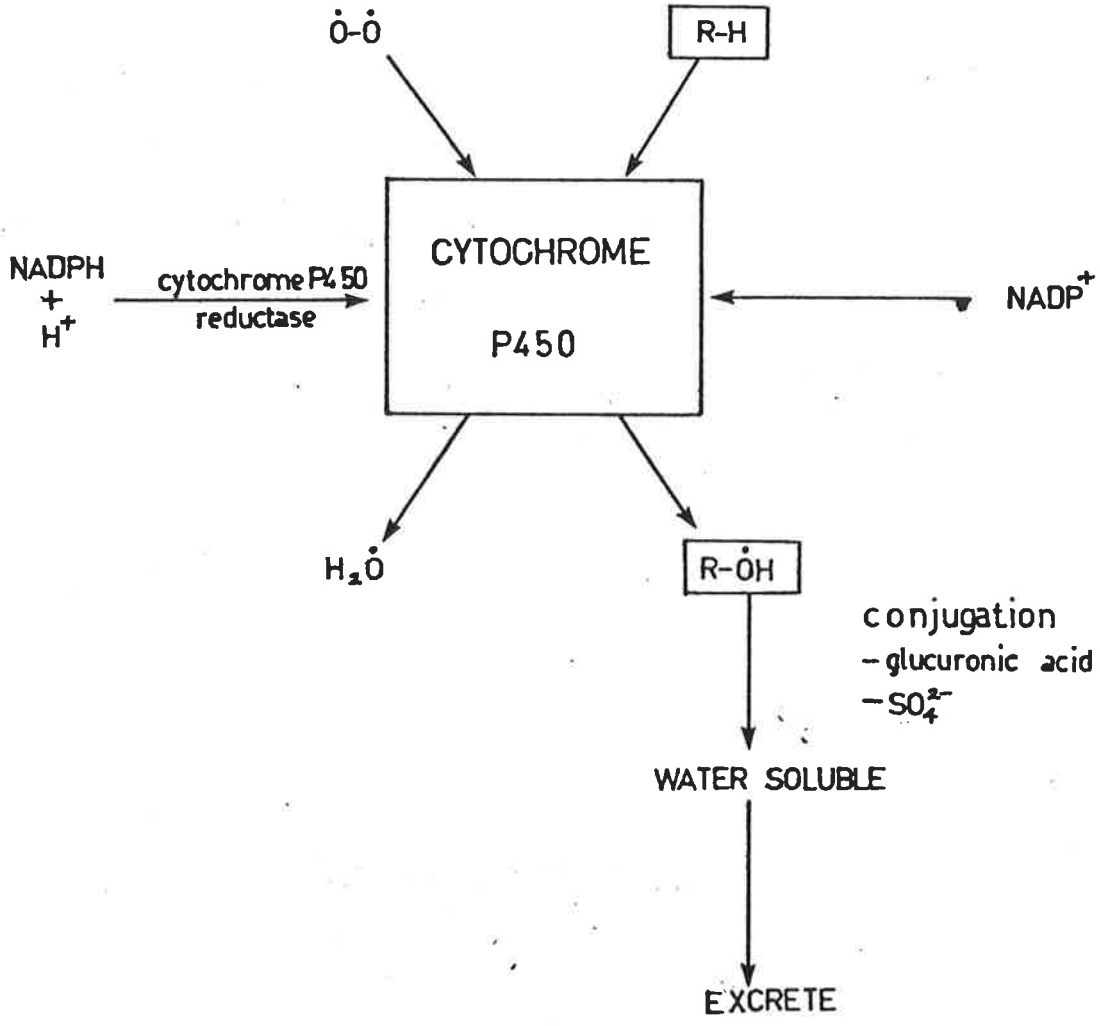
1.5 MECHANISM OF REPRESSION OF ALA-SYNTHASE BY HEME

Although there is general agreement that heme plays an important role in the physiological control of ALA-

FIGURE 1.4

THE GENERAL MECHANISM OF THE MICROSOMAL MONOOXYGENASE (OR MIXED FUNCTION OXYGENASE) SYSTEM

R-H = steroids, drugs, foreign chemicals



synthase synthesis (Granick, 1966; Tyrrell and Marks, 1972; Ohashi and Kikuchi, 1972 and Tomita *et al.*, 1974), there is no such consensus on the mechanism of heme action, and several different theories have been proposed.

Granick (1966) originally proposed that heme prevents the induction of ALA-synthase by repression of transcription. However, later studies by Sassa and Granick (1970) and Tyrrell and Marks (1972) suggested that, contrary to this, heme exerted its effect at a post-transcriptional stage. This conclusion was based on the fact that in chick embryo liver cells treated with actinomycin D, the half life of AIA induced ALA-synthase was longer (5.2 h) than in cells treated with hemin (3.6 h). However, the half-life of ALA-synthase in hemin treated cells was about the same as that in cells treated with cycloheximide (Tyrrell and Marks, 1972). Granick and Beale (1978) have proposed therefore, that hemin inhibits some post-transcriptional step, possibly in the processing of mRNA for ALA-synthase.

Whiting (1976) and Brooker (personal communication) have shown that hemin (at 10 μM) does not inhibit *in vitro* synthesis of ALA-synthase, in a chick embryo liver or wheat germ cell free extract respectively. These results suggest that hemin does not act at a translational step.

In contrast an entirely different explanation for heme action was proposed by Ohashi and Kikuchi (1972), Hayashi *et al.* (1972) and Yamauchi *et al.* (1980); that, in adult chickens, and rats, heme exerts its effect by controlling transport of cyto-plasmically synthesized ALA-synthase into the mitochondria. However, heme does not affect ALA-synthase

transport in embryonic chick livers (Tomita *et al.*, 1974; Tyrrell and Marks, 1972; Sassa and Granick, 1970).

Moreover, Paterniti and Beattie (1979) have suggested that high concentration of hemin (10 μM) inhibits rat liver mitochondrial ALA-synthase activity. At present however, it is difficult to reconcile this viewpoint with available evidence to the contrary (Whiting, 1976).

It should be noted however, that these mechanisms need not be mutually exclusive and it is possible that heme may exert its effect at several levels.

Because ALA-synthase synthesis is prevented by heme, this suggests that normally an intracellular pool of heme may exist to regulate the level of the enzyme. There is some experimental evidence for the existence of such a heme pool [10 - 100 nM, Granick *et al.* (1975)], but its location and concentration in the cell is not known, nor what role, if any, it plays in the control of ALA-synthase.

1.6 PROPOSED MECHANISM OF INDUCTION OF ALA-SYNTHASE

In Granick's original hypothesis (1966) porphyrinogenic drugs were regarded as competitors with heme for a site on an apo-repressor protein. Thus, a drug would displace heme from the apo-repressor and cause de-repression of ALA-synthase gene transcription. But although inducers have been characterised according to chemical structures, lipid solubility and steric considerations, no common structural features have emerged which would be consistent with this

hypothesis.

Sassa and Granick (1970) later suggested that different porphyria inducing drugs may act directly on either the transcription or translation of ALA-synthase. The half-life of ALA-synthase in chick embryo liver cells measured in the presence of cycloheximide was not altered by the porphyria inducing drugs AIA or DDC indicating that these drugs do not affect the rate of degradation of the enzyme. Actinomycin D however increased the apparent half-life of ALA-synthase and this effect was augmented by the addition of AIA. This increase was not observed with DDC. These results were taken as an indication that AIA caused a marked increase in the level of the enzyme by either increasing the lifetime of the mRNA for ALA-synthase or increasing its translational activity. DDC was suggested to act primarily by causing increased transcription of ALA-synthase mRNA.

The observation by Tyrrell and Marks (1972) however, suggested that both AIA and DDC acted at the level of transcription. They demonstrated that both cycloheximide and actinomycin D suppressed AIA induction of ALA-synthase. However, cells pretreated with a combination of AIA and cycloheximide for 5 h, washed and re-incubated in fresh medium (without AIA or cycloheximide) exhibited an immediate increase in ALA-synthase activity. This increase was enhanced by the addition of actinomycin D but was completely blocked when the cells were re-incubated in fresh medium containing cycloheximide. In contrast when the cells were pretreated with AIA and actinomycin D for 5 h, washed and re-incubated, there was little increase in ALA-synthase

activity. They obtained similar results with DDC.

Although, their results obtained with DDC were compatible with the postulated mechanism of DDC induction of ALA-synthase by Sassa and Granick (1970), the data on AIA effects are conflicting. Sassa and Granick (1970) suggested that AIA acted at the level of translation while the data of Tyrrell and Marks (1972) suggested that it acted at the level of transcription. Different concentrations of AIA were used. Sassa and Granick, (1970) used 60 µg/ml of AIA, while Tyrrell and Marks (1972) used 300 µg/ml. Whether this could be the reason for the conflicting data is not known.

Tomita *et al.* (1974) using chick embryo liver organ culture showed that the half-life of ALA-synthase, after the addition of actinomycin D, was the same whether or not the incubation medium contained AIA. The results are in apparent conflict with those of Sassa and Granick (1970); this could be due to the difference between organ and cell culture of chick embryo liver but there is not evidence on this point.

De Matteis (1970) suggested that heme repression is the sole control of ALA-synthase and that the drug-mediated induction of ALA-synthase could involve perhaps only the depletion of the regulatory heme pool. This would result in de-repression of the synthesis of ALA-synthase. Drugs may cause such a depletion of the intracellular heme pool by either inhibiting the synthesis of heme or enhancing the rate of cellular utilization.

1.7 POSSIBLE MECHANISM FOR REDUCING INTRACELLULAR HEME LEVELS

a) Decreased heme synthesis

This mechanism is exemplified by drugs such as DDC and griseofulvin. De Matteis and Abbritti (1973a) suggested that the primary role of DDC is to inhibit the activity of ferrochelatase which catalyses the incorporation of iron into heme protoporphyrin IX. They proposed that the regulatory heme pool would be depleted as a consequence of inhibition of heme synthesis and utilization of the unconjugated heme for hemoprotein synthesis. However, this proposal was contradicted by Granick *et al.* (1975) who showed that desferrioxamine mesylate (DES), an iron chelator and inhibitor of ferrochelatase, did not induce ALA-synthase. This might imply that over the experimental period turnover of hemoproteins is too low for heme depletion to be of any significance or that the lowering of the heme concentration per se is not sufficient to cause induction, and that the drugs must therefore have additional effects, e.g., increasing rate of turnover of hepatic hemoproteins. Rikind (1979) has also come to a similar conclusion by using sub-inducing levels of DDC which suppressed ferrochelatase without causing any increase in ALA-synthase synthesis.

b) Increased heme utilization

Certain chemicals (e.g., phenobarbital) are known to stimulate the synthesis of the apo-protein of cytochrome P-450 (Correia and Meyer, 1975), thereby producing a protein which has an unoccupied binding site for heme. The filling of this site would serve to deplete

cellular free heme and lead to induction of ALA-synthase.

c) Degradation of cytochrome P-450 heme

The hemoprotein cytochrome P-450 figures prominently in this mechanism. It is the major hepatic hemoprotein comprising up to 10% of microsomal protein and its synthesis utilises the major fraction of hepatic heme (Estrabrook *et al.*, 1970).

Certain xenobiotic substrates (e.g., AIA, allobarbital, secobarbital, allylisopropylacetylcarbamide) of the MFO system selectively destroy the heme moiety of cytochrome P-450 but not that of cytochrome b_5 or other hepatic hemoproteins (De Matteis, 1970, 1971a, 1973b; Meyer and Marver, 1971; Levin *et al.*, 1972; 1973). Thus cytochrome P-450 heme is apparently more labile than the heme of other hepatic hemoproteins.

Wada *et al.* (1968) and Waterfield *et al.* (1969) first described the decline in cytochrome P-450 following administration of AIA to mice and rabbits respectively. The cytochrome P-450 was measured utilizing an assay of the heme moiety associated with the cytochrome (reduced - carbonmonoxide spectrum). Therefore, the reported decline in the quantity of cytochrome P-450 is really an indication of the decline in the quantity of cytochrome P-450 with an associated heme moiety. Levin *et al.* (1972) treated rats with phenobarbital prior to administration of AIA and showed that the decline in cytochrome p-450 was accompanied by a characteristic brown-green discolouration of the whole liver, particularly intense in the microsomal fractions. In

further studies, [^{14}C-ALA] was given to rats prior to administration of AIA; radioactivity lost from the prelabelled cytochrome P-450 heme was recovered in the isolated 'green pigment' fractions (De Matteis, 1970; 1971a; Unseld and De Matteis, 1975; Farrell and Schmid, 1979). This result indicated that AIA destroyed the heme moiety of cytochrome P-450. Based on these results De Matteis (1971a) postulated a mechanism for AIA induction of ALA-synthase as follows: AIA destroys the heme moiety of cytochrome P-450 leaving the cytochrome P-450 apo-protein intact. The apo-protein subsequently accepts unconjugated liver heme resulting in lowering of the free heme levels. De Matteis (1973) suggested that this mechanism might account for the marked increase in ALA-synthase activity.

Corriea and Meyer (1975) and Correia *et al.* (1979) demonstrated that cytochrome P-450 apo-protein has the capacity to remain intact without a heme moiety and can form the holocytochrome by interacting with unconjugated heme. The authors have suggested that interaction of AIA wil the heme moiety of cytochrome P-450 may be a mechanism of heme removal. These workers also presented evidence that the 'green pigment' isolated from the microsomal fractions of AIA-treated rats is formed from the covalent attachment of AIA to the heme moiety of cytochrome P-450 (Ortiz de Montellano *et al.*, 1978). Tephly *et al.* (1979) reported the isolation of a green porphyrin like pigment from mice and rats treated with DDC, and De Matteis *et al.* (1980) have reported that this green pigment is in fact N.methylporphyrin which is different from the green pigment

isolated after AIA treatment.

As there is no evidence to indicate whether the destruction of cytochrome P-450 heme is a primary effect of the drug or an unrelated secondary effect, the cytochrome P-450 heme destruction theory has not yet been proposed as a general mechanism of drug induction of ALA-synthase.

Moreover, there is evidence which argues against the idea that increased degradation of cytochrome P-450 heme is essential for the induction of ALA-synthase.

1) The saturated analogue of AIA, 2-propyl-2-isopropylacetamide (PIA) was reported not to cause destruction of cytochrome P-450 heme and yet was equally effective as AIA in inducing porphyria in the chick embryo (De Matteis, 1971b; Krupa *et al.*, 1974).

2) Although the porphyrinogenic drug phenobarbital was reported to affect cytochrome P-450 heme the effect was apparently to stimulate the synthesis of cytochrome P-450 synthesis (Padmanaban *et al.*, 1973; Rajamanickam *et al.*, 1975). A model of ALA-synthase regulation was advanced in which the level of cytochrome P-450 apo-protein determined the induction of ALA-synthase via a positive control mechanism (Padmanaban *et al.*, 1973). No suggestion was made concerning the mechanism by which the drugs may stimulate cytochrome P-450 synthesis.

One of the more recent proposals by Granick and Beale (1978) is that inducing chemicals and/or their metabolites have two different simultaneous actions both of which are necessary for ALA-synthase induction to occur. One action

is to cause an increased synthesis of mRNA for ALA-synthase. The other action is to cause a depletion of free heme; as stated earlier this could occur in several ways. The chemical may (a) inhibit one or several steps of the heme biosynthetic chain (b) cause an increase in heme utilisation (c) cause an increase in heme degradation. Heme was proposed to inhibit some aspect of mRNA processing or transport rather than transcription. Granick and Beale (1978) have thus suggested that neither drug effect alone (a direct effect on gene control or heme decrease) is sufficient to induce ALA-synthase.

To summarize, there appear to be two possibilities to explain drug mediated induction of ALA-synthase. The first is that the inducing drug increases transcription of ALA-synthase gene and, in addition causes depletion of heme which was acting to modulate the expression of ALA-synthase mRNA. However, no compelling evidence for a direct gene controlling action exists. The second possibility is that heme repression is the sole control and that the drug-mediated induction of ALA-synthase involves only the depletion of the regulatory heme thus allowing transcription of ALA-synthase.

1.8 AIMS OF THE WORK IN THESIS

The aim of this work is to elucidate the molecular control of heaptic ALA-synthase and relate these findings to an explanation of the human hepatic porphyrias. The present investigation is concerned particularly with control

of drug induction of ALA-synthase. While the basic elements of control of ALA-synthase in liver (induction by drugs and repression by heme) are known, the precise biochemical mechanisms of these effects are not fully understood at the molecular level. A number of hypotheses on the action of inducers have been discussed earlier in this chapter, and to distinguish experimentally between these proposals is desirable. These consideration, then provide the rationale for experiments described in the following chapters.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Experimental animals

Fertilised hen eggs (White Leghorn) were obtained from the Department of Agriculture, Parafield Research Poultry Station, Adelaide, Australia, and maintained in a humidified cabinet at 37°C for 17 - 18 days prior to use.

2.1.2 Compounds tested as modifier of ALA-synthase activity

The following compounds were gratefully received as gifts: 2-allyl-2-isopropylacetamide (AIA) from Hoffman-La Roche, Nutley, N.J., U.S.A. and deferoxamine methane sulphonate (DES) from CIBA-GEIGY (Australia), N.S.W., Australia. 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) was purchased from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.

2.1.3 Chemicals used in ALA-synthase activity assay

The following chemicals were obtained from Sigma Chemical Co., St. Louis, MO., U.S.A.; Pyridoxal phosphate, levulinic acid, dithioerythritol, sodium succinate, glycine, coenzyme A, δ-aminolevulinic acid (ALA)-hydrochloride, and ATP. Acetylacetone was purchased from BDH Chemical Ltd. Dowex AG50W-X8 (H^+ form, 200 - 400 mesh) cation exchange resin was purchased from Bio-Rad Laboratories, Richmond, California.

2.1.4 Isotopes

The following radiochemicals were obtained from the Radiochemical Centre, Amersham: [2,3-^{14}C]-succinate (10 mCi/mmol), [3,5-^{3}H(N)]-ALA (13 Ci/mmol) and L-[4,5-^{3}H])-leucine (105 Ci/mol).

2.1.5 Compounds and medium used in cell suspension experiments

Crystalline bovine insulin, hydrocortisone, hyaluronidase, collagenase (Type I), Bt_2cAMP, 3-isobutyl-1-methylxanthine, cycloheximide, cordycepin, herparin (sodium salt), recrystallized lyophilised trypsin, and actinomycin D were obtained from Sigma Chemical Co., St. Louis, MO., U.S.A. Eagle's basal medium was from Commonwealth Serum Laboratories, Melbourne, Australia.

2.1.6 General reagents

General chemicals were routinely obtained from BDH Chemical Ltd., and were of AR grade.

2.1.7 Buffers

PEST buffer: 0.1 mM pyridoxal 5'-phosphate, 0.1 mm EDTA, 0.25 M sucrose and 5 mM Tris-Cl, pH 7.4.

PET buffer: 0.1 mM pyridoxal 5'-phosphate, 0.01 mM EDTA and 5 mM Tris-Cl, pH 7.4.

Hemolysis buffer: 0.185 M ammonium chloride, 0.017 M Tris base and 0.01 M potassium bicarbonate, pH 7.4.

2.1.8 Solutions

[CaMg]-EDTA: This was prepared as described by Sinclair and Granick (1977). Equimolar amounts of $CaCl_2$, $MgCl_2$ and disodium EDTA were dissolved separately, mixed, and neutralized with NaOH.

Ehrlich's reagent: The stock solution contained 0.3% $HgCl_2$, 12.7% perchloric acid, 75% glacial acetic acid. This was stable indefinitely at 4°C. The reagent was prepared by adding 1.81% p-dimethyl-amino-benzaldehyde and this was stable for 4 weeks at 4°C.

2.2 METHODS

2.2.1 Preparation of bacterial succinyl CoA synthase

This enzyme was kindly supplied by Mr. I.A. Borthwick and was prepared from *E. coli* (Crooke's strain) by a method based on that of Ramaley *et al.* (1967). Cells were grown and the enzyme was extracted by sonic extraction, acetone fractionation and ammonium sulfate fractionation as described by Ramaley *et al.* (1967). The pellet from the ammonium sulphate precipitation was resuspended in 0.05 M potassium phosphate - 0.05 M KCl, pH 7.2 and dialysed against this buffer for 12 h. The dialysed enzyme was stored overnight at -15°C then clarified by centrifugation for 15 min at 10,000 xg. The preparation was stored in a small aliquot in -15°C. This procedure yielded an enzyme preparation with a specific activity of approximately 15 µmoles succinhydroxamic acid/mg protein/30 min when assayed

by the method of Kaufman (1955).

2.2.2 Estimation of protein

Protein in the homogenate, mitochondria or microsomal suspension was precipitated with 10% trichloroacetic acid and the precipitate was washed once in 5% trichloroacetic acid and solubilized in 0.1 M NaOH. Protein was estimated by the method of Lowry (1951) using bovine serum albumin as standard.

2.2.3 Preparation of tissue samples

The livers from 17 day old chick embryos were removed onto ice, and rinsed in PEST buffer. Each liver was quickly blotted, weighed, and homogenized gently in PEST buffer (10 ml/g liver) using a Potter Elvhjem glass homogenizer with a motor driven teflon pestle (3 passes). Care was taken to avoid frothing during homogenization.

Nuclei and cell debris were pelleted by centrifugation at 800 xg for 10 min. The resultant supernatant was centrifuged at 10,000 xg for 15 min to pellet the mitochondria. The mitochondrial pellet was resuspended in PET buffer (1 ml/g liver) and ruptured by ultra-sonication for 4 x 5 sec bursts using a Branson Ultrasonifier. Triton X-100 was added to a final concentration of 0.5%. The sonication/Triton step was found to be important to ensure complete rupture of the mitochondria and ready access of substrates to the enzyme as previously reported by Granick and Urata (1963) and Yoda *et al.* (1975).

To determine ALA-synthase activity in an homogenate, the liver was homogenized and sonicated in the PET buffer (1 ml/g liver) and Triton X-100 was added to a final concentration of 0.5%. This crude homogenate was then assayed for ALA-synthase activity.

2.2.4 Colorimetric assay for ALA-synthase in homogenate or mitochondrial suspensions

To measure ALA synthase activity the method of Poland and Glover (1973) was modified to include 10 mM [CaMg[-EDTA and 30 mM sodium levulinate as previously used by Sinclair and Granick (1977). This assay estimated colorimetrically the amount of ALA formed. The reaction mixture (100 µl) contained 50 mM Tris-Cl, 0.25 mM pyridoxal phosphate, 100 mM glycine, 10 mM succinate, 10 mM [CaMg]-EDTA, 30 mM sodium levulinate, 1 mM dithioerythritol, 15 mM ATP, and 0.25 M NaCl, pH 7.4. 2.0 ml of 1.5 x reagent mixture was thawed and 1.6 mg of CoA and 80 µl of *E. coli* succinyl CoA synthase (1 unit/µl) was added. Each assay mixture contained 100 µl of 1.5 x reagent mixture containing the succinyl CoA generating system and 0 - 50 µl of mitochondrial suspension or homogenate. The final reaction volume was adjusted with PET buffer to 150 µl.

The reaction was initiated by the addition of enzyme, and the mixture was incubated at 37°C for 60 min in a shaking water bath. 100 µl of cold 10% (w/v) trichloroacetic acid was added to stop the reaction, and the mixture was held on ice for 10 min. Precipitated protein was removed by centrifugation at 1,000 xg for 10 min and the super-

natant retained for the determination of ALA content as follows: 150 µl of the supernatant was mixed with 75 µl of warm (80°C) 1 M sodium acetate/acetyl acetone mix (10:1, v/v) and the pH was adjusted to 4 - 6 using 10 N HCl. The mixture was heated for 10 min at 80°C, and then allowed to cool. 225 µl of Ehrlich's reagent was then added and after 5 min, the absorbance at 555 nm was measured. To determine low levels of ALA-synthase activity (such as are found in normal livers) the sample was scanned from 600 to 500 nm using a Varian Superscan 3 double beam spectrophotometer (sensitivity 0.05) instead of reading the absorbance at 555 nm using a single beam spectrophotometer. The amount of ALA produced was calculated using a molar extinction coefficient of 58 $mM^{-1}cm^{-1}$.

ALA-synthase activity was expressed as units/mg mitochondrial or homogenate protein. One unit of ALA-synthase is defined as that amount of enzyme which catalyses the production of 1 nmole of ALA in 60 min at 37°C. All assays were carried out in duplicate and corrected for a blank to which trichloroacetic acid was added at zero incubation time. In some experiments a 'no enzyme' blank was also included but this was invariably lower than the zero time blank.

When aminoacetone pyrrole was also likely to be significant in comparison with ALA-synthase (such as is found in normal uninduced livers), 50 µl of 0.5 M Na_2HPO_4/1 N NaOH (1:3, v/v) was added to the mixture after heating at 80°C for 10 min. The pH was adjusted to 7 (as determined by pH paper). To this 1 ml of CH_2Cl_2 was added, mixed and cent-

rifuged at 500 xg for 5 min. 150 µl of the supernatant was mixed with 150 µl of Ehrlich's reagent (Poland and Glover, 1973) and absorbance at 555 nm was recorded.

Determination of ALA-synthase activity in cells: At the end of the appropriate incubation period, aliquots (25 ml) of the cell suspension were centrifuged at 500 xg for 5 min at 4°C. The cell pellet was homogenized and sonicated in 0.6 ml of assay mixture and Triton X-100 was added to a final concentration of 0.5%. Aliquots of 0.5 ml were incubated for 60 min in the colorimetric assay of ALA-synthase as described above in a final volume of 1.5 ml.

2.2.5 Radiochemical assay for ALA-synthase activity

Cation exchange column: Dowex AG50W-X8 resin (H^+ form, 200 - 400 mesh) was converted to the Na^+ form by extensive washing in 1 M sodium acetate, pH 3.9. The resin was packed in pasteur pipette columns (4 x 0.7 cm) and was washed with 5 ml of 1 M NaOH until pH 9.0, with water (approximately 10 ml) until pH 7.0, and then with 5 ml of 0.1 M sodium acetate buffer, pH 3.9, until the resin was equilibrated. Regeneration of the resin after each assay was by washing with 5 ml of 1 M sodium acetate, pH 8.5, and then repeating the in-column procedure as described earlier.

Assay solutions: The reagents for the ALA-synthase assay were stored frozen (at -15°C) and stock solutions were stable for several months.

Reagent mixture: This was 5 x concentrated containing: 250 mM Tris-Cl, (pH 7.4), 50 nM glycine, 100 mM $MgCl_2$, 75 mM ATP, 5 mM [CaMg]-EDTA, 150 mM sodium levulinate, 1.25 M NaCl and 5 mM dithioerythritol.

The concentrations of Tris, pyridoxal phosphate and [CaMg]-EDTA in the reagent cocktail were calculated such that upon addition of 90 µl of mitochondrial suspension or homogenate in PET buffer, the final assay concentrations were 27 mM, 0.25 mM and 7 mM respectively.

Succinate solution: A 5 mM solution of sodium succinate was buffered with 50 M Tris-Cl, pH 7.4, and to that was added [2,3-^{14}C]-succinate to a specific activity of 6.6 µCi/µmole and 33 µCi/ml. This solution was stored in aliquots at -15°C.

To remove possible low levels of impurities in the radioactive succinate which might contaminate the isolated ALA, the original material was diluted to 0.1 ml in 5.0 M sodium acetate, pH 3.9 and was applied to a Dowex AG50W-X8 ion exchange column (pre-equilibrated with the same buffer) and was washed through with water. The [2,3-^{14}C]-succinate was adjusted to pH 7.4 with 0.1 M NaOH (Condie and Tephly, 1980) and then was added to the 5 mM succinate solution to the required specific activity.

Carrier solutions: 10 mM ALA in 1 M sodium succinate in 1.0 M sodium acetate, pH 4.6.

Column buffers: 1.0 M sodium acetate buffer (pH 4.6), 0.1 M sodium acetate buffer (pH 3.9), methanol:0.1 M sodium acetate (2:1 v/v) (pH 3.9), 10 mM HCl, 1.0 M sodium acetate (pH 8.5).

Assay mixture: 1.0 ml of 5 x reagent mixture was thawed and 12 µl of 100 mM pyridoxal phosphate solution, 3 mg of CoA and 66 µl of *E. coli* succinyl CoA synthase (1 unit/µl) was added. This was sufficient to generate in the assay at least 60 µmole of succinyl-CoA in 30 min as measured by the method of Kaufman (1955).

Each assay mix contained: 30 µl of 5 x reagent mixture containing the succinyl-CoA generating system, 30 µl succinate solution, and 0 - 90 µl of mitochondrial suspension. The final reaction volume was adjusted with PET buffer to 150 µl.

Assay Procedure: The assay mixture was incubated in eppendorf tubes at 37°C for 30 - 60 min in a shaking water bath, and the reaction was terminated by the addition of 100 µl of 10% trichloroacetic acid, 15 µl of 10 mM ALA and 15 µl of 1 M sodium succinate. After 10 min on ice the tubes were centrifuged at full speed on a eppendorf centrifuge and both the pellet and supernatant fractions were retained. The pellet was washed by resuspending in 0.5 ml of cold 5% trichloroacetic acid, centrifuged and was discarded. The supernatant was combined with the original supernatant for further fractionation (if necessary this supernatant could be stored frozen).

To the pooled supernatant fraction (0.75 ml) was added 1.5 ml of 1.0 M sodium acetate buffer, pH 4.6, and the pH was adjusted to 3.7 - 4.0 with glacial acetic acid (2 - 5 drops), using 5 µl of sample and narrow range pH paper as an indicator. This step was important to ensure that amino acids do not bind to the ion exchange column

(Ebert *et al.*, 1970). The pH adjusted solution was placed onto a Dowex AG50W-X8 ion exchange column (4 x 0.7 cm, pre-equilibrated with 0.1 M sodium acetate buffer, pH 3.9), and sequentially washed with 5 ml of 0.1 M sodium acetate buffer, pH 3.9, 5 ml of methanol:0.1 M sodium acetate buffer, (pH 3.9) (2:1 v/v) pH 3.0, and 5 ml of 10 mM HCl. ALA was eluted with 5 ml of 1.0 M sodium acetate, pH 8.5, and was immediately adjusted to pH 4.5 (as determined by pH paper) with HCl (3 drops of 10 M HCl in the collection tube was usually sufficient for this).

ALA pyrrole was formed in this solution by adding 0.2 ml of acetylacetone, loosely capping the tubes, and immersing in a boiling water bath for 15 min. This solution was then rapidly cooled and ALA pyrrole was extracted in 6 ml (x 3) of ethylacetate (saturated with 1 M sodium acetate, pH 4.6). The ethylacetate extract was evaporated to dryness at 60°C in a rotary evaporator (or under a stream of dry air) and the residue was dissolved in 3 ml of Triton X-100 containing toluene scintillation fluid. The [^{14}C]-ALA was determined by liquid scintillation spectroscopy. Protein in the mitocondrial suspension or homogenate was estimated by the procedure of Lowry *et al.* (1951) as described in Section 2.2.2.

Enzyme activity was expressed as pmoles of ALA formed per mg of mitochondrial protein per 60 min.

2.2.6 Determination of total porphyrins in cells

After incubation for appropriate times, samples of the cell suspension were centrifuged at 500 xg

for 5 min and the cell pellet was rinsed with 1 ml of buffer (0.15 M NaCl, 0.02 M Tris, pH 7.4). Total porphyrins in the cells were extracted with 1 ml of a mixture of 1 N $HClO_4$ and methanol (1:1, v/v) and quantitated using a Perkin Elmer model 203 Fluorescence Spectrophotometer equipped with a red light insensitive photomultiplier R-106 as described by Granick *et al.* (1975). The instrument was daily calibrated with rhodamine B (40 ng/ml) in ethylene glycol and total porphyrins were determined using proto-porphyrin IX as standard (Granick *et al.*, 1975).

For determination of protein content, the $HClO_4$/ methanol insoluble material was rinsed with 5 ml of ethanol, then with 5 ml of ethanol-ether (3:1, v/v), dried and protein determined by the method of Lowry (1951) as described in Section 2.2.2.

2.2.7 Determination of microsomal heme oxygenase activity in cells

At the end of the appropriate incubation period, aliquots (50 ml) of the cell suspension were centrifuged at 500 xg for 5 min. Each cell pellet was homogenized in 5 ml of PEST buffer, pH 7.4, and sonicated for 20 sec. Cell debris was removed by centrifuging twice at 2,000 xg for 5 min, and mitochondria were isolated from the supernatant by re-centrifuging at 10,000 xg for 15 min. Microsomes were obtained from the post-mitochondrial supernatant by centrifuging at 105,000 xg for 60 min. The microsomal pellet was suspended in 2.6 ml of 0.1 M potassium phosphate buffer, pH 7.4, by

sonication for 20 sec. Heme oxygenase activity from the microsomes was estimated by the method of Maines and Sinclair (1977). To 500 µl of microsomes containing 3 mg of protein/ml were added 50 µl of rat liver microsomal supernatant (12 mg protein/ml) with the following compounds to the indicated final concentrations: 1 mm $MgCl_2$, 4 mM $NADP^+$, 0.8 mM glucose 6-phosphate, 0.25 units of glucose 6-phosphate dehydrogenase, 225 µg of bovine serum albumin and 20 µM hematin. The final volume was 750 µl. The mixture was incubated at 37°C for 30 min, and the reaction was terminated by immersion of the flasks in ice. Bilirubin formed was extracted by addition of 1.5 ml of chloroform. The chloroform extract was scanned between 350 and 650 nm, with the chloroform extract of an incubation mixture containing all components of the incubation mixture minus the hematin serving as the reference. The difference in absorption between 450 and 530 nm was used for calculation of the bile pigments formed, using the excitation co-efficient 58 $mm^{-1}cm^{-1}$.

2.2.8 Procedure for washing cells to remove exogenously added inducer: After incubation of the cells for the appropriate time, aliquots of cell suspension were removed and centrifuged at 175 xg for 3 min. The packed cell pellet was quickly rinsed with 1 ml of fresh medium and then gently resuspended in the original volume of medium containing either AIA, DES or no additions, as appropriate. The cells were incubated at 37°C on a gyrotary shaker (150 cycles/min) for 10 min and then centrifuged at 175 xg for 3 min. The washing procedure was repeated twice more to ensure complete removal of the

inducing drug.

CHAPTER THREE

SELECTION OF EXPERIMENTAL PROCEDURES FOR ASSAY OF ALA-SYNTHASE

3.1 INTRODUCTION

ALA-synthase, a pyridoxal-phosphate requiring enzyme, promotes the condensation of succinyl-CoA and glycine to form ALA (Granick and Urata, 1963) as previously described. The intracellular concentration of this enzyme is normally low (Granick and Urata, 1963), but a wide variety of xenobiotics and steroids are known to increase markedly its synthesis in mammalian (De Matteis, 1971) and avian liver cells (Granick, 1963; Granick *et al.*, 1975; Sassa and Kappas, 1977; Morgan *et al.*, 1977; Tomita *et al.*, 1974). However, the fact that ALA-synthase can be present in tissues at either very low or extremely high levels has posed some problems in the design of a reliable and sensitive assay. When present at relatively high levels, enzyme activity has been measured using a colorimetric assay (Mauzerall and Granick, 1956; Marver and Tschudy, 1966; Poland and Glover, 1973; Sinclair and Granick, 1977) in which ALA produced *in vitro* is converted to a pyrrole by condensation with acetylacetone and quantitated using Ehrlich's reagent. In crude homogenates, another enzyme, aminoacetone synthase, promotes the condensation of acetyl CoA and glycine, producing aminoacetone, which also forms a pyrrole with acetylacetone (Marver *et al.*, 1966; Irving and Elliott, 1970). The activity of this enzyme represents only a small (and insignificant) proportion of measured total ALA-synthase activity when the level of the latter enzyme is high. But under basal conditions, when ALA-synthase levels are very low, aminoacetone synthase may

represent a significant proportion of the measurable activity (Irving and Elliott, 1970). Therefore although the colorimetric assay is accurate for high levels of ALA-synthase, nmoles of ALA produced, (Mauzerall and Granick, 1956), it is not sufficiently sensitive for measuring basal activity or activity in very small samples.

To overcome this problem, radiochemical assays were developed (Kikuchi *et al.*, 1958; Lewis *et al.*, 1967; Ebert *et al.*, 1970; Irving and Elliott, 1970; Strand *et al.*, 1972; Ohashi and Sato, 1973; Briggs and Condie, 1976) to enable a more sensitive measurement of ALA-synthase. These assays are sufficiently sensitive for use in normal tissues (Ebert *et al.*, 1970) or for minute amounts of material (Strand *et al.*, 1972) and are based on the incorporation of [2,3-^{14}C]-succinate into ALA; the sensitivity is largely a function of the specific activity of the isotope.

Several methods for the colorimetric and radiochemical assays of ALA-synthase have been described and used extensively. To help clarify the colorimetric and radiochemical assay procedures a standard protocol for the measurement of ALA-synthase from chick embryo liver mitochondrial suspension was developed and is described here.

3.2 RESULTS

3.2.1 Standardization of the colorimetric assay for ALA-synthase activity using authentic ALA

In the majority of experiments described in later chapters, where relatively large tissue samples were available, the already described (Materials and Methods) colorimetric assay was used. The validity of this method was checked by applying the colorimetric procedure to samples containing a known amount of authentic ALA. As shown in Fig. 3.1, it gives a linear response to increasing amounts of ALA upto a maximum of 90 nmoles.

3.2.2 Application of the colorimetric assay for ALA-synthase activity to chick embryo livers

Further experiments using the colorimetric method as described in Materials and Methods for estimation of ALA-synthase activity were carried out to establish standard incubation conditions for the assay of mitochondrial ALA-synthase activity from chick embryo liver.

Mitochondria were isolated from chick embryo livers as described in Materials and Methods and the effect of various incubation times and protein concentrations on the colorimetric assay of ALA-synthase was determined. Fig. 3.2 and Fig. 3.3 show the results of experiments designed to establish the times of incubation and protein concentrations for which the assay gives a linear response. As shown, the assay was linear for 60 min and up to 4 mg of mitochondrial protein.

The effect of an exogenous succinyl-CoA generating

FIGURE 3.1

Standardization of the colorimetric assay using chemically prepared ALA.

—— amount standard ALA added (95% pure)

■ amount calculated using E_{mm} = 58 for the Ehrlich's pyrrole color salt

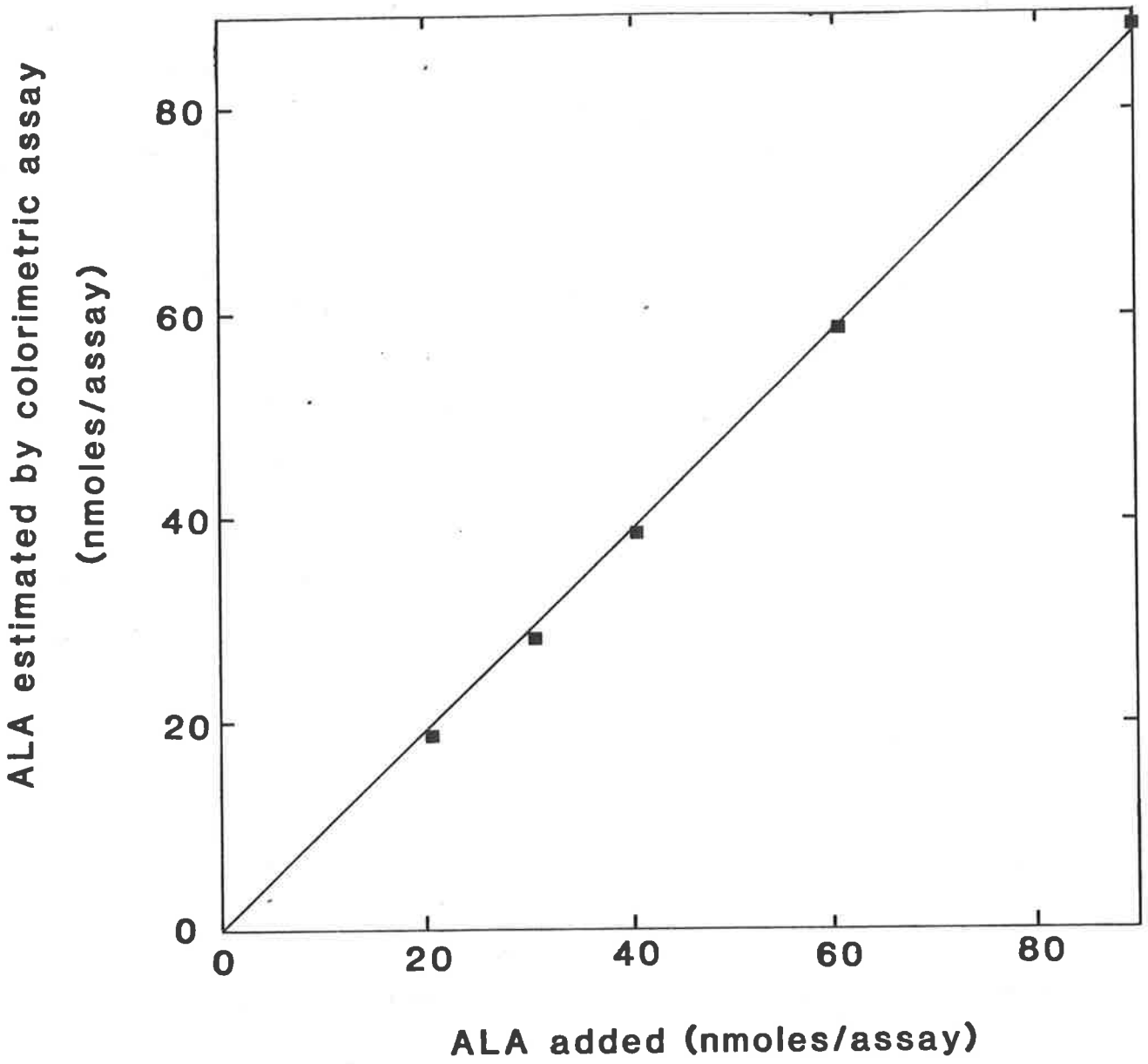

ALA estimated by colorimetric assay
(nmoles/assay)
0
20
40
60
80
0
20
40
60
80
ALA added (nmoles/assay)

FIGURE 3.2

Estimation of ALA-synthase activity in chick embryo liver mitochondria by the colorimetric assay as described in Materials and Methods: Variation of ALA formed with incubation time. 3 mg of mitochondrial protein was used/assay.

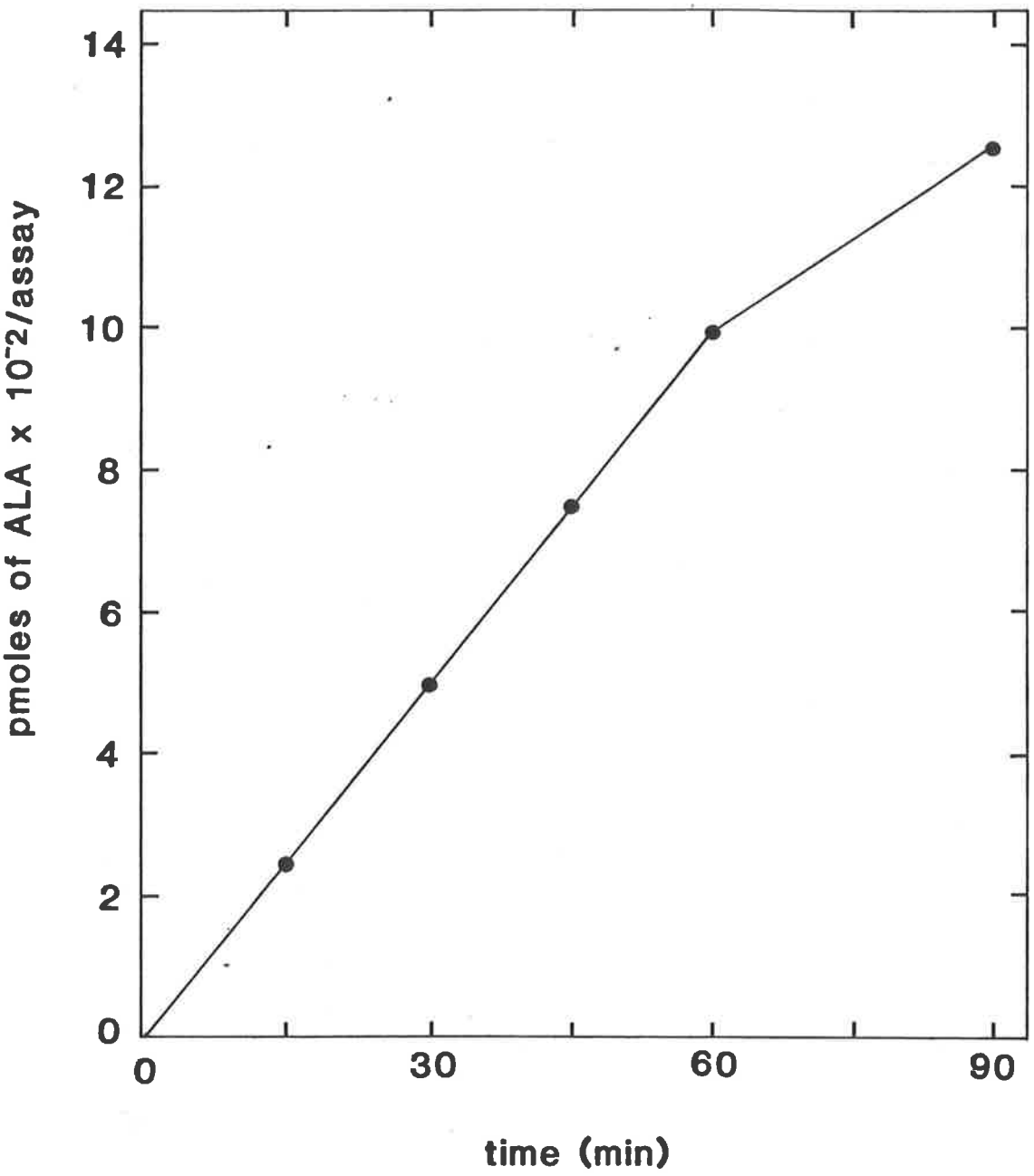
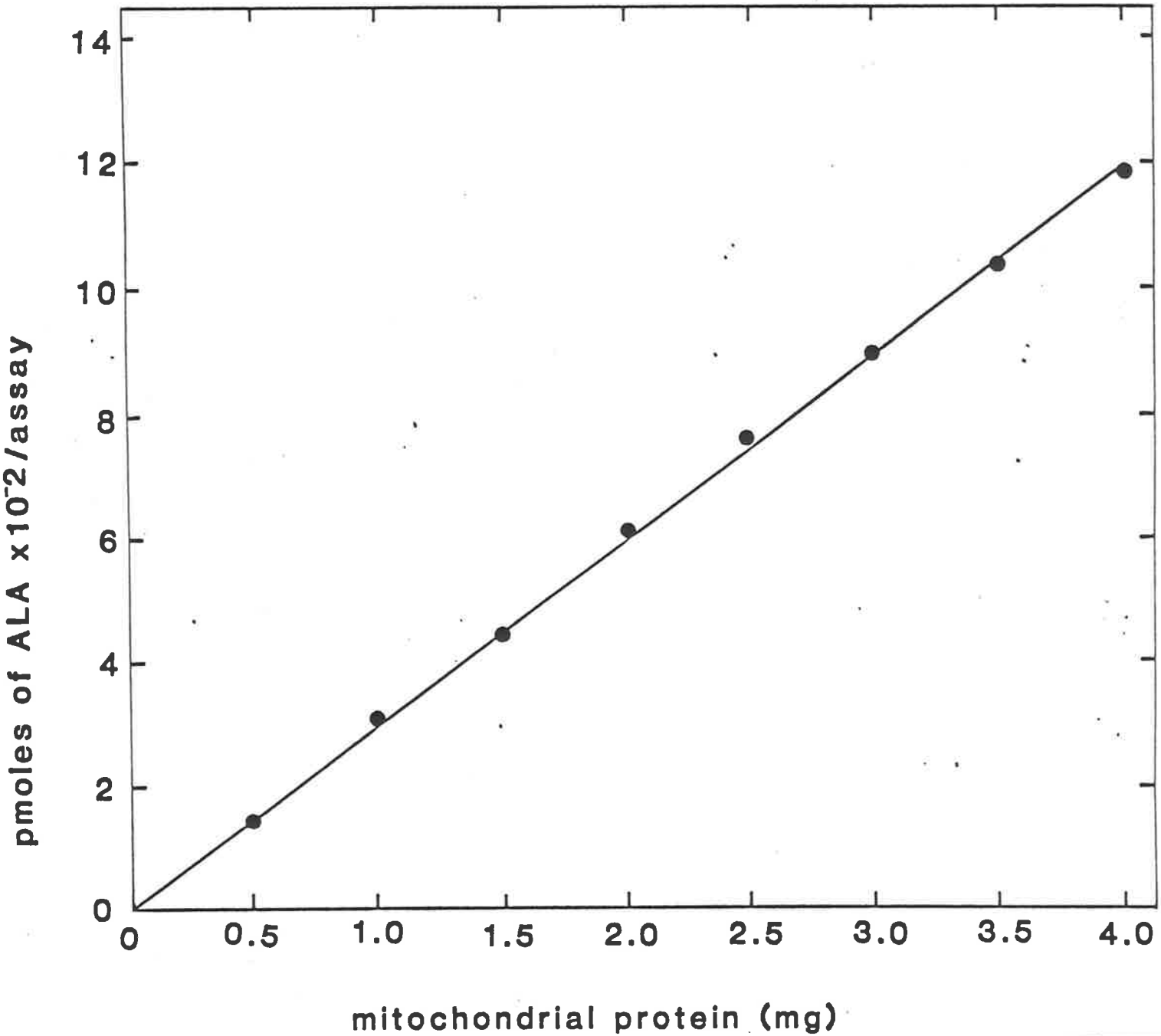

14
12
10
8
6
4
2
0
pmoles of ALA x 10^-2/assay
0
30
60
90
time (min)

FIGURE 3.3

Estimation of ALA-synthase activity in chick embryo liver mitochondria by colorimetric assay (30 mins) as described in Fig. 3.2: Variation of ALA formed with amount of mitochondrial protein added.


14
12
10
8
6
4
2
0
pmoles of ALA x10-2/assay
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
mitochondrial protein (mg)

system on the estimation of ALA-synthase activity by colorimetric assay was examined. The activity of ALA-synthase in mitochondria was determined in the presence or absence of succinyl-CoA generating system in the assay mixture. The results showed that omission of the succinyl-CoA generating system resulted in underestimation (18%) of the ALA-synthase activity when mitochondria which had been stored frozen were used. No such requirement was observed when a freshly prepared mitochondrial suspension was used. The succinyl-CoA generating system was absolutely required when ALA-synthase activity from mitochondrial freeze-dried extract was determined.

3.2.3 Standardization of the radiochemical assay and its application to the chick embryo livers

At the time when the work in this thesis started it was thought that the radiochemical assay would be needed and hence the following work was carried out on the assay. In fact it proved to be unnecessary and the radiochemical assay was not subsequently utilised. The study of the method is included here for completeness.

The effect of various incubation times and protein concentrations on the radiochemical assay of mitochondrial ALA-synthase was determined. Mitochondria were prepared from chick embryo livers as described in Material and Methods and aliquots were assayed for ALA-synthase activity using the radiochemical assay procedure described.

Using a chick embryo liver mitochondrial preparation, incorporation of [^{14}C]-succinate into ALA proceeded

TABLE 3.1

ALA-SYNTHASE ACTIVITY IN CHICK EMBRYO LIVER AS DETERMINED BY RADIOCHEMICAL AND COLORIMETRIC ASSAY

Tissue	ALA-synthase activity (pmoles ALA formed/mg mitochondrial protein/h)	
	Radiochemical Assay	Colorimetric Assay
Chick embryo liver mitochondria	305	300

Mitochondria were prepared, as described in Materials and Methods from 17 day chick embryo liver and ALA-synthase activity was assayed using 184 µg of mitochondrial protein in a 30 min radiochemical assay and 828 µg in a 60 min colorimetric assay.

linearly for at least 45 min (Fig. 3.4) and the incorporation was proportional to the amount of preparation added between 50 µg and at least 750 µg of mitochondrial protein (Fig. 3.5). As little as 10 µl of sample corresponding to approximately 5 mg wet weight of liver could be readily assayed. Recovery of ALA was determined for the ion exchange and the ethylacetate extraction steps separatedly as previously reported by Irving and Elliott (1969) and described in Materials and Methods (Chapter Two), and then for the complete assay procedure as described below.

[3,5-^{3}H(N)]-ALA (1 µCi) plus 150 nmoles of unlabelled ALA was diluted with 2.25 ml of 1 M sodium acetate buffer, pH 4.6, adjusted to pH 3.7 - 4.1 with glacial acetate acid (as described in Materials and Methods) and was applied to the Dowex AG50W-X8 ion exchange column. The column was washed as described and ALA was eluted with 5 ml of 1 M sodium acetate, pH 8.5. An aliquot of this eluate was counted; 90 - 93% of the added radioactivity was recovered from the column. Recovery from ethylacetate extraction was determined by forming the pyrrole from 1 µCi [^{3}H]-ALA plus 150 nmoles unlabelled ALA in 5 ml of 1 M sodium acetate buffer, pH 4.6. Following this, the solution was extracted, as described, with ethylacetate and an aliquot was counted, the recovery of [^{3}H]-ALA was 85 - 90% of that added initially. Overall recovery of ALA through the complete assay procedure was determined by adding 1 µCi [^{3}H]-ALA and 150 nmoles unlabelled ALA to an assay mixture in which [^{14}C]-succinate was replaced by unlabelled succinate. Mitochondrial suspension was added and the reaction immediately stopped

FIGURE 3.4

Effect of incubation time on the incorporation by mitochondria of [^{14}C]-succinate into ALA; mitochondria were prepared from chick embryo livers as described in Materials and Methods and aliquots were assayed for ALA-synthase activity for varying times using the radiochemical assay procedure described. 184 µg of chick embryo liver mitochondrial protein was used. Enzyme activity was expressed as pmoles [^{14}C]-ALA produced/mg mitochondrial protein.

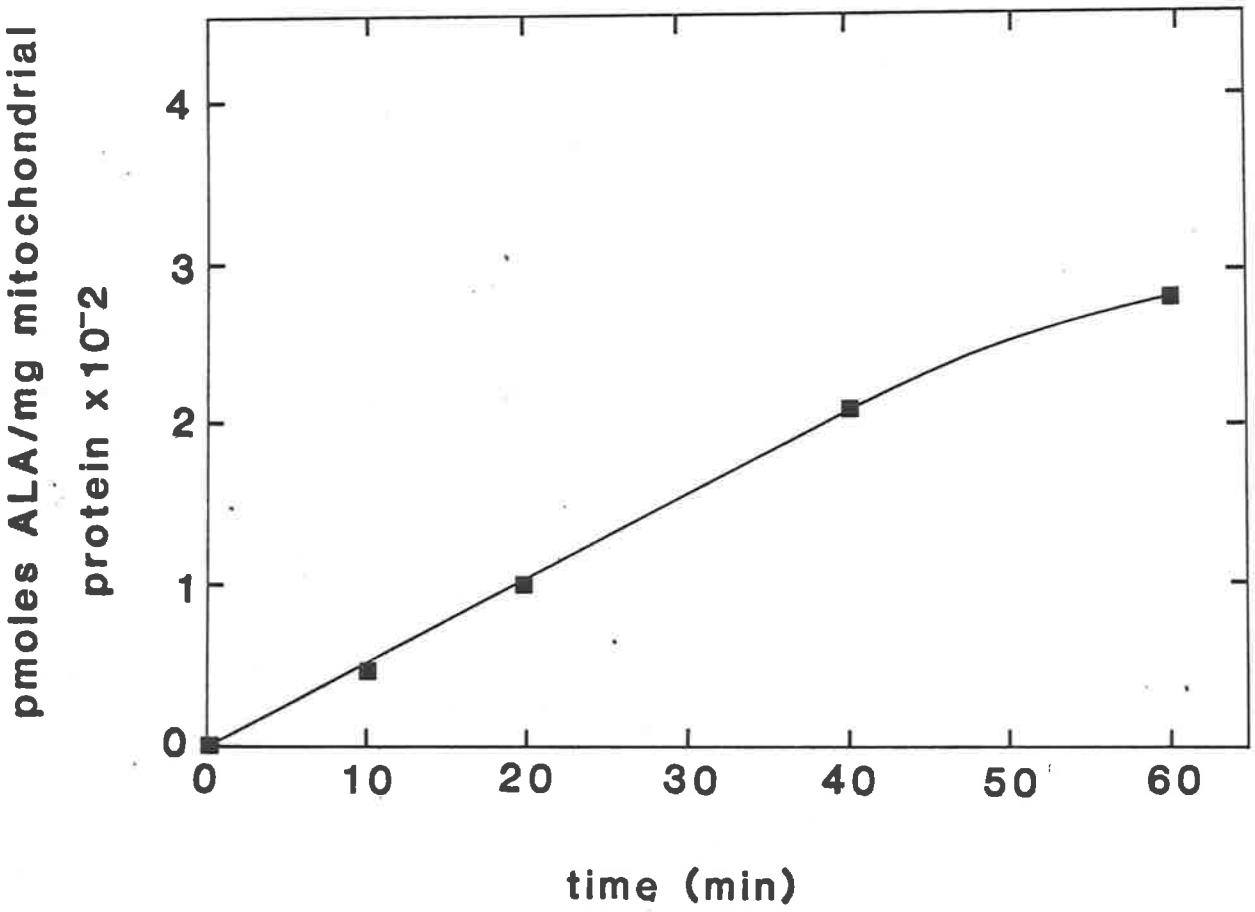
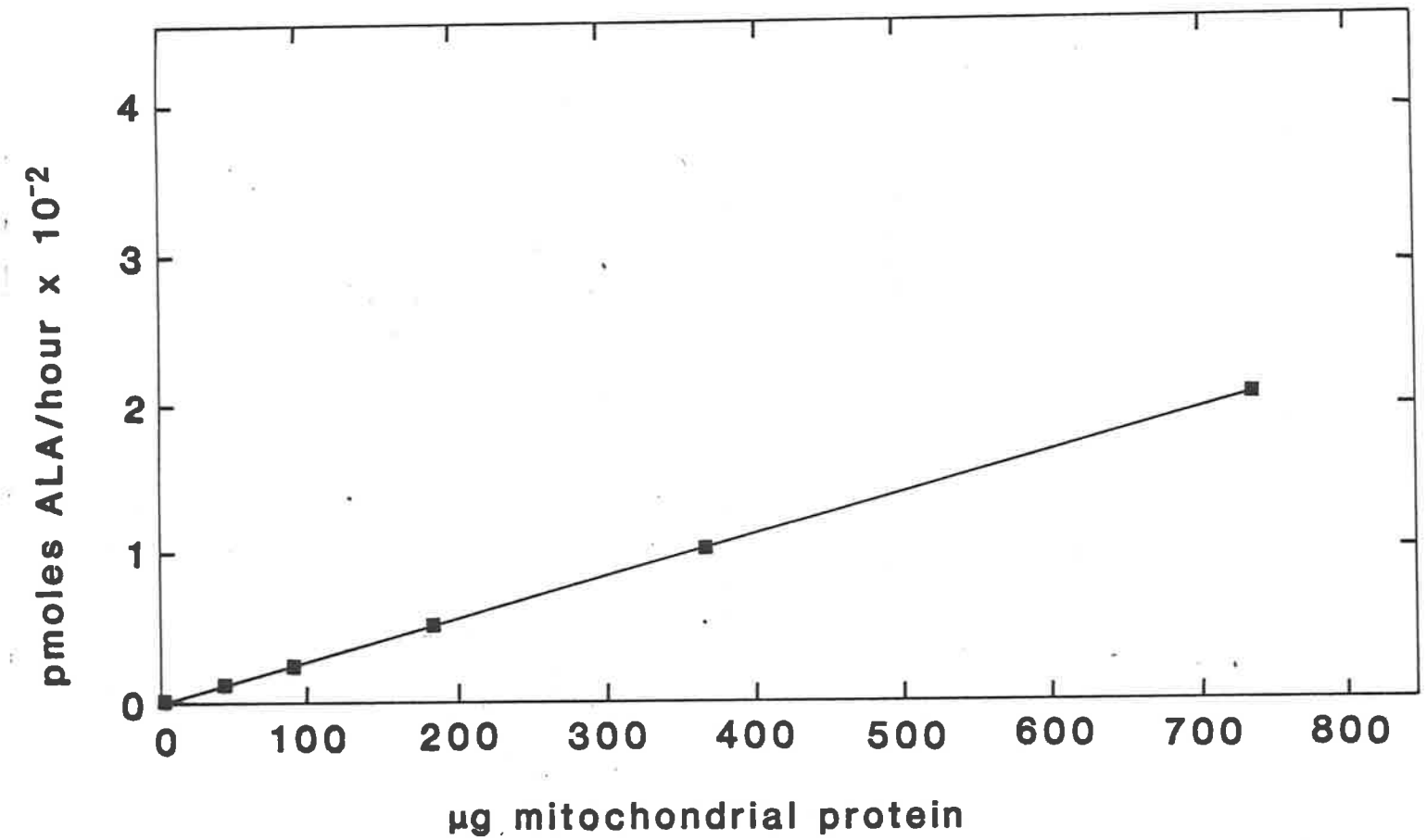

pmoles ALA/mg mitochondrial protein x10⁻2
0
1
2
3
4
0
10
20
30
40
50
60
time (min)

FIGURE 3.5

Effect of varying mitochondrial protein concentrations on the activity of ALA-synthase *in vitro* using the radiochemical assay procedure. Mitochondria were prepared from chick embryo livers as described in Materials and Methods and various aliquots were assayed for ALA-synthase activity by incubation for 30 min using the radiochemical assay procedure. 46 - 736 µg of chick embryo liver mitochondiral protein was used. Activity was expressed as pmoles [^{14}C]-ALA produced/h.


pmoles ALA/hour x 10^{-2}
0
1
2
3
4
0
100
200
300
400
500
600
700
800
µg mitochondrial protein

by addition of 100 µl of 10% trichloroacetic acid. The assay was then processed as described and an aliquot of the pooled ethylacetate extract was counted; recovery of [^{3}H]-ALA was 82 - 85% of the input.

The activity of ALA-synthase in chick embryo liver mitochondria is shown in Table 3.1. Activity was almost identical with that determined by the colorimetric assay.

With a specific activity of 6.6 µCi/µmole of [^{14}C]-succinate, 1 nmole of ALA produced would contain 13,000 cpm. Background radioactivity (as determined by a zero time sample) was 80 - 120 cpm. The limit of sensitivity of this assay (200 cpm over background) therefore represented 15 pmoles of ALA; this is at least 30 times more sensitive than the colorimetric assay. In fact, because less material could be assayed using this procedure, the overall assay sensitivity was much greater than the colorimetric assay and could be increased further by increasing the specific radioactivity of the succinate solution. The useful range of this assay was between 15 - 5,000 pmoles of ALA produced during the period of incubation.

3.3 DISCUSSION

The experiments described in this chapter were to provide information necessary to define standard procedures for storing and assaying chick embryo liver ALA-synthase. The results here show that the standard colorimetric method described in the Materials and Methods gives satisfactory

assay of ALA-synthase up to 4 mg of chick embryo livers mitochondrial protein. The results also established that mitochondria can be rapidly frozen and stored at -15°C without major losses in activity up to 7 days. The radiochemical assay described provides a sensitive method for measuring low levels of ALA-synthase and therefore makes it suitable for studies on the regulation of heme biosynthesis in small tissue samples.

Calcium and magnesium saturated EDTA and sodium levulinate were used in colorimetric and radiochemical assays described in Materials and Methods to inhibit ALA-dehyratase activity without depleting the pool of Mg^{++} which is necessary for generation of succinyl CoA. Sinclair *et al.* (1977) have previously reported similar advantages of using [CaMg]-EDTA and sodium levulinate in their colorimetric assay procedure for determining the activity of ALA-synthase from monolayer culture of chick embryo hepatocytes. Omission of the succinyl-CoA generating system resulted in underestimation of the mitochondrial ALA-synthase activity when mitochondria were stored frozen and thawed just before use. No such requirement was observed when freshly prepared mitochondria were assayed for ALA-synthase activity. The succinyl-CoA generating system was absolutely required when ALA-synthase activity from an extract of freeze-dried mitochondria was measured. Similar results have previously been reported by Ebert *et al.* (1970) and Strand *et al.* (1972).

In some cases of radiochemical assay described here, the final step of ALA-pyrrole formation and extraction

proved to be unnecessary. This was the case, for instance, when partially purified enzyme preparations (mitochondrial freeze-dried extracts) were being assayed. In such an instance, an aliquot from the eluate of Dowex AG50W-X8 column was dissolved in 3 ml of Triton-X100 containing toluene scintaillation fluid and was counted directly by liquid scintaillation spectroscopy. A similar adaption to the radiochemical assay has previously been reported by Ebert *et al.* (1972). As stated earlier, because the level of ALA-synthase activity in isolated chick embryo liver cells were not known, both the radiochemical and the colorimetric assays were standardized; the former having the advantage of sensitivity and the latter being rapid although less sensitive as described in the introduction to the chapter. Subsequent studies showed that the colorimetric assay gave satisfactory results for ALA-synthase activity in isolated chick embryo liver cells. Hence, in all the further studies this assay was employed.

CHAPTER FOUR

PREPARATION OF ISOLATED CHICK EMBRYO LIVER CELLS AND OPTIMISATION OF CONDITIONS FOR ACCUMULATION OF PORPHYRINS AND INDUCTION OF ALA-SYNTHASE

4.1 INTRODUCTION

Hepatic ALA-synthase can be induced by various chemicals in a variety of systems as follows: whole animals Granick and Urata, 1963, isolated perfused rat liver (Bock *et al.*, 1971), cultured liver cells of chick embryo (Granick, 1966); in suspensions of adult rat liver cells (Edwards and Elliott, 1974), organ culture of finely chopped chick embryo liver (Tomita *et al.*, 1974). Studies on the regulation of ALA-synthase have been greatly facilitated by the introduction of a technique for culturing monolayers of chick embryonic liver cells (Granick, 1966). A drawback of this system, however, was the variability of fetal bovine serum which was used to supplement the culture medium (Honn *et al.*, 1975). In addition, the presence of heme in such sera had been shown to be inhibitory to the formation of ALA-synthase (Granick *et al.*, 1975). It was for this reason that Granick *et al.* (1975) modified the avian liver cell culture method so that the cells were maintained for part of the time in a chemically defined, serum-free, medium. With this modification, chick embryo liver cells were grown in a medium containing fetal bovine serum for the first 24 h, and then the medium was replaced with medium containing insulin but no serum. Induction of ALA-synthase was then studied during the next 24 h in the absence of serum. Insulin supplementation of the growth medium was necessary for the cells to respond to chemicals and synthesize ALA-synthase. However since in this technique chick embryo liver cells were incubated in

the presence of fetal bovine serum during the first 24 h, it was possible that cells might have absorbed substances from the serum which could affect the rate of ALA-synthase formation. Therefore, Sassa and Kappas (1977) refined the culture technique further to completely avoid the use of serum and studied the induction of ALA-synthase in cultured liver cells maintained in chemically defined medium.

Studies from different workers (Creighton and Marks, 1972; Marks *et al.*, 1973; Krupa *et al.*, 1973; Taub *et al.*, 1976) indicate the following order of sensitivity to porphyrin-inducing drugs: chick embryo liver cells in monolayer culture > 17 day-old chick embryo > adult chicken (and other avian species) > mammals (e.g., mice, rat, rabbits and guinea pigs).

As already stated the chick embryo liver monolayer culture system has played a key role in studies on ALA-synthase control and with the development of chemically defined synthetic media is likely to remain an important tool. Nevertheless it has certain disadvantages - the culture materials are expensive and the system imposes a 10 - 12 h delay before experiments can be done. It should be pointed out that despite the widely used term chick embryo liver monolayer 'cell culture' the hepatocytes (which are the cells of interest in these studies) do not multiply but become attached to the tissue culture plates and often become overgrown with fibroblasts predominantly. The precise value of allowing hepatocytes to attach to the substratum has not been clearly defined. If suspensions of chick embryo liver cells could be used immediately

after dispersion of the liver tissues, it would eliminate the need for cell culture and the ability to pipette homogenous cell suspensions would have obvious advantages. Previously, Edwards and Elliott (1974) had reported induction of ALA-synthase in isolated cells of adult rat liver in suspension. An investigation was therefore made as to whether isolated cells could be prepared from chick embryo liver and used for studies on ALA-synthase induction. This chapter describes the setting up and standardization of the experimental system for induction of ALA-synthase in a suspension of isolated chick embryo liver cells in chemically defined serum-free medium.

4.2 RESULTS

4.2.1 Preparation of isolated chick embryonic liver cell suspensions

Several methods for preparing cell suspensions from intact mammalian tissues have been described (Anderson, 1953; Branster and Morton, 1957; Howard and Pesch, 1968; Berry and Friend, 1969; Edward and Elliott, 1974; Granick *et al.*, 1975; Sassa and Kappas, 1977). Cell suspensions prepared using different disaggregation procedures were compared to determine the yields of isolated cells and their viability as indicated by tryphan blue and fluorescein diacetate staining.

Comparison of methods for preparing cell suspensions

Mechanical preparation: This procedure was based on the method of Anderson (1953) and Branster and Morton (1957). 17 to 18 day old chick embryos were perfused to remove red blood cells. The perfusion was accomplished by slitting the embryo open by a median ventral section, inserting a 22-gauge syringe needles into the heart and forcing through 20 ml of 0.25 M sucrose buffered with 0.01 M Tris-HCl, pH 7.4 and 150 units of heparin. The liver was then removed, cut into pieces and dispersed with about 20 - 30 strokes of the pestle in a loose fitting potter-Elvehjem homogenizer.

Cells prepared using mechanical separation failed to exclude tryphan blue indicating damage to cell membranes.

Separation with trypsin: Livers of 17 to 18 day old embryos were perfused as described above except that 20 ml of Eagle's glucose salt solution, minus calcium and magnesium, and 150 units of heparin was used as the perfusion medium. Ten livers prepared in this way were chopped into fragments (1 to 2 mm) with a razor blade and treated with 7.5 ml of 1% sterile trypsin at 37° for 15 min as described by Granick *et al*. (1975) in a gyrorotatory water bath. Then 0.1 mg of varidase (an inhibitor of trypsin) was added, and the incubation was continued for a further 15 min during which time the material was intermeittently pipetted in and out until no lumps or strands were visible.

This method gave a good yield of isolated cells (1 ml

of packed cells from 4 g of liver) but the majority of these failed to exclude tryphan blue.

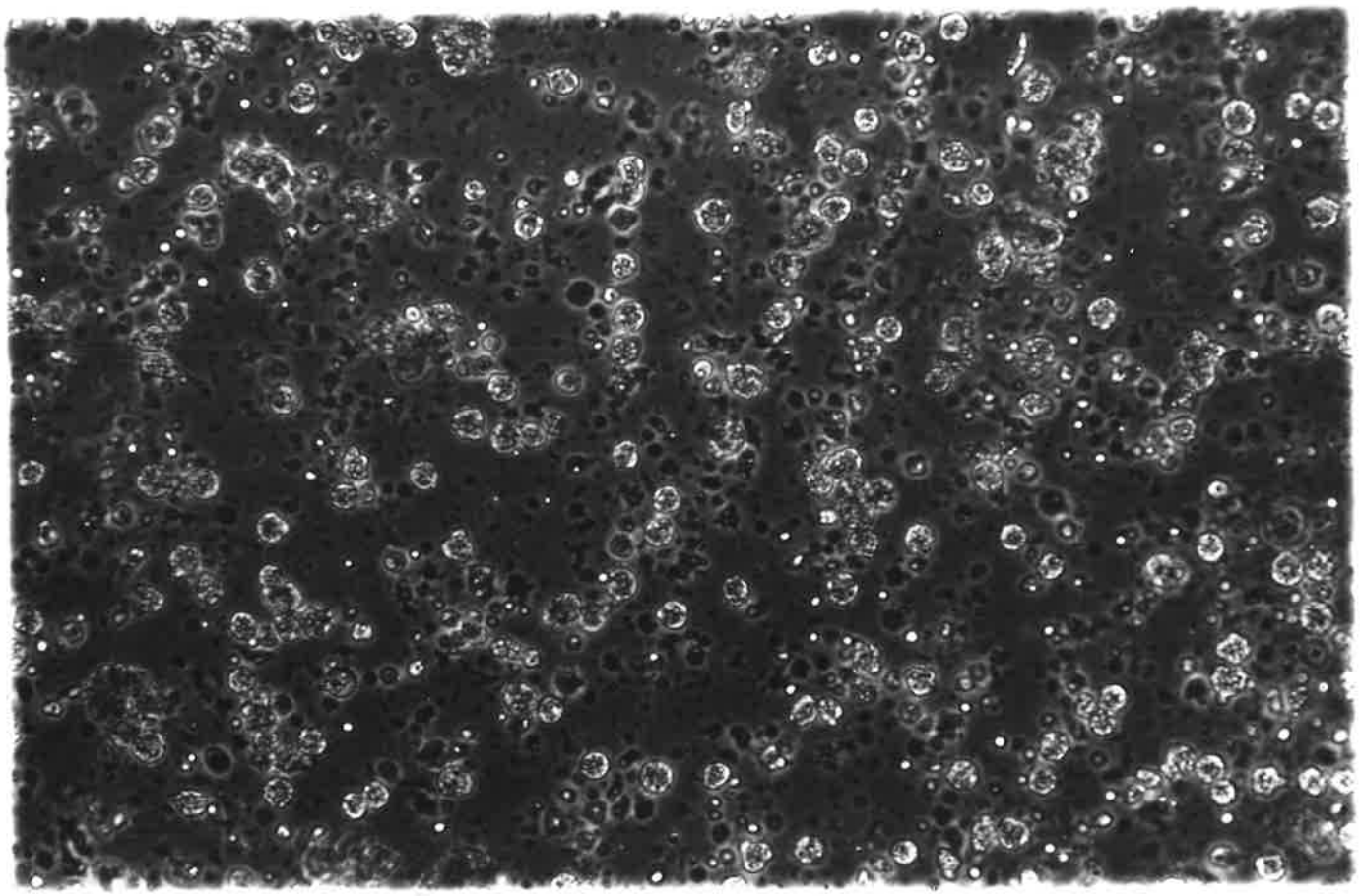
Separation with collagenase or hyaluronidase alone: This was done as described above except that after perfusion liver slices were treated with 0.05% collagenase or hyaluronidase in Hank's salt solution, pH 7.4, containing 4 mM $CaCl_2$ and 4 mM $MgCl_2$.

These methods produced relatively low yields of isolated cells (about 20% by weight of original tissue) but about 95% of these cells excluded tryphan blue.

Separation with collagenase and hyaluronidase: The procedure used was modified from that of Berry and Friend (1969) for the preparation of rat liver cell suspension and Sassa and Kappas (1977) for the preparation of hepatocytes of chick embryo. Livers of ten unperfused 17 day old chick embryos were removed and cut as thinly as possible with a stainless steel razor blade in 20 ml of Ca^{++} and Mg^{++}-free Hank's salt solution, pH 7.4, containing 0.5 mM ethylene glycol bis(β-aminoethyl ether) N,N'-tetra-acetic acid and 150 units of heparin. After 15 min at room temperature, the sedimented liver slices were transferred to 20 ml of Hank's salt solution, pH 7.4, containing 4 mM $CaCl_2$, 4 mM $MgCl_2$, 0.05% hyaluronidase and 0.05% collagenase and were shaken at 37° in a gyrotory water bath shaker (150 cycles/min) for 50 min. During this shaking the liver slices were intermittently drawn up and down in a large bore pasteur pipette. The cell suspension was centrifuged at 150 xg for 3 min and the supernatant was discarded. In order to hemolyse contaminating red blood cells, 20 ml of

FIGURE 4.1

Phase contrast photomicrograph of an isolated suspension of chick embryo liver cells. Cells were prepared as described in Materials and Methods, using collagenase and hyaluronidase, followed by hemolysis with $NH_4Cl/HCO_3/Tris$.



sterile NH_4Cl/HCO_3/Tris solution (0.185 M NH_4Cl, 0.01 M $KHCO_3$, and 0.017 M Tris base, pH 7.4) was added to the sedimented cells. The suspension was again pipetted up and down a few times, allowed to stand for 20 min at room temperature, and then centrifuged at 175 xg for 3 min. The yield was about 1 - 2 ml of packed cells from 4 g of liver.

Cells prepared with collagenase and hyaluronidase gave an acceptable yield of viable cells. Of the recovered intact cells 97% excluded tryphan blue. The cell preparations after hemolysis were essentially colorless and free of erythrocytes (Fig. 4.1). Thus on the basis of the above experiments it was concluded that cell suspensions prepared with collagenase and hyaluronidase were the most suitable for further studies. The cell pellet was suspended in 100 ml of Eagle's basal medium containing glutamine (2 mM) and supplemented with streptomycin 10 mg and pencillin G (10,000 units).

4.2.2 Incubation of cell suspensions

Aliquots (50 ml) of the liver cell suspension, containing approximately 2 - 3 x 10^7 cells per ml were transferred to 300 ml capacity flasks. The flasks were continiously gassed by humified CO_2:air (5:95, v/v). The pH was maintained at pH 7.4 by regulating the gas flow rate and the suspension was shaken at 37° in a gyrotory water bath (150 cycles per min).

Inducers or other compounds were added to the cell suspension in a small volume of either 0.9% NaCl solution

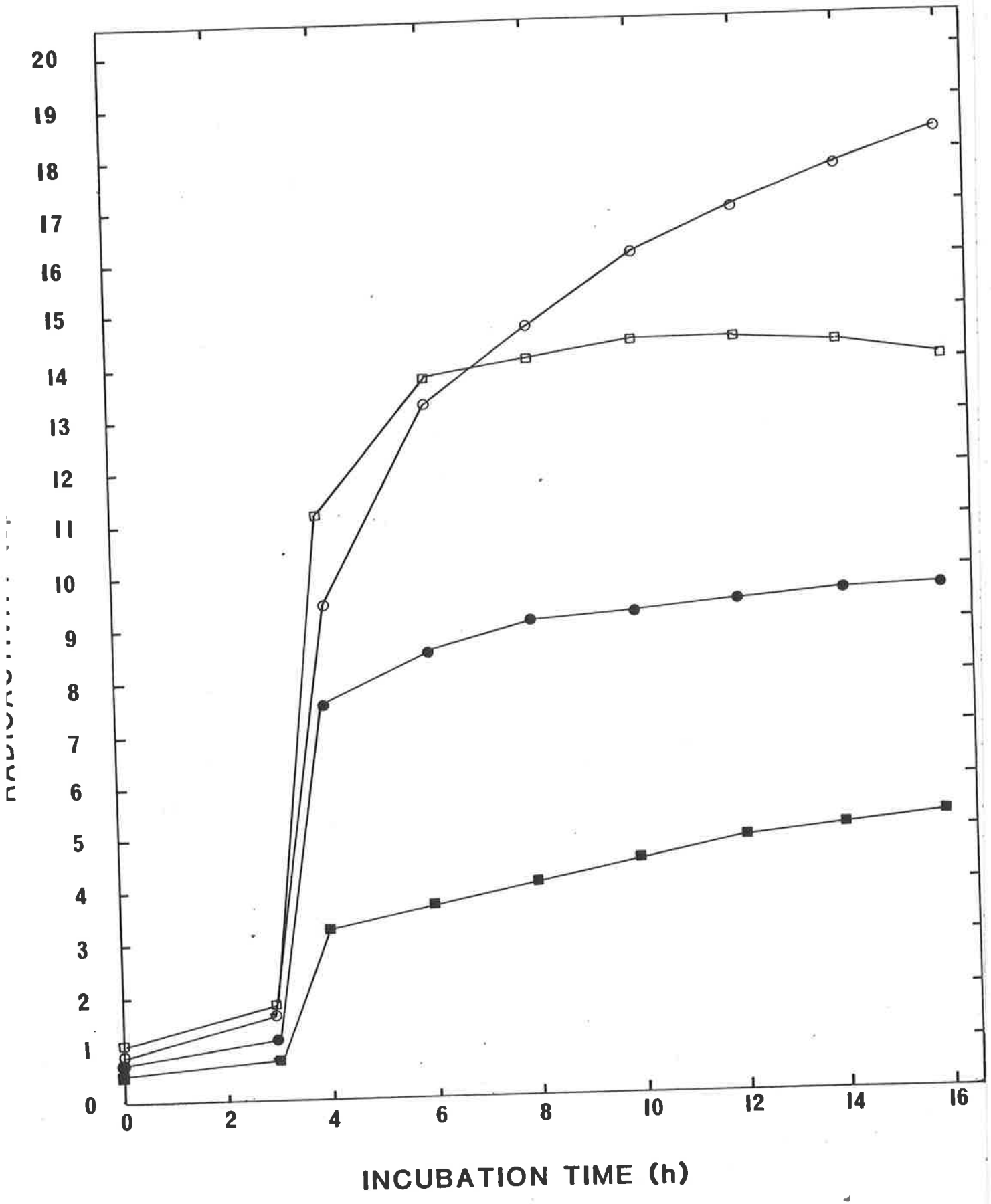
or redistilled ethanol. Appropriate controls showed that the ethanol concentration used had no effect on cell viability. In all cases a similar volume of saline or ethanol was added to control incubations.

Effect of incubation of cell viability: As judged by phase contrast microscopy (Fig. 4.1) the cells were essentially all dissociated from each other and retained their morphology for at least 8 - 10 h. About 97% of cells were viable at the start of incubation as measured by tryphan blue and fluorescein diacetate staining. After 8 - 10 h of incubation the isolated cells tended to aggregate together to form clumps. The cell viability fell by approximately 1% per h under the described incubation conditions.

The effect of shaking rate on endogenous protein synthesis: The effect of increasing shaking rates on the incorporation of [^{3}H]-leucine into the chick embryo liver cellular protein was examined. Results (not shown) indicated that reducing the rate of shaking from 150 to 50 rpm or increasing the rate of shaking from 150 to 300 rpm resulted in 60% and 30% reduction respectively in the rate of incorporation of [^{3}h]-leucine into the total cellular protein over a 15 h incubation period. Preliminary studies indicated that Bt_2cAMP, hydrocortisone, insulin, AIA and DES are needed for optimal induction of ALA-synthase (Section 4.2.6). When these were included similar results to those described above were obtained (Fig. 4.2). The observed decrease in protein synthesis by isolated cells with the increased rate of shaking (300 rpm) over 16 h

FIGURE 4.2

Effect of increasing shaking rates on the metabolic state of the cells as judged by the incorporation of [^{3}H]-leucine into the chick embryo liver cellular protein. Cells were incubated in Eagle's basal media supplemented with AIA (500 µg/ml), Bt_2cAMP (50 µM), insulin (1.0 µg/ml), hydrocortisone (0.05 µg/ml), DES (500 µg/ml), [^{3}H]-leucine (2 µCi/10 ml) and were shaken at 37°C in Gyrotory water bath shaker (50 cycles/min, ■ ; 75 cycles/min, ● ; 150 cycles/min, ○ and 300 cycles/min □). Aliquots were taken after different incubation times and were processed for the determination of radioactivity incorporated into total protein using the method of Ballard *et al.* (1974). Protein from the 1 ml aliquots was precipitated with 10 ml of 10% TCA at 60°C and collected on GF/A filters. The precipitate was washed (x 2) with 5% hot (60°C) TCA, dried under a heat lamp for 15 min and counted for radioactivity using Triton-toluene scintillation fluid.


20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
16
INCUBATION TIME (h)

period could be the result of cell damage due to shearing forces generated at such high rpm; a reduced speed of shaking possibly resulted in limited availability of oxygen for respiration, perhaps due to cell aggregation. No attempt was made to study this further. The shaking speed of 150 rpm was found to be optimum for the further studies. Similar results on the effect of shaking rate on general protein synthesis were obtained when AIA and DES were omitted.

4.2.3 Induction of protoporphyrin synthesis in isolated chick embryo liver cells

As described earlier, ALA-synthase is the rate limiting step in porphyrin biosynthesis and in preliminary studies the intracellular accumulation of total porphyrins in isolated chick embryo liver cells was used as a convenient, although indirect, measure of the amount of ALA-synthase present. Isolated liver cells were incubated as described in Table 4.1 and total intracellular porphyrins were determined spectrofluorometrically as described in Materials and Methods. The results (Table 4.1) show that the addition of AIA to these cells caused a marked increase in the intracellular concentration of porphyrins. This occurs because upon elevation of ALA-synthase levels the activity of ferrochelatase, which catalyses the incorporation of Fe^{++} into heme, becomes rate limiting. Heme synthesis can be inhibited by addition of an iron chelator deferoxamine methanesulphonate (DES) (Sassa and Granick, 1970). Addition of this agent to an AIA induced chick embryo liver cell

TABLE 4.1

Isolated liver cells were incubated in the serum-free Eagle's basal medium containing glutamine. All additions were made at 0 h and intracellular porphyrin levels were determined spectrofluorometrically 10 h after incubation as described in Materials and Methods. Values are the mean of two to four separate determinations.

Additions (μg/ml)	Intracellular porphyrin level
	pmol/mg protein/10 h
1. No addition	6.0
2. DES(500) alone	16.4
3. DES(500) + AIA(500)	29.1
4. DES(500) + AIA(500) + Bt_2cAMP (50 μM)	117.4
5. DES(500) + AIA(500) + Bt_2cAMP (50 μM) + insulin (1.0)	103.2
6. DES(500) + AIA(500) + Bt_2cAMP (50 μM) + hydrocortisone (0.05)	127.1
7. DES(500) + AIA(500) + Bt_2cAMP (50 μM) + insulin (1.0) + hydrocortisone (0.05)	146.7
8. As (7) DES omitted	115.1
9. As (7) AIA omitted	16.8

suspension caused a synergistic increase in the accumulation of porphyrins (Table 4.1) even when AIA was used at its maximum effective concentration. Isolated cells synthesized 29.1 pmol of porphyrins/mg cellular protein over 10 h in the presence of AIA and DES compared with 6.0 pmol/mg protein in the absence of AIA and DES. The level of porphyrins was stimulated a further four fold by the addition of Bt_2cAMP (50 µM). Optimal induction of porphyrin accumulation was observed when both insulin and hydrocortisone were present (Table 4.1). Individually, these hormones had only a marginal effect on porphyrin synthesis but together they significantly increased porphyrin accumulation. Additional experiments showed that when different concentrations of insulin (0.5, 1.0, 2.0 µg/ml) and hydrocortisone (0.025, 0.05, 1.0 µg/ml) and Bt_2cAMP (50 µM), maximal porphyrin levels were obtained with 1 µg/ml of insulin and 0.05 µg/ml of hydrocortisone (results not shown). The supplementation of tri-iodothyronine (1.0 µg/ml) to the medium did not effect the accumulation of porphyrins significantly (results not shown).

4.2.4 Optimization of the concentrations of AIA, Bt_2cAMP and DES required for maximal induction of porphyrin synthesis

The effect of increasing concentrations of Bt_2cAMP on porphyrin accumulation was examined. Liver cells were incubated as described in Fig. 4.3 and varying concentrations of Bt_2cAMP were added. The results (Fig. 4.3) show that maximum levels of intracellular porphyrins

FIGURE 4.3

Total intracellular porphyrin levels in response to increasing concentrations of Bt_2cAMP in the chick embryo liver cell suspensions. Liver cells were prepared and incubated in Eagle's basal medium containing AIA (500 µg/ml), insulin (1.0 µg/ml), hydrocortisone (0.05 µg/ml) and DES (500 µg/ml). Porphyrin levels in the cells were determined spectrofluorometrically 10 h after incubation as described in Materials and Methods. Values are the mean of four determinations.

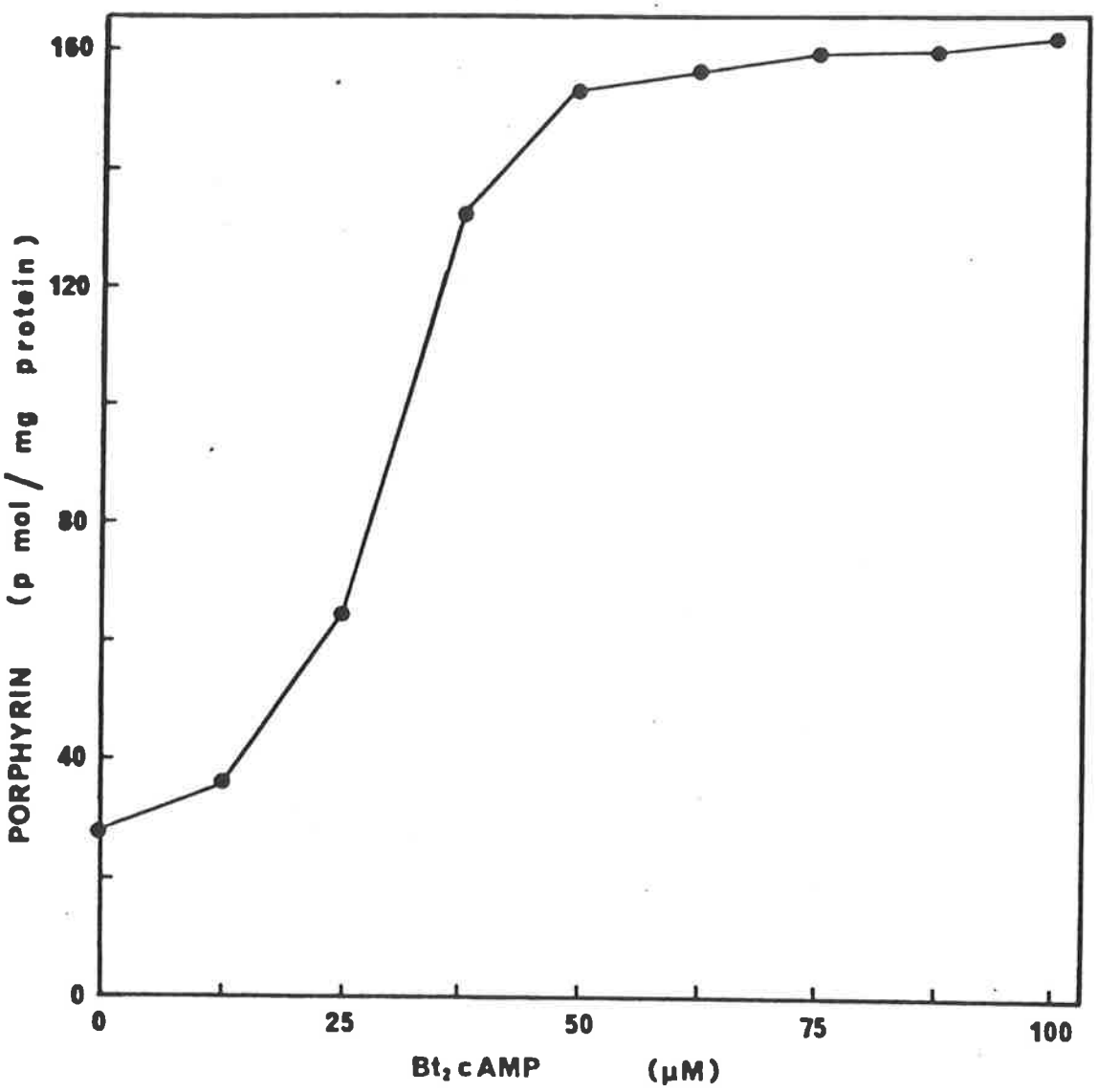

160
120
80
40
0
PORPHYRIN (p mol / mg protein)
0
25
50
75
100
Bt2 cAMP (μM)

FIGURE 4.4

Total porphyrin accumulation in response to increasing concentrations of AIA in the chick embryo liver cell suspensions. Liver cells were incubated in Eagle's basal medium containing Bt_2cAMP (50 µM), insulin (1.0 µg/ml), hydrocortisone (0.05 µg/ml) and DES (500 µg/ml). Porphyrin levels were estimated spectrofluorometrically 10 h after incubation as described in Materials and Methods. Values are the mean of two determinations.

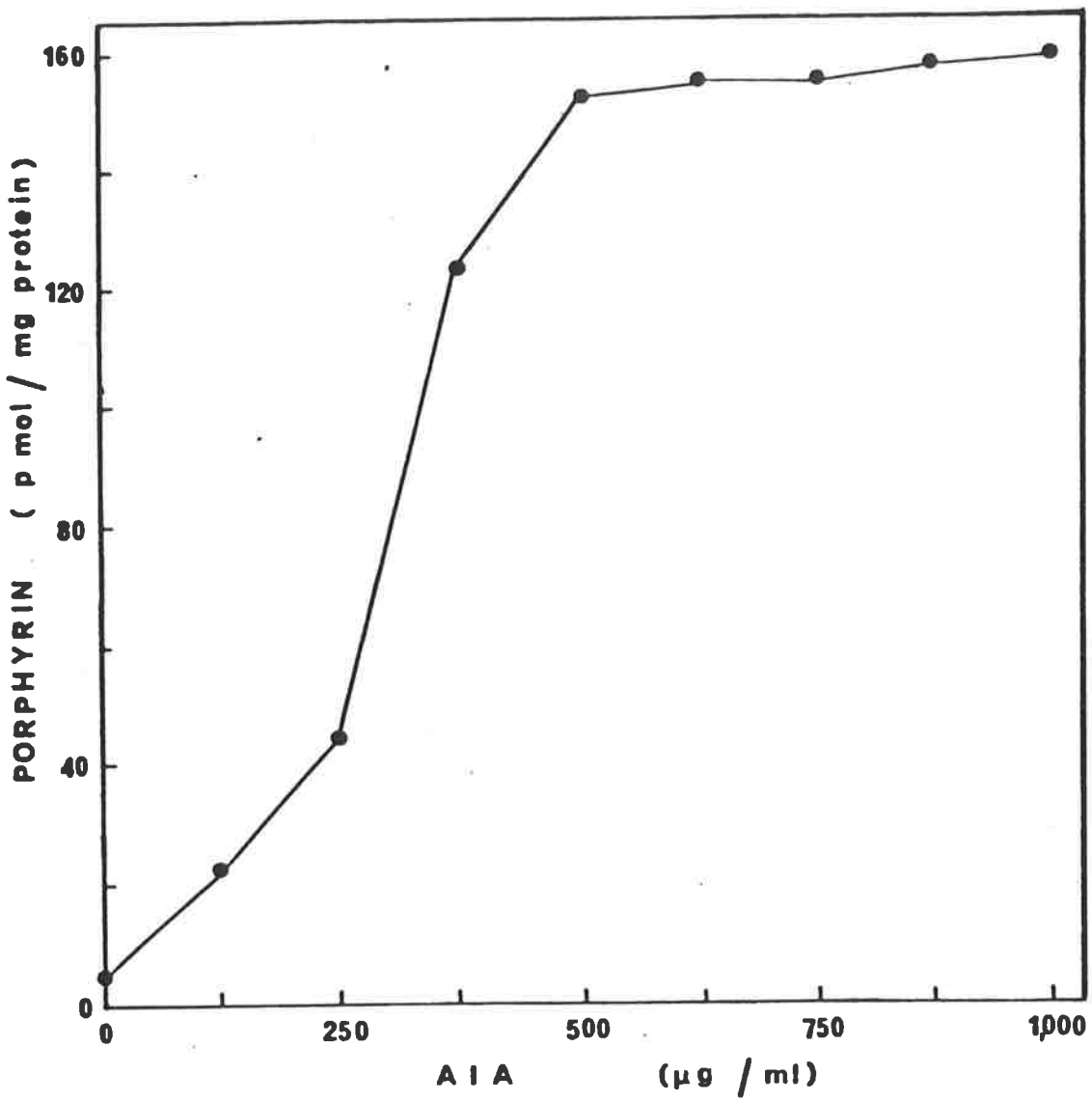
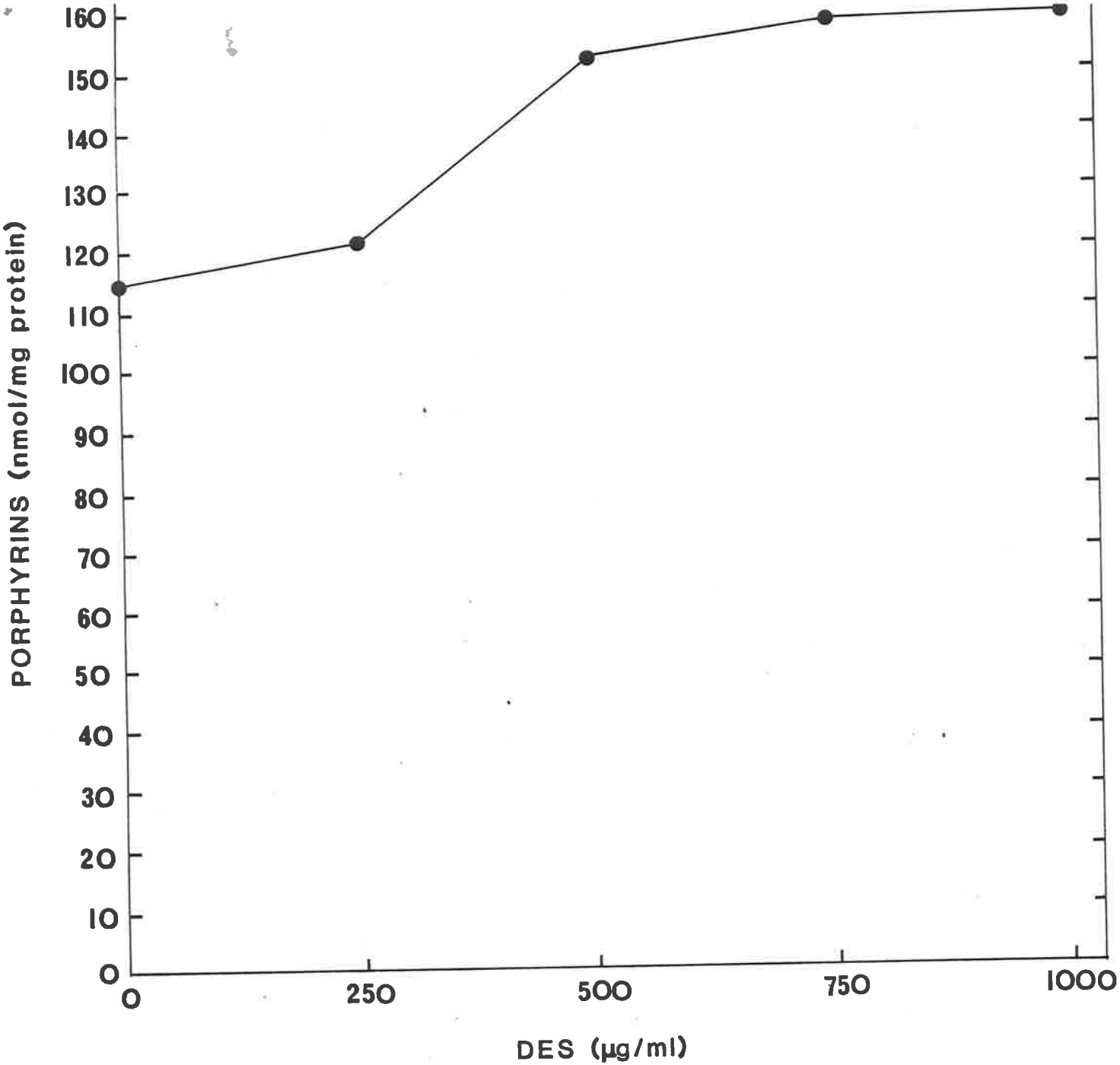

160
120
80
40
0
PORPHYRIN (p mol / mg protein)
0
250
500
750
1000
A I A (µg / ml)

FIGURE 4.5

Effect of increasing concentrations of DES on the intracellular porphyrin accumulation in chick embryo liver cell suspensions. Liver cells were incubated in Eagle's basal medium supplemented with AIA (500 µg/ml), Bt_2cAMP (50 µM), insulin (1.0 µg/ml) and hydrocortisone (0.05 µg/ml). Porphyrin levels in the cells were estimated spectrofluorometrically 10 h after the incubation as described in Materials and Methods. Values are the mean of two determinations.


PORPHYRINS (nmol/mg protein)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
250
500
750
1000
DES (µg/ml)

occurred at 50 μM Bt_2cAMP.

To optimize the concentration of AIA required for the induction of porphyrin synthesis, chick embryo liver cells were incubated with varying concentrations of AIA as described in Fig. 4.4. In the presence of 50 μM Bt_2cAMP, the optimal level of AIA was found to be about 500 μg/ml. To determine the effect of varying concentrations of DES on the intracellular porphyrin levels in the presence of AIA (500 μh/ml) and Bt_2cAMP (50 μM), the cells were incubated as described in Fig. 4.5. The optimum level of DES required for maximum accumulation of porphyrins was found to be about 500 μg/ml.

4.2.5 Effect of Bt_2cAMP on ALA-synthase synthesis

ALA-synthase induction (rather than total intracellular porphyrin accumulation) was investigated using the conditions optimal for porphyrin accumulation. Isolated liver cells were incubated in the presence of Bt_2cAMP and AIA; the results in Fig. 4.6 show an immediate induction of ALA-synthase and this continued for about 6 h. With control suspensions incubated in the presence of ALA but absence of Bt_2cAMP there was almost no increase in the level of ALA-synthase for 8 h (Fig. 4.6) after which a slow increase occurred. Separate experiments (not shown) showed that cAMP added as the sodium salt (50 μM) was nearly as effective as Bt_2cAMP in permitting this induction by AIA. Addition of 3-isobutyl-1-methylxanthine (MIX), an inhibitor of cyclic nucleotide phosphodiesterase activity and hence of cAMP breakdown (Beavo *et al.*, 1970),

FIGURE 4.6

Effect of Bt_2cAMP on ALA-synthase synthesis. The liver cells were incubated in Eagle's basal media containing AIA (500 µg/ml), insulin (1.0 µg/ml), hydrocortisone (0.05 µg/ml), DES (500 µg/ml) and ALA-synthase was estimated in cell homogenates prepared after various time intervals as described in the Materials and Methods. Further additions were: no Bt_2cAMP (○); 50 µM Bt_2cAMP at 0 h (●); 50 µM Bt_2cAMP at 0 h and extra 50 µM Bt_2cAMP at 4 h (△); 50 µM Bt_2cAMP with MIX (30 µg/ml) at 0 h (▲).

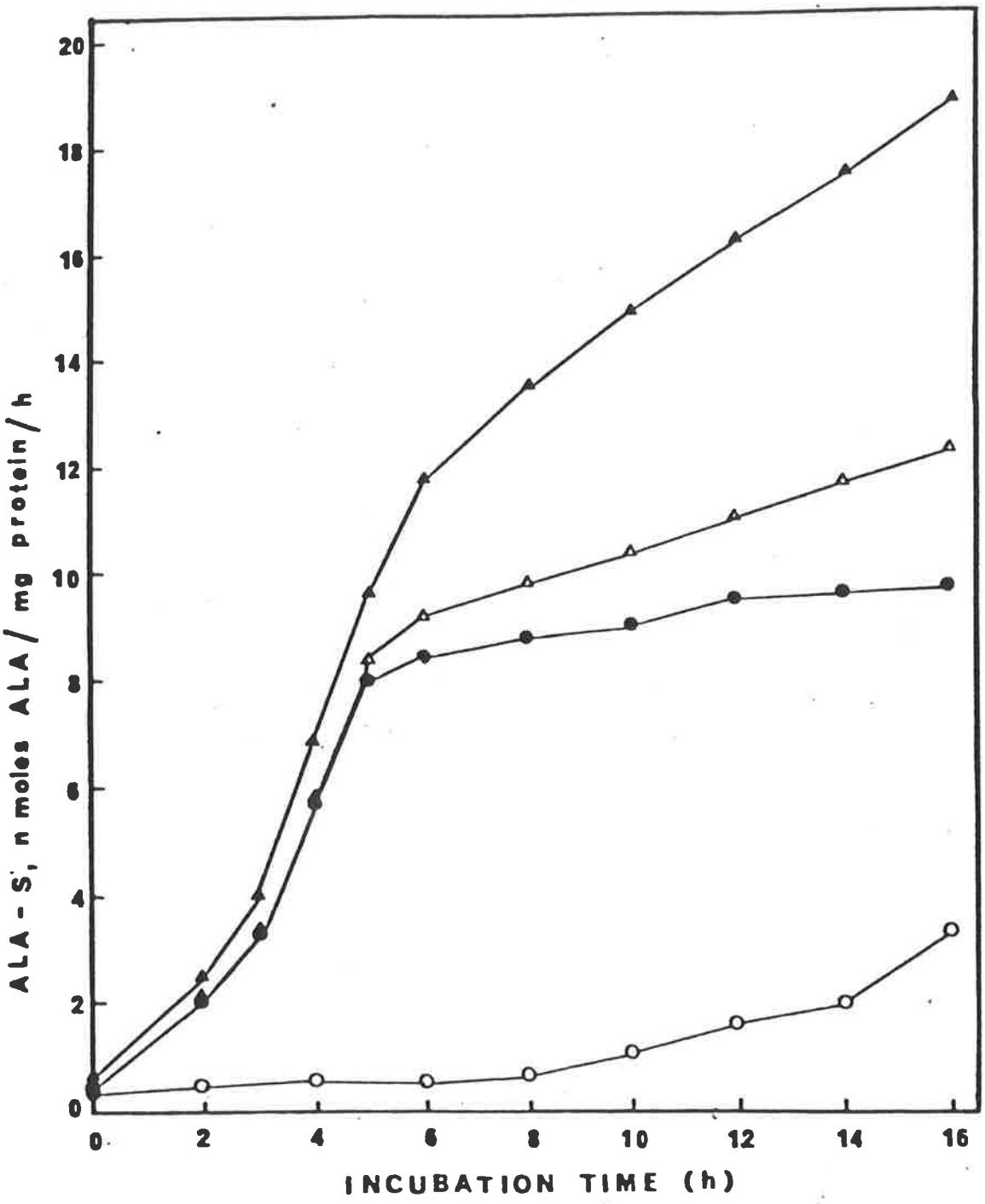

ALA - S, n moles ALA / mg protein / h
INCUBATION TIME (h)
0
2
4
6
8
10
12
14
16
18
20

FIGURE 4.7

Effect of Bt_2cAMP, hydrocortisone and insulin on general protein synthesis. The liver cells were incubated in Eagle's basal media supplemented with AIA (500 µg/ml), DES (500 µg/ml) and the incorporation of L-[4,5-^{3}H]-leucine into total cellular protein after various intervals was determined as described in Materials and Methods. Further additions at 0 h were: hydrocortisone (0.05 µg/ml) (○); insulin (1.0 µg/ml) (●); Bt_2cAMP (50 µM) (△); hydrocortisone (0.05 µg/ml), insulin (1.0 µg/ml) and Bt_2cAMP (50 µM) together (▲). For the measurement of protein synthesis in cells, an aliquot (25 ml) of cell suspension was incubated with 25 µCi of L-[4,5-^{3}H]-leucine (specific radioactivity 105 Ci/mol) added at zero time, and duplicate 1 ml samples were taken for the determination of radioactivity incorporated into total protein using the method of Ballard *et al.* (1974) as described in Fig. 4.2.

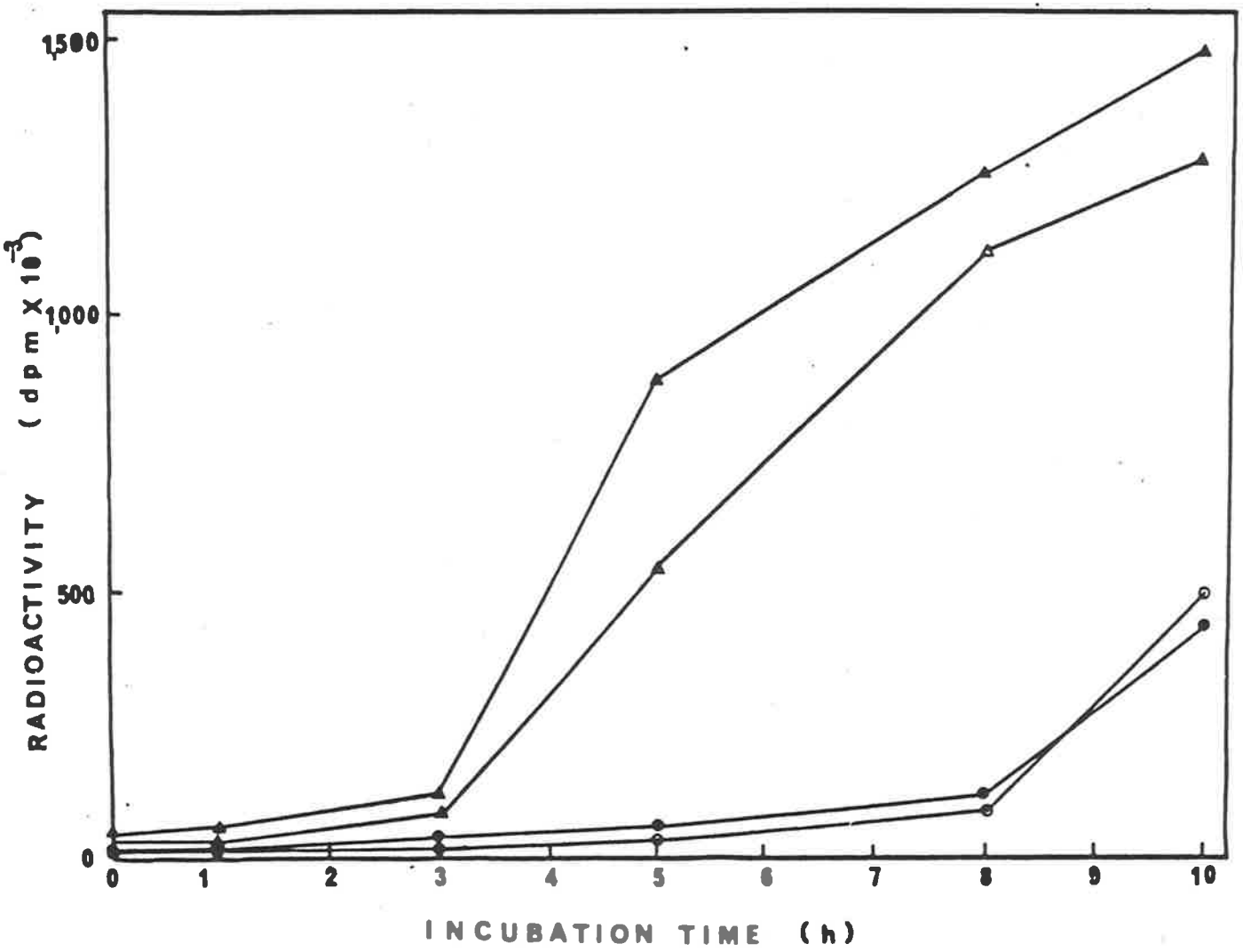

1,500
1,000
500
0
RADIOACTIVITY (dpm X 10⁻³)
0
1
2
3
4
5
6
7
8
9
10
INCUBATION TIME (h)

FIGURE 4.8

Induction of ALA-synthase and intracellular porphyrin accumulation by DDC. Isolated liver cells were incubated in Eagle's basal medium supplemented with Bt_2cAMP (50 μM), insulin (1.0 μg/ml). ALA-synthase activity (O) and intracellular porphyrin levels (●) were estimated as described in Materials and Methods. DDC at 25 mg/ml was sonciated in safflower oil and then added to media to a final concentration of 25 μg/ml. The media was further sonciated to give a homogenous suspension of DDC.

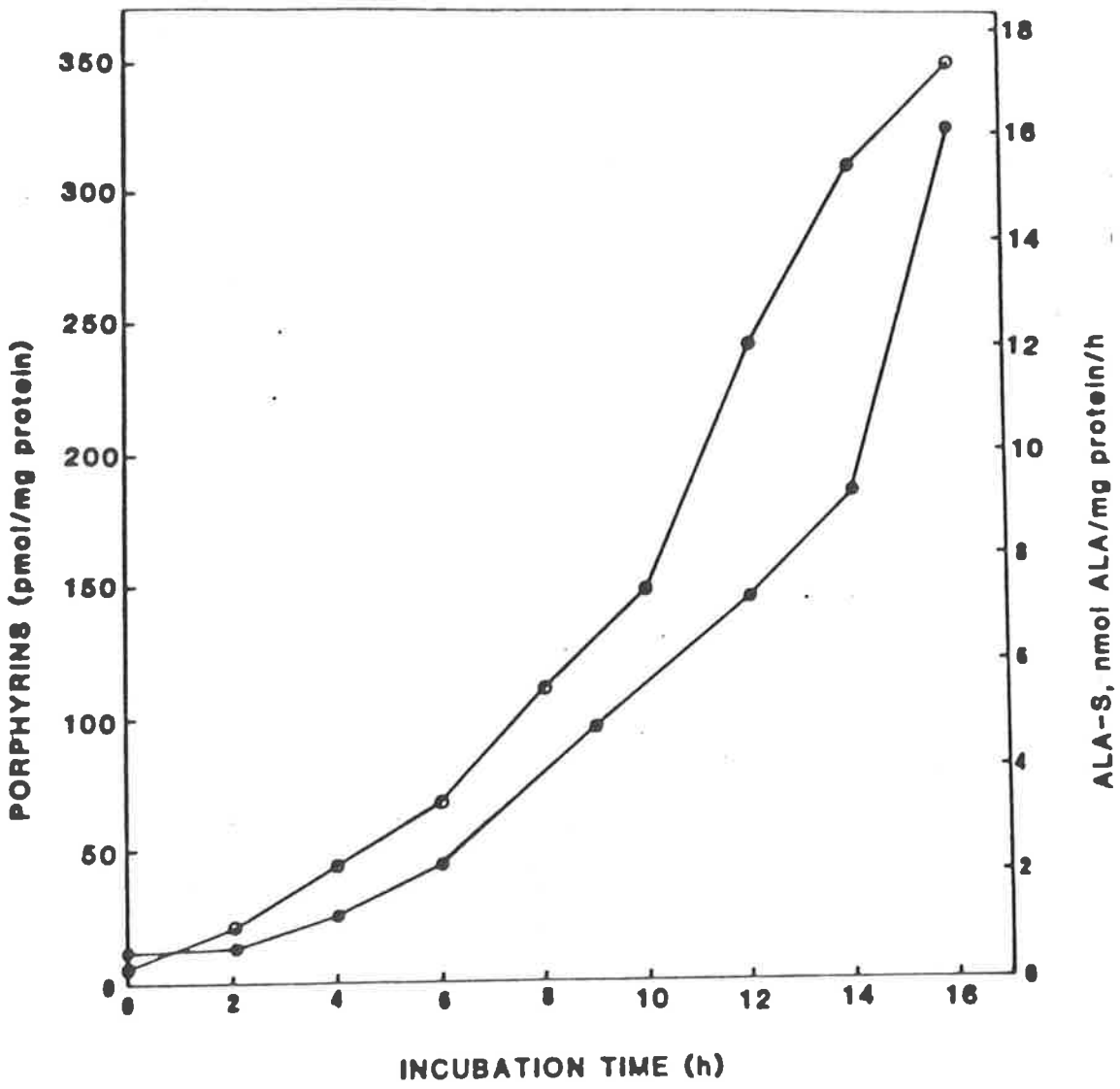
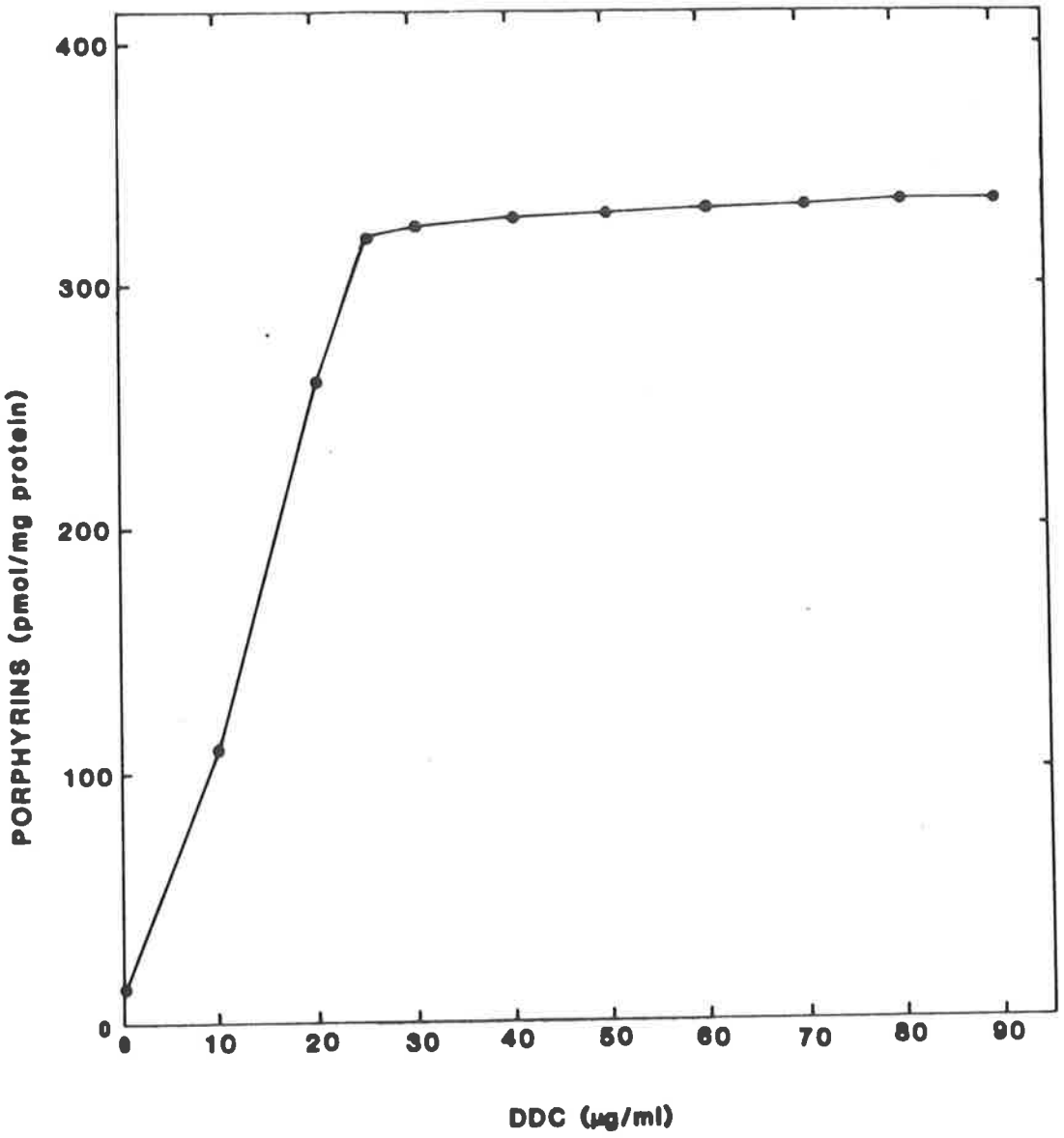

PORPHYRINS (pmol/mg protein)
ALA-S, nmol ALA/mg protein/h
INCUBATION TIME (h)
0
50
100
150
200
250
300
350
2
4
6
8
10
12
14
16
18

FIGURE 4.9

Effect of increasing concentrations of DDC on the levels of porphyrin in a suspension of chick embryo liver cells. Liver cells were incubated and porphyrin estimated as described in Fig. 4.8.


400
300
200
100
0
PORPHYRINS (pmol/mg protein)
0
10
20
30
40
50
60
70
80
90
DDC (µg/ml)

caused a further increase in the levels of ALA-synthase with activity reaching 19 nmol ALA/mg cellular protein/h measured after 16 h (Fig. 4.7). When additional Bt_2cAMP was added at 4 h (in the absence of MIX), only a small additive effect was seen (Fig. 4.7).

4.2.6 Effect of Bt_2cAMP, hydrocortisone and insulin on general protein synthesis in isolated chick embryo liver cells

Although induction of ALA-synthase by cells under optimal inducing conditions occurred immediately (Fig. 4.6), the incorporation of L-[4,5-^{3}H]-leucine into total cellular protein was delayed by 3 h, (Fig. 4.7). In the absence of Bt_2cAMP but in the presence of AIA, DES, and either hydrocortisone or insulin, this lag increased to about 8 h (Fig. 4.7).

4.2.7 Induction of ALA-synthase and porphyrin accumulation by DDC in a suspension of chick embryo liver cells

Following the achievement of ALA-synthase induction in isolated cells by AIA, experiments were carried out to see whether another commonly used inducing drug DDC, was also effective in this system.

A suspension of chick embryo liver cells in the presence of Bt_2cAMP (50 µM), insulin (1 µg/ml) and hydrocortisone (0.05 µg/ml) was treated with DDC to a final concentration of 25 µg/ml; the DDC was dissolved in ethanol or dimethyl sulphoxide. Relatively low levels of

induction of ALA-synthase (4-fold) was observed and DDC crystallised from the media. Tomita *et al.* (1974), Fischer *et al.* (1976) and Kawanishi *et al.* (1978) have also previously reported low levels of induction using DDC in the same way. In an attempt to add the drug to the media such that it would not precipitate, DDC (25 mg/ml) was added as a sonicated dispersion in safflower oil to give 25 µg DDC/ml of media. This resulted in a 25-fold increase in ALA-synthase synthesis over a 16 h period (Fig. 4.8). Increased porphyrins in the cells paralleled the change in enzyme levels. The level of induction was 6-fold greater than those previously reported in the literature. This was probably due to the high localised concentration of drug permitted by the sonic dispersion in oil. Safflower oil alone did not cause induction. To dtermine the optimum level of DDC required for maximum induction of porphyrin synthesis, isolated liver cells were incubated in the presence of varying concentrations of DDC; 25 µg/ml was found to be optimum (Fig. 4.9).

4.3 DISCUSSION

The work in this chapter establishes that freshly prepared isolated chick embryo liver cells can be used directly for studying the effect of porphyrinogenic drugs on the heme biosynthetic pathway. The system has advantages in that it is simple, it eliminates the need for tissue culture and, perhaps most importantly, it permits pipetting

of the cell suspension; since the cells from many livers can be pooled, this eliminates variability in cell number and sensitivity to drugs which may occur in other systems.

Total porphyrin levels in the isolated liver cells were increased by AIA and DDC and this increase was enhanced by, although was not dependant upon, the presence of both insulin and hydrocortisone. However, a point of caution must be added here. Separate experiments showed that 30% of total porphyrins were lost from the cells to the medium during the first 12 h of induction. Optimisation of conditions using intracellular porphyrin levels, although convenient, was therefore not a strictly accurate measure of the induction of ALA-synthase. The optimal levels of insulin, hydrocortisone and Bt_2cAMP were therefore confirmed by measuring ALA-synthase activity directly. By comparison, in chick embryo tissue culture studies, cells incubated in the presence of these hormones remained poorly responsive to AIA for about 24 h (Granick *et al.*, 1975; Sassa and Kappas, 1977). When hormones were added to cells cultivated for 24 h in serum-free media lacking hormones, it took a further 24 h before maximal induction of ALA-synthase could be obtained (Sassa and Kappas, 1977).

Perhaps the most important finding, at least from an experimental viewpoint, is that induction of ALA-synthase by AIA is completely dependent on the presence of Bt_2cAMP over the first 6 h of incubation. Some induction begins after about 8 h in the absence of Bt_2cAMP but only to a small extent. The induction of ALA-synthase in the presence of Bt_2cAMP is immediate and reaches a maximum

level of about 9 nmol of ALA/mg protein/h. This value doubled in the presence of MIX, an inhibitor of cAMP breakdown.

However, although ALA-synthase induction by AIA in the presence of Bt_2cAMP was immediate, the isolated cells showed a lag of about 3 h in total cellular protein synthesis, as measured by the incorporation of L-[4,5-^{3}H]-leucine into trichloroacetic acid precipitable material. The reason for this lag is not known but it is extended in the absence of Bt_2cAMP.

There is some confusion about the requirement for cAMP in the induction of ALA-synthase. Using chick embryo liver cultures, previous workers [Granick, (1966); Granick *et al.* (1975); Tomita *et al.* (1976); Sassa and Kappas (1977); Morgan *et al.* (1977)] have reported some induction of ALA-synthase by drugs in the absence of cAMP. But cAMP has been reported by these authors to enhance this induction. No clear requirement for cAMP in the induction of ALA-synthase and porphyrin synthesis is reported by these authors.

One exception to above is the report of Edwards and Elliott (1974) who showed an absolute dependence upon Bt_2cAMP for ALA-synthase induction by adult rat liver cell suspensions. Recently Scott and Edwards (1981) have shown that induction of ALA-synthase by AIA in monolayer culture of adult rat hepatocytes was also dependent on the presence of cAMP.

In agreement with the results described in this chapter Marks *et al.* (1979) have reported that in cultured

chick embryo liver cells several agents which are reported to increase intracellular cAMP levels, viz. glucagon, sodium fluoride, cAMP or its dibutyryl derivative, 3-isobutyl-1-methylxanthine and papaverine all enhanced drug induced porphyrin biosynthesis. On the other hand, agents which are reported to decrease intracellular cAMP levels, viz. alloxan and imidazole, diminished drug induced porphyrin accumulation.

Although, the mode of action of cAMP in these cells is unclear its effect is likely to be a more general effect on cell viability (as measured by general protein synthesis experiments) than a specific modulation of ALA-synthase induction by porphyrinogenic drugs. No attempt has been made to study this.

CHAPTER FIVE

STUDIES ON THE MECHANISM OF DRUG INDUCTION OF ALA-SYNTHASE

5.1 INTRODUCTION

As mentioned in the general introduction (Chapter One), immunological studies have shown that in experimentally induced porphyria, the synthesis of ALA-syntahse occurs *de novo* (Whiting and Granick, 1976; Whiting, 1976; Brooker *et al.*, 1980) and heme, the final product of the pathway, inhibits enzyme induction (Granick, 1966; Tyrrell and Marks, 1972; Tomita *et al.*, 1974; Sassa and Kappas, 1977). Although there is general agreement that heme plays an important role in the physiological control of ALA-synthase activity (Granick, 1966; Ohashi and Kikuchi, 1972; Tyrrell and Marks, 1972; Tomita *et al.*, 1974), there is no concensus as to the mechanism by which drugs override this and induce enzyme synthesis. A number of theories have been proposed and these were discussed in detail in the general introduction. To repeat the main point, however, Granick's (1966) hypothesis for the induction of ALA-synthase envisaged porphyrinogenic drugs to compete with the primary regulator heme for a site on an oporepression protein. Heme would therefore be dissociated from the apo-repressor, resulting in derepression of the operator gene (using a prokaryote analogy) and thus initiate transcription of ALA-synthase mRNA. But this theory is difficult to reconcile with the wide structural diversity of inducing drugs all acting as analogues of heme. More telling is the evidence of Granick *et al.* (1975) that there is no apparent linear relationship between the inducing drug concentration and heme inhibition. Sassa and Granick (1970) suggested that different inducing drugs may act at different

levels, either involving increased transcription (DDC) or translation (AIA) of ALA-synthase. In contrast to this proposal, subsequent studies by Tyrrell and Marks (1972) suggested that both AIA and DDC involve increased transcription of ALA-synthase. Recently, Brooker *et al.* (1980) have shown increased translation of hepatic polyribosomes and isolated poly A^+ mRNA for ALA-synthase following induction of chick embryos *in ovo* by AIA plus DDC.

Granick and Beale (1978) have proposed that inducing chemicals have two different simultaneous actions. The first action is to increase functional mRNA of ALA-synthase. The second postulated action of the inducing drugs is to reduce heme levels. When both actions are present, the synthesis of ALA-synthesis is proposed to occur. Neither effect alone (mRNA increase or heme decrease) is sufficient on this theory to induce ALA-synthase. In this theory, heme is postulated to act post-transcriptionally (in their diagram it is shown as affecting the processing of a pre-mRNA).

An alternative and distinctly different mechanism was suggested by De Matteis (1970); that chemical inducers promote the degradation of intracellular heme, thereby reducing the feedback control on ALA-synthase.

To summarize, there appeared to be two possibilities. The first that inducing drug increases mRNA for ALA-synthase presumably by activating gene transcription and in addition causes abolition of heme repression. However, no unequivocal evidence for a direct gene controlling action of these drugs exists. The second possibility is that heme

repression is the sole control and that drugs simply eliminate this. In the former case one might predict that the presence of the drug would be essential for continued induction of ALA-synthase. In the second case, once cellular heme has been depleted, ALA-synthase induction should continue indefinitely even after removal of the drug, provided that endogenous heme synthesis is prevented. The experiments described in this chapter were designed to ask the question of whether the continued presence of porphyrinogenic drugs (AIA or DDC) is necessary for continued induction of ALA-synthase once the process of induction had been initiated.

5.2 RESULTS

5.2.1 Effect of an iron chelating agent (DES) on the induction of ALA-synthase

The aim of this experiment was to ask the question whether inhibition of heme synthesis by itself is sufficient to cause induction of ALA-synthase in isolated chick embryo liver cells.

Isolated chick embryo liver cells were prepared as described in Materials and Methods and DES (500 µg/ml) was added to the cells either alone or with AIA (500 µg/ml) or DDC (25 µg/ml). The activity of ALA-synthase and the levels of porphyrin was measured after 10 h. The results in Table 5.1 show that DES caused approximately 3-fold increase in total porphyrin levels, but did not induce

TABLE 5.1

EFFECT OF AN IRON CHELATING AGENT (DES) ON THE INDUCTION OF ALA-SYNTHASE BY DRUGS

Isolated chick embryo liver cells were incubated in Eagle's Basal Medium supplemented with Bt_2cAMP (50 μM), insulin (1 μg/ml) and hydrocortisone (0.05 μg/ml). Further additions were made at 0 h. After incubation the cells were sonicated, and the ALA-synthase activity and total intracellular porphyrins accumulated was determined as described in Materials and Methods.

Values are the mean of four separate determinations.

Additions (μg/ml)	ALA-synthase nmol ALA/mg protein/h	Total intracellular porphyrin levels pmol/mg protein/10 h
None	0.30	6.0
DES (500)	0.31	16.8
AIA (500)	7.50	115.1
DDC (25)	15.50	ND[1]
AIA (500) + DES (500)	9.40	146.7
DDC ((25) + DES (500)	15.51	ND

[1]ND Not determined

ALA-synthase. Separate experiments which are described in Chapter Six (Section 6.2.1) have shown that DES, an iron chelator, supresses heme synthesis in these cells by inhibiting incorporation of iron into protoporphyrin to synthesize heme. The above results therefore suggest that in isolated chick embryo liver cells inhibition of ferrochelatase alone is not sufficient to induce ALA-synthase at least over the measured 10 h period. This is in agreement with the results of Sinclair and Granick (1975).

ALA-synthase was also measured in homogenates after the cells were incubated for 8 h with AIA alone or AIA plus DES. The experiment showed that when the chelator (DES) was added in addition to AIA, it caused a 40% increase in the synthesis of ALA-synthase than obtained by AIA alone (Table 5.1). But when another inducing drug DDC was used, DES was found not to be synergistic in inducing ALA-synthase synthesis (Table 5.1). This result is in agreement with Rifkind (1979) who showed that, like DES, DDC also inhibits ferrochelatase activity; AIA has no effect.

5.2.2 Maintenance of ALA-synthase induction in the absence of inducers by DES

The purpose of the next experiments was to ask whether the presence of the drug AIA is necessary for continued induction of ALA-synthase once the process has been initiated. To test it, isolated chick embryo liver cells were incubated in the presence of AIA. After 3 h, which is sufficient time to initiate measurable

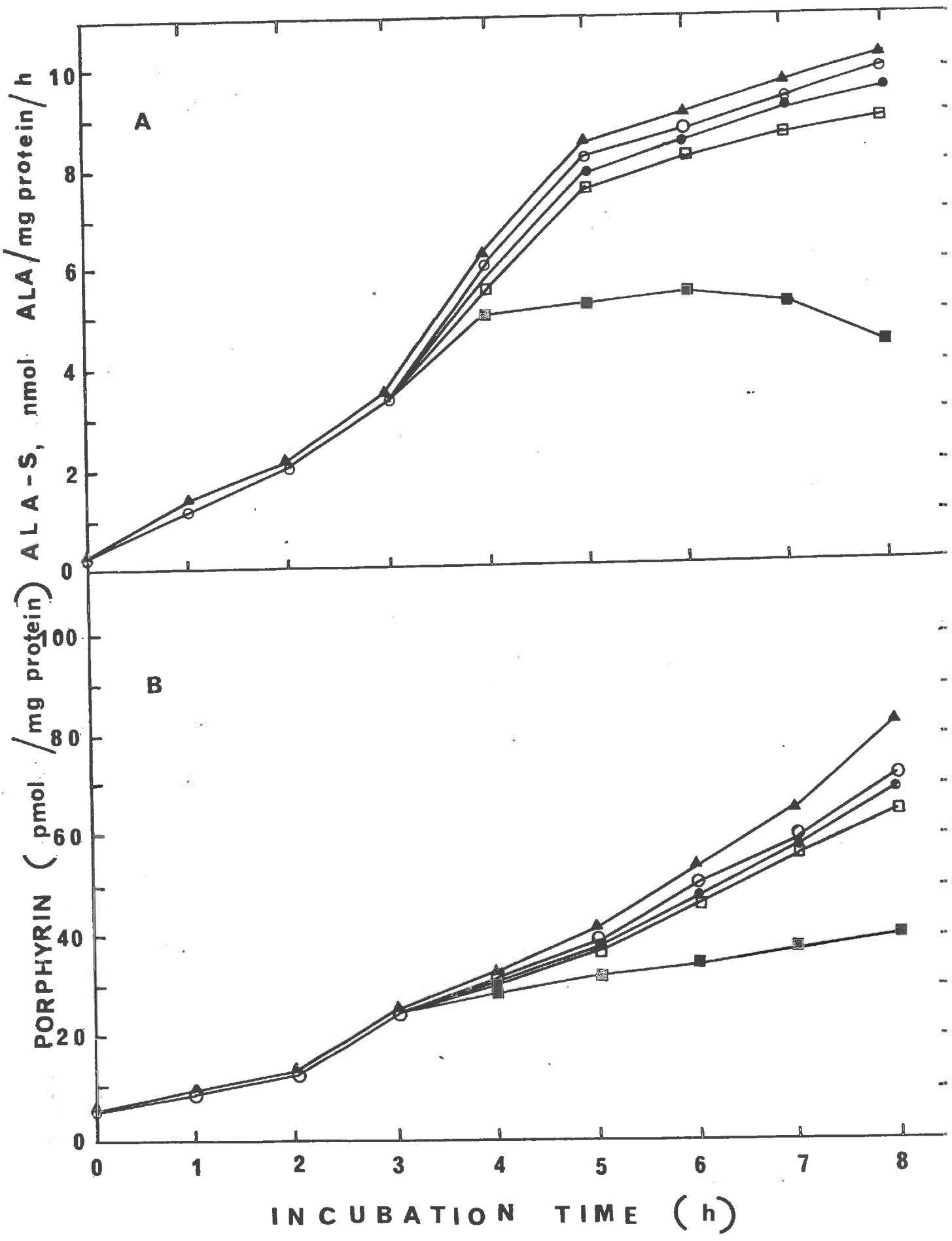
induction of ALA-synthase, cells were washed (as described in Materials and Methods) to remove AIA and were then reincubated in fresh medium lacking AIA. Appropriate controls showed that the cell washing procedure did not effect cell viability as judged by fluorescein diacetate staining, and did not effect the incorporation of L-[4,5-^{3}H]-leucine into total cellular protein (results not shown).

ALA-synthase activity [Fig. 5.1(A)] and total porphyrin levels [Fig. 5.1(B)] were then followed. The lower curve in Fig. 5.1(A) shows that washing the cells and resuspending in fresh media lacking AIA causes an immediate fall in the rate of enzyme accumulation. There is no further increase in ALA-synthase by 1 h after washing. This is not the result of the washing procedure alone for when cells are identically treated but re-incubated with AIA, induction of enzyme is unimpaired. The brief rise in ALA-synthase following washing and in the absence of additional inducer might be attributed to the continued presence of mRNA; this assumes of course that mRNA is limiting, a likely but not proven assumption. Surprisingly, when induced cells were identically treated but re-incubated with DES in the absence of added inducer enzyme induction proceeded for at least a further 5 h and at a rate approaching that of the unwashed control. These experiments suggest that once induction of ALA-synthase is established, inducer is no longer required and implies that heme accumulation switches off the induction. Similar results were obtained by measuring total porphyrins accumulation Fig. 5.1 (B) instead of ALA-synthase activity Fig. 5.1(A).

FIGURE 5.1

DES MEDIATED MAINTENANCE OF ALA-SYNTHASE INDUCTION IN THE ABSENCE OF INDUCER AIA

Isolated chick embryo liver cells were incubated in regular media supplemented with AIA (500 µg/ml) for 3 h, washed with fresh media as described below and re-incubated in fresh media containing the following additions: AIA (500 µg/ml) and DES (500 µg/ml) (○); AIA (500 µg/ml) (●); DES (500 µg/ml) (□); no additions (■); and control without washing (▲). ALA-synthase (A) and intracellular total porphyrin levels (B) were determined after various incubation times, as described in Materials and Methods. Values are the mean of four separate determinations.


A
B
ALA-S, nmol ALA/mg protein/h
PORPHYRIN (pmol /mg protein)
INCUBATION TIME (h)
0
2
4
6
8
10
20
40
60
80
100
1
3
5
7

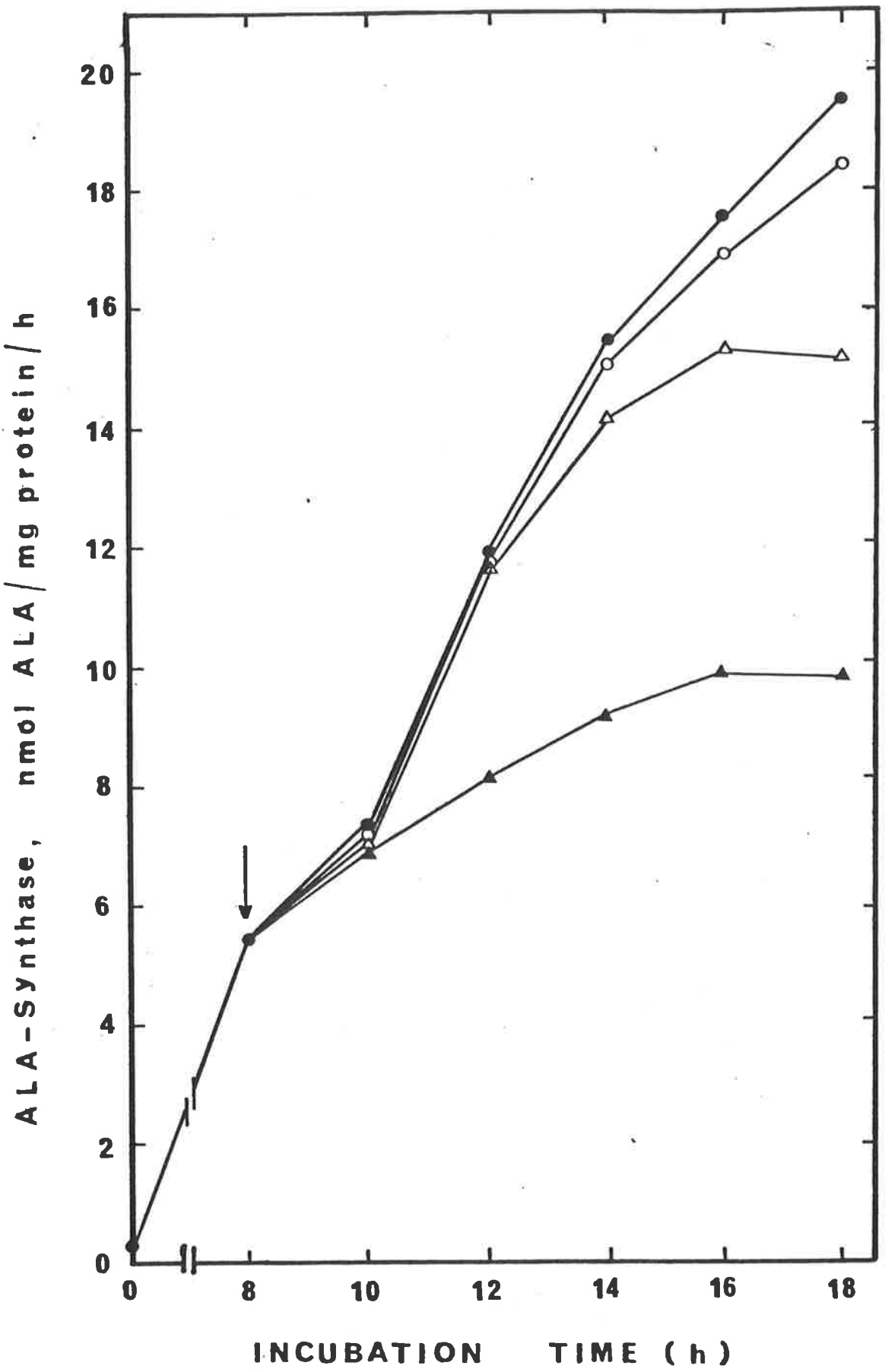
5.2.3 Maintenance of ALA-synthase induction by DES in the absence of inducer DDC

To repeat this experiment using DDC as the inducing drug instead of AIA, chick embryo liver cells were treated with DDC for 8 h after which the drug was removed by washing in the presence of DES, and the cells were re-incubated in fresh medium lacking the drug but containing DES. Fig. 5.2 shows that under these conditions, DES alone in the absence of inducer promoted full enzyme induction for a further 6 - 8 h at a rate approaching that of the unwashed control confirming the results obtained when AIA was used as the inducer (Fig. 5.1). When DDC was readded after cell washing, ALA-synthase induction continued for at least 10 h (Fig. 5.2). However, when cells were reincubated in fresh medium lacking DDC as well as DES, ALA-synthase induction ceased within 1 h. The result, like that of AIA, implies that heme accumulation switches off ALA-synthase induction. Separate experiments showed that the levels of induction maintained by DES in the absence of inducers (AIA or DDC) was approximately the same as the induction obtained when sub-optimal concentrations of AIA (250 µg/ml) or DDC (10 µg/ml) were added to the media at 0 h respectively in the presence of DES and ALA-synthase levels were measured over the same time period.

FIGURE 5.2

DES MEDIATED MAINTENANCE OF ALA-SYNTHASE INDUCTION IN THE ABSENCE OF INDUCER DDC

Isolated chick embryo liver cells were incubated in regular media (see in Table 5.1) supplemented with DDC (25 µg/ml) for 8 h, washed with fresh media containing DES as described in Fig. 5.1 except that the medium used to wash away the cells also contained sonicated safflower oil (0.5 µl/ml) and re-incubated in fresh media containing the following additions (↓): DDC (25 µg/ml) (○); DES (1 mg/ml) (△); no additions (▲); and control without washing (●). ALA-synthase activity was determined after various incubation times as described in Materials and Methods. Values are the mean of four separate determinations.


ALA-Synthase, nmol ALA / mg protein / h
0
2
4
6
8
10
12
14
16
18
20
0
8
10
12
14
16
18
INCUBATION TIME (h)

5.3 DISCUSSION

The results described in this chapter show that DES, an iron chelator and inhibitor of ferrochelatase caused a 3-fold increase in total porphyrin levels but did not induce ALA-synthase in isolated chick embryo liver cells. This suggests that inhibition of heme synthesis is not sufficient to cause induction of ALA-synthase. As described in Section 4.2 Sinclair and Granick (1975) have previously shown that DES did not induce ALA-synthase in cultured chick embryo hepatocytes. Rifkind (1979) has also come to a similar conclusion by using sub-inducing levels of DDC which suppressed ferrochelatase without causing any increase in ALA-synthase synthesis. Sinclair and Granick (1975) have shown that the chelator caused a synergistic increase in the action of AIA, DDC and other inducers even when the drugs were used at their maximum inducing concentrations. The synergistic increase was attributed to the combined effect of (i) induction of ALA-synthase by the chemicals and (ii) the prevention by the chelator of subsequent heme synthesis. Thus the feedback control by heme on the synthesis of ALA-synthase is prevented and ALA-synthase continues to accumulate (Sinclair and Granick, 1975). The results described in this chapter show that although DES acted synergisticly with AIA in inducing ALA-synthase when DDC was used as the inducing drug, DES was found not to be synergistic. These results are in contrast to those reported by Sinclair and Granick (1975) where DDC and DES were shown to be synergistic in inducing ALA-

synthase synthesis. The differences in results could be explained by the low level of DDC used by Sinclair and Granick (1975) in their experiments. The new method used to add DDC to the medium has been described earlier in Section 4.2.7.

The results described here also show for the first time that after the initial induction event by AIA or DDC, continued ALA-synthase induction and total porphyrin accumulation can be maintained by the inhibitor of heme synthesis (DES) in the absence of added inducers. It might be argued that in fact the inducing drug is still present in low concentration after washing the cells. This of course is very probable but nonetheless the results show that washing causes a rapid termination of induction. An alternative is that small amounts (sub-inducing) of AIA or DDC act synergistically with DES to promote continued induction of ALA-synthase. These levels of AIA or DDC by themselves may not be effective. But it must be emphasized that in the isolated cells levels of induction maintained by DES in the absence of inducers were the same as the induction obtained when 250 μg/ml of ALA or 10 μg/ml of DDC were added to the media at 0 h respectively in the presence of DES and ALA-synthase levels were measured over the same time period. On this basis it seems reasonable to conclude that after washing the cells then so far as induction of ALA-synthase goes, there is effectively no drug present.

It might also be argued that DES acts both as an inducing drug and iron chelator. This seems unlikely

since the results described in this chapter show that DES did not induce ALA-synthase and suggests that DES merely acted to block the conversion of protoporphyrin to heme on this hypothesis it is necessary to assume either that the heme pool has a low rate of turnover or that removal of heme is not enough to induce ALA-synthase. If heme depletion alone is enough then the pool must turn over slowly, such that it does not significantly diminish in amount even after heme synthesis has been inhibited for several hours.

The results described above appear to be compatible with the Granick and Beale (1978) hypothesis and show that after initiation of induction by AIA or DDC, the synthesis of ALA-synthase could be maintained in the absence of drugs and suggests that this is dependent upon the concentration of intracellular heme being kept low. But the results are incompatible with the theory that drugs cause continuous stimulation of the genome, thereby leading to the induction of ALA-synthase.

However, a note of caution must be added at this point. These experiments provide only indirect evidence that the level of intracellular heme is the primary control of ALA-synthase induction. More definitive proof of this proposal awaits an accurate measurement of the concentration of intracellular heme. Clearly until more direct evidence is available the conclusions in this complex system must be viewed as tentative.

CHAPTER SIX

CONTROL OF THE INDUCTION OF ALA-SYNTHASE BY HEMIN

6.1 INTRODUCTION

Granick (1966), Sassa and Granick (1970) and Tyrrell and Marks (1972) have shown that hemin, when added to cultured chick embryo liver cells, inhibited the induction of ALA-synthase by AIA. Hemin injected intravenously into animals was also found to inhibit induction of ALA-synthase (Marver, 1969). Moreover, Strand *et al.* (1972) and Granick *et al.* (1975) in cultured chick embryo liver cells, and Padmanaban *et al.* (1973) in rats, have shown that only hemin and not any intermediate of the heme biosynthetic pathway inhibited induction of ALA-synthase and they suggested that heme was a major regulator of hepatic heme biosynthesis.

As described in Chapter Five, drug induced depletion of intracellular heme has been suggested (De Matteis, 1970; Meyer and Marver, 1971) as a possible mechanism of induction of ALA-synthase. However, there is no real agreement as to the manner in which the various inducing drugs may cause heme depletion. As described earlier, there are several proposals. (a) Inducers may block an enzyme of the heme biosynthetic pathway (De Matteis, 1973; Sinclair and Granick, 1974; Sinclair and Granick, 1975). (b) Compounds with allylic groups are suggested to cause destruction of the heme of cytochrome P-450, and by reconsitituion of the apo-protein, causes depletion of free heme (Abbritt and De Matteis, 1971; Levin *et al.*, 1973). (c) Certain chemcials (e.g., phenobarbital) may stimulate the synthesis of the apo-protein of cytochrome P-450, a protein with a high affinity for heme, and therefore cause depletion of intracellular heme

(Correia and Meyer, 1975). (d) Heme depletion may occur by increasing the activity of the degradative enzyme, heme oxygenase in the liver phagocytic cells (Meier and Meyer, 1976). In support of heme mediated control in isolated chick embryo liver cells it has been shown in the previous chapter that the continued presence of a chemical inducer is not required after initiation of induction provided that DES is present and the probable effect of the latter is to inhibit heme synthesis. This result raises the possibility that if the concentration of cellular heme could be reduced even in the absence of inducing drugs, synthesis of ALA-synthase may occur.

The experiments described in this chapter are to re-examine more closely the effect of heme on ALA-synthase control. The experiments were undertaken because despite the large number of demonstrations that heme represses induction of ALA-synthase there is a lack of a systematic study of the effects of different heme concentrations on the system and especially lacking is information on the time course of heme inhibition.

6.2 RESULTS

6.2.1 Repression of drug mediated induction of ALA-synthase by endogenous heme synthesized from added ALA or protoporphyrin

The purpose of the experiments described below was to study the control of ALA-synthase synthesis

by hemin in a suspension of chick embryo liver cells. The cells were incubated as described in Materials and Methods and the effect of exogenously added ALA or protoporphyrin on ALA-synthase induction was examined. The results described in Table 6.1 show that the addition of ALA or protoporphyrin to AIA induced liver cells in suspension resulted in marked inhibition of ALA-synthase synthesis measured over an 8 h period. However, added ALA or protoporphyrin did not inhibit induction over this period when AIA induced cells were supplemented with DES (Table 6.1). This result suggests that in the absence of DES the added ALA or protoporphyrin was converted to heme which then prevented the induction of ALA-synthase. However, in the presence of DES, formation of heme is presumed to be prevented and in its absence no inhibition of ALA-synthase synthesis occurred.

When DDC was used as the inducing drug, ALA (25 μM) or protoporphyrin (1.0 μM) did not inhibit the induction of ALA-synthase over a 14 h period (Table 6.2). Even when higher concentrations of ALA (50 μM) or protoporphyrin (2.0 μM) were added, there was only a slight effect on the induction of ALA-synthase (Table 6.2). These results agree with the data of De Matteis (1973) and suggest that DDC prevented the synthesis of heme from added ALA or protoporphyrin; therefore in the absence of endogenously synthesized heme no inhibition of ALA-synthase induction occurred. This conclusion is supported by the data of Rifkind (1979) who showed that DDC inhibited the activity of chick embryo liver ferrochelatase *in vitro*.

TABLE 6.1

INHIBITION BY EXOGENOUSLY ADDED ALA OR PORPHYRIN OF SYNTHESIS OF ALA-SYNTHASE INDUCED BY AIA

Isolated chick embryo liver cells were incubated in Eagle's basal medium supplemented with Bt_2cAMP (50 µM), insulin (1 µg/ml) and hydrocortisone (0.05 µg/ml). Further additions were made at 0 h. After 8 h incubation the cells were sonicated and the ALA-synthase activity determined as described in Materials and Methods. The results are the means of three separate determinations.

Additions (µg/ml)	ALA-synthase nmol ALA/mg Protein/h
None	0.31
AIA (500)	7.50
AIA (500) + Hemin (2 µM)	0.30
AIA (500) + ALA (50 µM)	2.73
AIA (500) + Protoporphyrin (1 µM)	1.84
AIA (500) + ALA (50 µM) + DES (500)	9.01
AIA (500) + Protoporphyrin (1 µM) + DES (500)	8.73

TABLE 6.2

INHIBITION BY EXOGENOUSLY ADDED ALA OR PROTOPORPHYRIN OF SYNTHESIS OF ALA-SYNTHASE INDUCED BY DDC

Isolated chick embryo liver cells were incubated in Eagle's Basal Medium supplemented with Bt_2cAMP (50 µM), insulin (1.0 µg/ml) and hydrocortisone (0.05 µg/ml). Further additions were made at 0 h and ALA-synthase activity was determined 14 h after incubation. Values are the mean of two to four separate determinations.

Additions (µg/ml)	ALA-synthase nmol ALA/mg protein/h
Safflower oil (0.5 µl/ml)	0.30
DDC (25)	15.50
DDC (25) + ALA (25 µM)	14.97
DDC (25) + ALA (50 µM)	13.03
DDC (25) + Protoporphyrin (1.0 µM)	14.99
DDC (25) + Protoporphyrin (2.0 µM)	12.11

6.2.2 Repression of drug mediated induction of ALA-synthase by exogenous hemin

Granick *et al.* (1975) have reported that hemin (0.1 to 0.3 μM), when added to the medium of cultured chick embryo liver cells repressed the AIA mediated induction of ALA-synthase by 50% after 20 h incubation. Recently, Sassa and Kappas (1977) reported that concentrations as little as 0.04 μM of exogenously added hemin repressed AIA mediated accumulation of protoporphyrin by approximately 50% in the chick embryo liver cells grown in serum-free medium over a 20 h period. Granick *et al.* (1975) also reported a linear correlation between the percentage repression of ALA-synthase and hemin concentration added to chick embryo liver cells in culture.

In order to show the hemin control of ALA-synthase in isolated chick embryo liver cells, the effect of increasing concentrations of hemin on ALA-synthase induction was examined. Enzyme activity was measured 8 h after induction by AIA in the presence of DES. DES was added to the medium to diminish endogenous heme production. The results in Fig. 6.1 show that concentrations as low as 50 nM of hemin inhibited by 50% the AIA-mediated induction of ALA-synthase over an 8 h period. Moreover, the results also show an unusual biphasic effect with different concentrations of hemin. Concentrations of hemin between 20 nM and 50 nM progressively inhibited AIA-mediated induction of ALA-synthase to approximately 50%, but concentrations greater than 200 nm were required for any additional inhibition.

Complete inhibition of enzyme synthesis over the 8 h period was only observed at hemin concentrations greater than 1 μM.

6.2.3 Time course of ALA-synthase induction in the presence of varying concentrations of hemin

To examine the effect of various concentrations of hemin on the time course of ALA-synthase induction, isolated chick embryo liver cells were incubated as described in Materials and Methods and varying concentrations of hemin were added at 0 h or 2 h. ALA-synthase was assayed after varying incubation times. The results Fig. 6.2(B) show that even at the lowest concentration used (20 nm), hemin added at zero time caused a complete inhibition of ALA-synthase induction for 2 h, after which time an increase in enzyme levels occurred.

The period of complete repression of enzyme synthesis increased with increasing hemin concentrations up to 50 nm. Between 50 and 200 nM hemin, the effect of ALA-synthase synthesis was unchanged, compatible with the plateau observed in Fig. 6.1. At a hemin concentration of 2 μM, enzyme synthesis was completely inhibited over the 8 h incubation period [Fig. 6.2(B)]. To confirm that this was not an effect of hemin on general protein biosynthesis, cells were incubated for 8 h in the presence of inducer, hormones, DES and different concentrations of hemin (up to 5 μM); general protein synthesis was determined by measuring the incorporation of L-[4,5-^{3}H]-leucine into trichloroacetic acid insoluble protein. The results (not shown) showed that hemin at least

FIGURE 6.1

EFFECT OF VARYING CONCENTRATIONS OF EXOGENOUS HEMIN ON ALA-SYNTHASE SYNTHESIS

Isolated chick embryo liver cells were incubated in regular media supplemented at 0 h with AIA (500 µg/ml), DES (500 µg/ml) and varying concentrations of hemin dissolved in 0.2 N KOH:ethanol, 1:1 (v/v). After 8 h incubation ALA-synthase activity was determined as described in Materials and Methods.

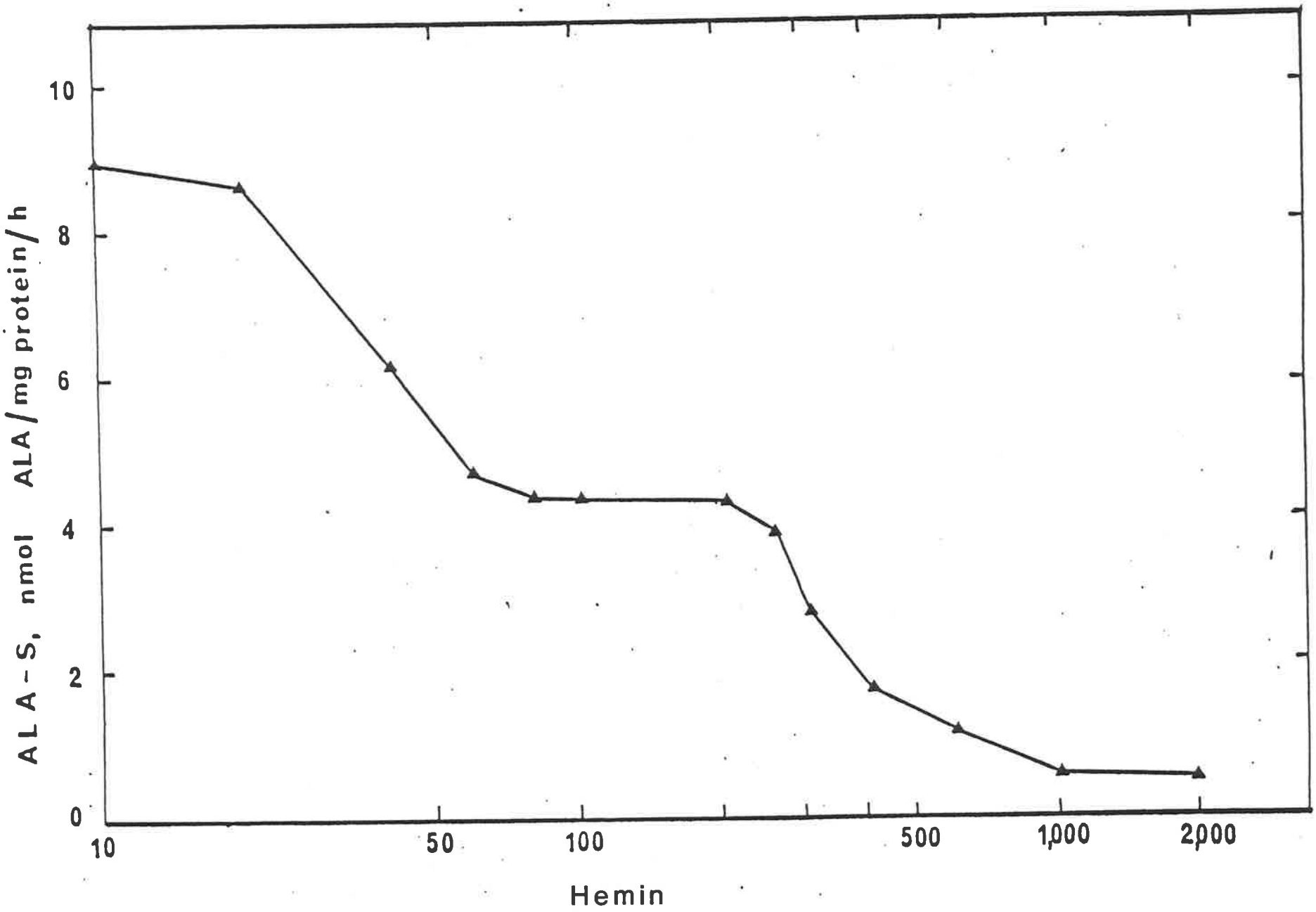
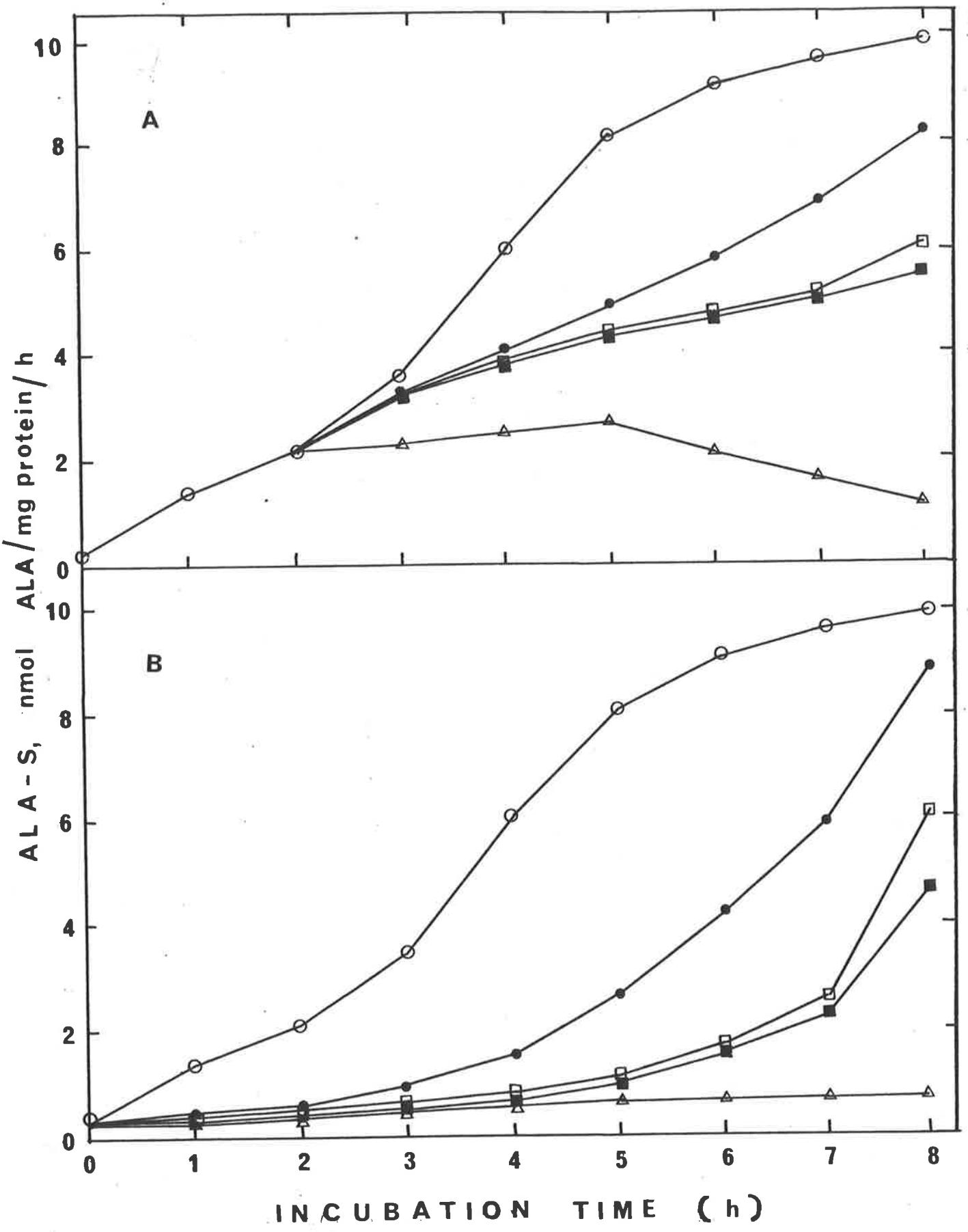

ALA-S, nmol ALA/mg protein/h
10
8
6
4
2
0
10
50
100
500
1,000
2,000
Hemin

FIGURE 6.2

TIME COURSE OF ALA-SYNTHASE INDUCTION IN THE PRESENCE OF VARYING CONCENTRATIONS OF HEMIN

Isolated chick embryo liver cells were incubated in regular media supplemented with AIA (500 µg/ml) and DES (500 µg/ml). Varying concentrations of hemin were added at 2 h (A) or 0 h (B) and ALA-synthase activity was determined after various incubation times. No addition (O), 20 nM hemin (●), 50 nM hemin (□), 200 nM hemin (■), 2 µM hemin (△).


A
B
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
ALA-S, nmol ALA/mg protein/h
INCUBATION TIME (h)

up to 5 μM did not affect general protein synthesis and suggest that the hemin repression of ALA-synthase induction is a specific effect.

To examine the effect of various concentrations of hemin on the time course of ALA-synthase induction after the induction is initiated, various concentrations of hemin were added 2 h after AIA. The results [Fig. 6.2(A)] show that even at the lowest concentration used (20 nM), hemin caused an 18% inhibition of induction over an 8 h period. Hemin concentrations from 50 nM to 200 NM showed a similar effect on the time course of ALA-synthase induction as observed in Fig. 6.1. At a hemin concentration of 2 μM enzyme synthesis was inhibited by 94% over an 8 h period. These results show that the isolated chick embryo liver cells are extremely sensitive to hemin.

6.2.4 Induction of hepatic ALA-synthase following the induction of heme oxygenase

Heme depletion has been proposed (De Matteis, 1970; Meyer and Marver, 1971) as a possible mechanism of enzyme induction. However, the mechanism of heme depletion is not understood and moreover it is not known whether in addition to their role in heme depletion, inducing drugs have also to promote mRNA production (other than by heme removal) as illustrated by Granick and Beale (1978). There is no real agreement as to the manner in which drugs may cause heme depletion. In support of heme mediated control, it has been shown in Chapter Five that the continued presence of a chemical inducer is not required after initiation of induction provided that cellular heme

accumulation is prevented.

If the concentration of intracellular heme is the sole controlling factor for ALA-synthase, then the implication is that if the concentration of cellular heme could be reduced in the absence of any inducing drug, synthesis of ALA-synthase might occur. One approach to this problem is based on the fact that heme is known to induce the degradative enzyme heme oxygenase (Maines and Sinclair, 1977) and (Bissell and Hammker, 1976). If heme in the medium were to be removed after this induction (by washing the cells) then the induced heme oxygenase might then eliminate remaining intracellular heme. (After carrying out this work attention was drawn to the fact that in a recent review Granick and Beale (1978) included a sentence pointing out the potential interest of such an experiment). This was tested as follows.

Chick embryo liver cells were treated with DES plus 0.5 μM hemin, a concentration previously shown (Maines and Sinclair, 1977) to induce the synthesis of heme oxygenase. After 11 h, the hemin was removed by extensive washing (the washing media contained DES) and replaced with DES alone to prevent endogenous heme synthesis. ALA-synthase was measured at 16 h and porphyrin levels were measured 22 h after washing. The results (Fig. 6.3) show that after 11 h, exogenous hemin had caused a 3-fold increase in heme oxygenase levels and, following the removal of exogenous hemin and replacement with DES alone, ALA-synthase activity increased more than 10-fold in 16 h. Intracellular porphyrin levels also increased 6-fold in 22 h. With lower concentrations

FIGURE 6.3

INDUCTION OF ALA-SYNTHASE FOLLOWING THE INDUCTION OF HEME OXYGENASE

A cell suspension was prepared from 17-day chick embryo livers as described in Materials and Methods, and incubated for 11 h in Eagle's basal medium supplemented with 2 mM glutamine, 50 µM Bt_2cAMP, 1 µg/ml insulin, 0.05 µg/ml hydrocortisone, 2 mg/ml DES and 0.5 µM hemin. The cells were then washed 3 times by resuspending and briefly incubating (10 min) in fresh media lacking hemin but containing DES, and finally suspended in the original volume of this media (↓). Mitochondria and microsome were prepared (Materials and Methods); ALA-synthase (■) and heme oxygenase activities (●) were estimated, and the values are the mean of 4 separate determinations. Heme oxygenase activity is expressed as nmol bilirubin produced/mg microsomal protein/h, and ALA-synthase activity as nmol ALA produced/mg mitochondrial protein/h.

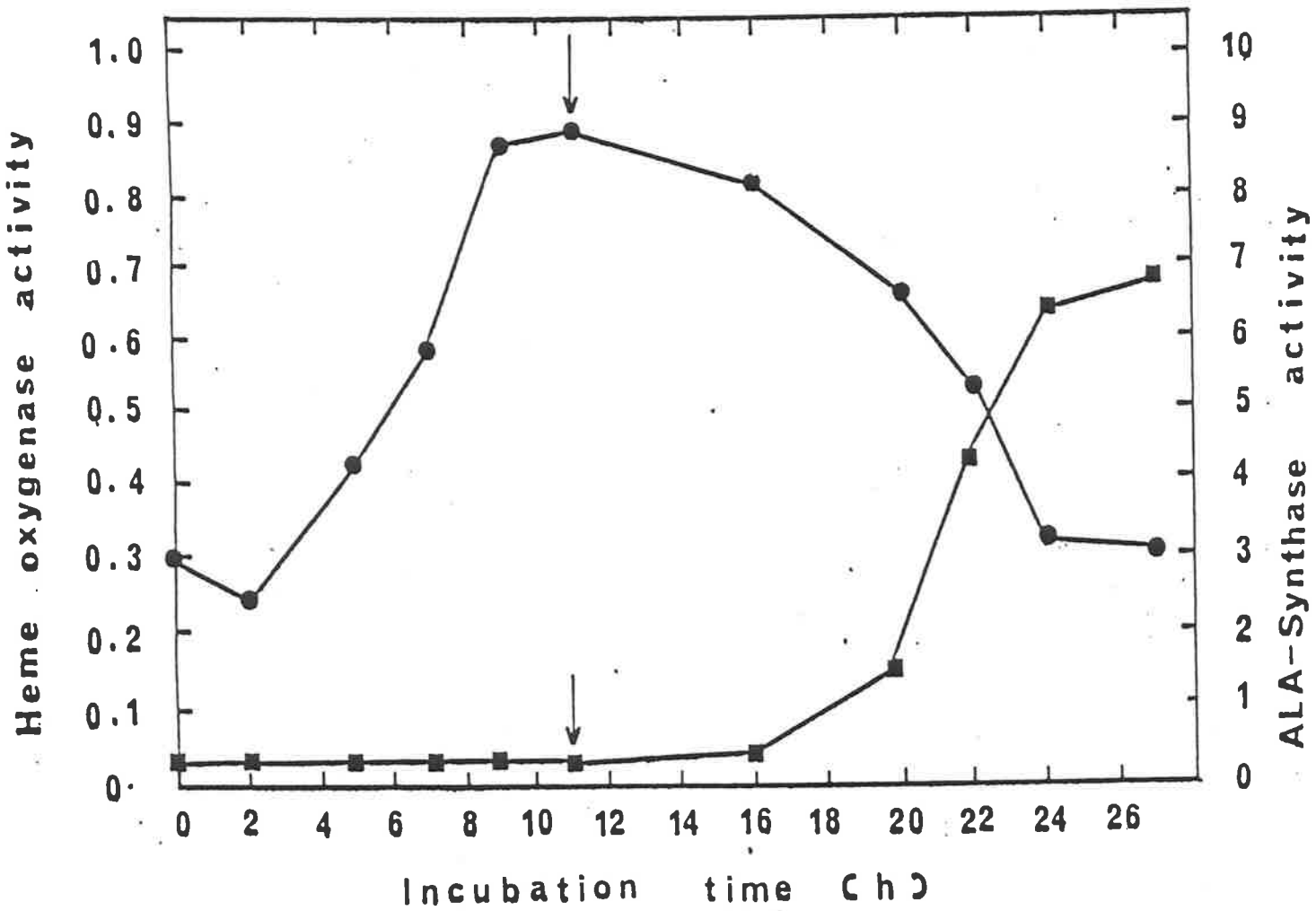

Heme oxygenase activity
ALA-Synthase activity
Incubation time (h)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
9
8
7
6
5
4
3
2
1
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26

TABLE 6.3

EFFECT OF VARYING CONCENTRATIONS OF HEMIN ON INDUCTION OF HEME OXYGENASE AND ALA-SYNTHASE

Chick embryo liver cells were prepared, incubated with varying concentrations of hemin and washed as described in Fig. 6.3. ALA-synthase and heme oxygenase activities and cellular prophyrin levels were estimated after the desired incubation times as described in Materials and Methods. Values are the mean of 4 separate determinations. Heme oxygenase activity at zero time was 0.3 nmol bilirubin/mg protein/h.

TABLE 6.3

Hemin conc^{n}. added at 0 h (nM)	Addition at 11 h (after washing)	Heme oxygenase activity at 11 h (nmol bilirubin/mg protein/h)	ALA-synthase activity at 16 h after washing (nmol ALA/mg protein/h)	Total porphyrin levels at 22 h after washing (pmol/mg protein)
No addition	No addition	0.32	0.31	6
No addition	DES	0.32	0.31	16
500	Hemin (500 nM) and DES	0.86	0.27	16
125	DES	0.33	0.30	17
250	DES	0.41	1.27	73
500	DES	0.89	7.19	144
1000	DES	ND[1]	5.85	132
2000	DES	ND[1]	5.14	116
5000	DES	0.90	3.80	92
500 (no DES)	DES	0.87	7.00	139

[1] - not determined.

of hemin, the response was greater, and at the optimum of 500 nM, there was a 23-fold increase in ALA-synthase and a 9-fold increase in porphyrins. Hemin below 250 nM was less effective, and at 125 nM did not promote induction of heme oxygenase or ALA-synthase (Table 6.3). These results clearly demonstrate the induction of ALA-synthase in the absence of a non-physiological chemical inducer. DES alone did not induce heme oxygenase or ALA-synthase, and induction of heme oxygenase by hemin alone was not affected by addition of DES (Table 6.3).

6.3 DISCUSSION

The above results describe the effect of varying concentrations of hemin on the time course of ALA-synthase induction and show that hemin concentrations as low as 20 nM completely inhibited AIA-induced ALA-synthase synthesis for a period of 2 h, followed by a recovery period in which the rate of enzyme synthesis approached control values in the absence of hemin. The degree of inhibition depended upon the time selected to measure it - 20 nM hemin inhibited for 2 h, less hemin may be required to inhibit for, say, 15 min.

These results are of particular importance with regard to the role of heme in ALA-synthase induction, since hemin concentrations as high as 50 μM have previously been used to define the site of action of heme (Tyrrell and Marks,

1972; Tomita *et al.*, 1974). The results in this chapter show that such amounts used in previously published studies on heme inhibition of induction did not include the time course and cannot be interpreted with any confidence. Furthermore, Sassa and Granick (1970) have estimated the intracellular concentration of heme in chick embryo liver cells to be of the order of 50 - 100 nM, a value that is shown in these experiments to be sufficient to completely inhibit ALA-synthase synthesis.

The results on the effect of varying concentrations of hemin on ALA-synthase induction also show that increasing concentrations of hemin extended the inhibitory period, but did not affect the subsequent rate of induction. Above 1 μM, hemin was completely inhibitory throughout the course of the experiment. These data demonstrate that AIA induced ALA-synthase synthesis is prevented by very low concentrations of heme and that this heme effect is reversible.

The biphasic shape of the hemin inhibition curve is surprising in that there is a plateau between 50 nM and 200 nM hemin, whereas a continuously increasing inhibition might be expected as reported by Granick *et al.* (1975) using monolayer culture of chick embryo liver cells. This effect could be due to the hemin concentration - dependent induction of heme oxygenase, (Bissell and Hammaker, 1976). As a consequence of this effect, the actual concentration of hemin necessary for inhibition of ALA-synthase induction may be even less than that observed in these experiments. However no attempt was made to test this explanation.

Perhaps the most significant finding in this chapter is the demonstration of induction of ALA-synthase in the absence of a chemical inducer suggesting that heme depletion may be the cause. This conclusion is supported by the data in Table 6.3 which shows that the final level of ALA-synthase was dependent upon the initial concentration of added hemin. These results conflict with the Granick and Beale (1978) hypothesis that drugs need two actions simultaneously for induction and support a one site theory of induction. Further, the demonstration that hemin completely repressed ALA-synthase induction at concentrations as low as 20 nM, suggests that heme control is likely to be a physiologically significant mechanism. To reconcile the fact with the conclusions of the heme oxygenase experiment one must make the assumption that turnover of cellular heme occurs at a low rate. If different metabolic compartments of heme exist (Granick *et al.*, 1975) then this assumption must be made for that compartment which represses enzyme induction. So far there is no direct independent evidence to prove this assumption and until such evidence is available the indications in this thesis that heme may be the sole controlling agent must be regarded only as provisional.

CHAPTER SEVEN

MECHANISM OF REPRESSION OF ALA-SYNTHASE BY HEMIN

7.1 INTRODUCTION

The results in the previous chapter reinforce the concept that heme is of central importance in the control of ALA-synthase synthesis, but although there is general agreement on this point (Granick, 1966; Tomita *et al.*, 1974; Tyrrell and Marks, 1972; Ohashi and Kikuchi, 1972), there is no such concensus on the mechanism of heme action. Many different schemes have been suggested and these are described in detail in the general introduction (Chapter One). To briefly repeat the main points however, Granick (1966) originally proposed that heme acts as a co-repressor for ALA-synthase transcription; chemical inducers were regarded as heme analogues that compete for the apo-repressor protein, thus preventing heme from repressing ALA-synthase transcription. However, later studies by Sassa and Granick (1970) and Tyrrell and Marks (1972) which were designed to test the validity of Granick's hypothesis suggested that, contrary to this transcriptional theory, heme exerted its effect at a post-transcriptional stage. Furthermore, Granick *et al.* (1975) and Sassa and Granick (1970) demonstrated that there was no linear relationship between the concentration of inducing drug and hemin inhibition. Ohashi and Kikuchi (1972), Hayashi *et al.* (1972) and Yamauchi *et al.* (1980) have proposed that in adult chickens, and rats, heme exerts its effect by controlling the transport of cytoplasmically synthesized ALA-synthase into mitochondria. However, heme does not affect ALA-

synthase transport in embryonic chick liver (Tomita *et al.*, 1974). Whiting (1976) reported that hemin treatment (10 μM) of pre-induced chick embryos *in ovo* for 3 h markedly decrease the translation of ALA-synthase from isolated polysomes in a chick embryo liver cell free extract, and Brooker (personal communication) has shown that hemin had no effect on the synthesis of ALA-synthase from poly A^+ RNA in a wheat germ cell free system.

The experiments described in this chapter examine more closely the mechanism of hemin inhibition of ALA-synthase synthesis in suspensions of chick embryo liver cells. This seemed worthwhile particularly because some previous studies have used concentrations of heme greatly in excess of those shown in the previous chapter to be necessary for inhibition.

7.2 RESULTS

7.2.1 Effect of RNA andprotein synthesis inhibitors on induction of ALA-synthase in isolated liver cells

It has been long known from the work of Sassa and Granick, (1970); Tyrrell and Marks, (1972); Tomita *et al.* (1974); Granick *et al.* (1975); Sassa and Kappas, (1977) that ALA-synthase induction in chick embryo monolayer culture and organ culture is inhibited by cyclo-heximide cordycepin and actinomycin D. Similar experiments with these inhibitors were carried out in the isolated chick

embryo liver cell system. Chick embryo liver cells were incubated in regular media containing AIA as described in Materials and Methods. The various inhibitors were added at 0 h and their effect on RNA synthesis, protein synthesis and the induction of ALA-synthase was studied.

Chick embryo liver cells (25 ml) were incubated in media containing 100 μCi of [5,6-^{3}H]-urdine and actinomycin D (2.5 μg/ml) or cordycepin (12.5 μg/ml). Total cellular RNA from 1 ml aliquots was precipitated with 10 ml of 10% TCA containing 0.5% unlabelled uridine and collected on GF/A filters. The precipitate was washed (x 2) with 5% TCA, dried under a heat lamp for 15 min and counted for radioactivity using Triton-toluene scintillation fluid.

Over a 10 h period, cordycepin inhibited by 98% and actinomycin D by 94% the incorporation of [5,6-^{3}H]-uridine into total cellular RNA. When added at zero time, these inhibitors completely prevented AIA-mediated induction of ALA-synthase over the 10 h period (results not shown). Therefore, RNA synthesis is required for the AIA-mediated induction of ALA-synthase.

Over a 10 h period cycloheximide (10 μg/ml) inhibited by 96% the incorporation of L-[4,5-^{3}H]-leucine into total cellular protein when added at 0 h. The incorporation of [^{3}H]-leucine into total cellular protein was determined as described in Fig. 4.2 (Chapter Four). Cychloheximide completely inhibited ALA-synthase induction over the same period.

Experiments were performed to determine the effect of these inhibitors when added after induction of ALA-synthase

was initiated. Cells were induced for 2 h with AIA, the inhibitors were added and ALA-synthase and total porphyrin levels in cells were estimated after various times (Fig. 7.1). Following addition of cordycepin and actinomycin D, the level of ALA-synthase activity increased slightly for a further 1 - 2 h, then remained at that value for an additional 5 h. The slight increase in enzyme level following inhibitor addition can presumably be attributed to translation of pre-existing mRNA. Addition of cycloheximide, caused the abrupt cessation of ALA-synthase production and the level of activity subsequently declined with a $t_{\frac{1}{2}}$ of 4 - 5 h (Fig. 7.1).

7.2.2 Comparison of the effects of hemin, cycloheximide and cordycepin on the induction of ALA-synthase and total intracellular porphyrin levels

The effect of cycloheximide and cordycepin (added at 2 h) were compared with the effect of varying concentrations of hemin on the time course of ALA-synthase induction and total porphyrin levels in the cells. After 2 h of AIA-mediated induction of ALA-synthase, different concentrations (50 nM or 2 μM) of hemin, or 10 μg/ml cycloheximide or 12.5 μg/ml cordycepin were added to the media and ALA-synthase and total porphyrins accumulation were measured after varying incubation times. The results (Fig. 7.2) showed that in the presence of 50 nM hemin, enzyme levels continued to increase for 90 min and then a plateau was reached. This plateau was probably a combination of enzyme synthesis and breakdown i.e., a steady state. A

FIGURE 7.1

EFFECT OF INHIBITORS OF RNA AND PROTEIN SYNTHESIS ON INDUCTION OF ALA-SYNTHASE (a) AND ACCUMULATION OF TOTAL PORPHYRINS (b)

The liver cells were incubated in Eagle's basal media containing AIA (500 µg/ml), insulin (1.0 µg/ml), hydrocortisone (0.05 µg/ml), DES (500 µg/ml), Bt_2cAMP (50 µM) and ALA-synthase and total porphyrins were estimated in cell homogenates prepared after various times as described in Materials and Methods. Further additions after 2 h incubation were: cycloheximide (10 µg/ml) (△); actinomycin D (2.5 µg/ml) (▲); cordycepin (12.5 µg/ml) (○) and no addition (●). Cordycepin was dissolved in the minimum volume of 10 mM HCl and was neutralized before adding to the medium.

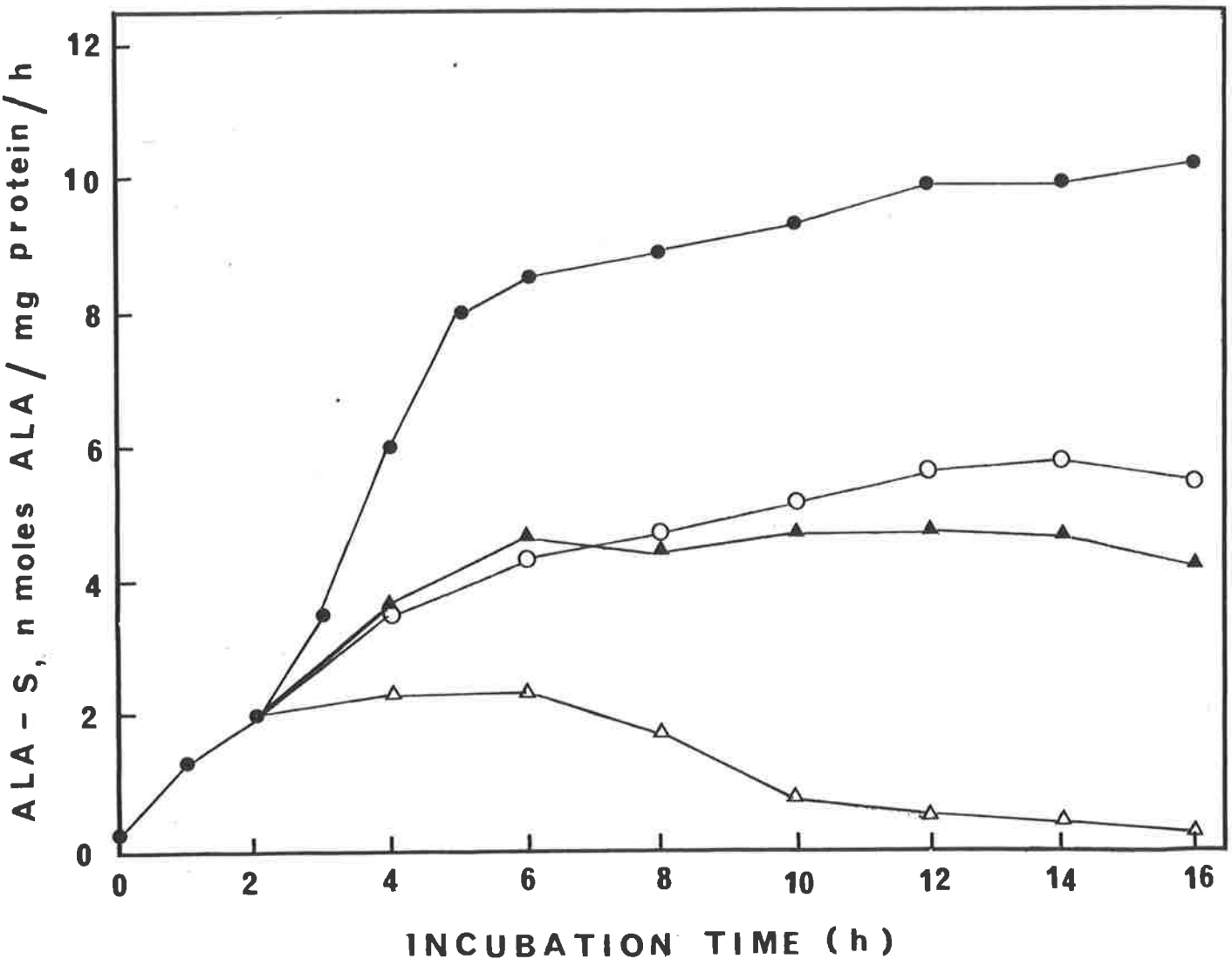
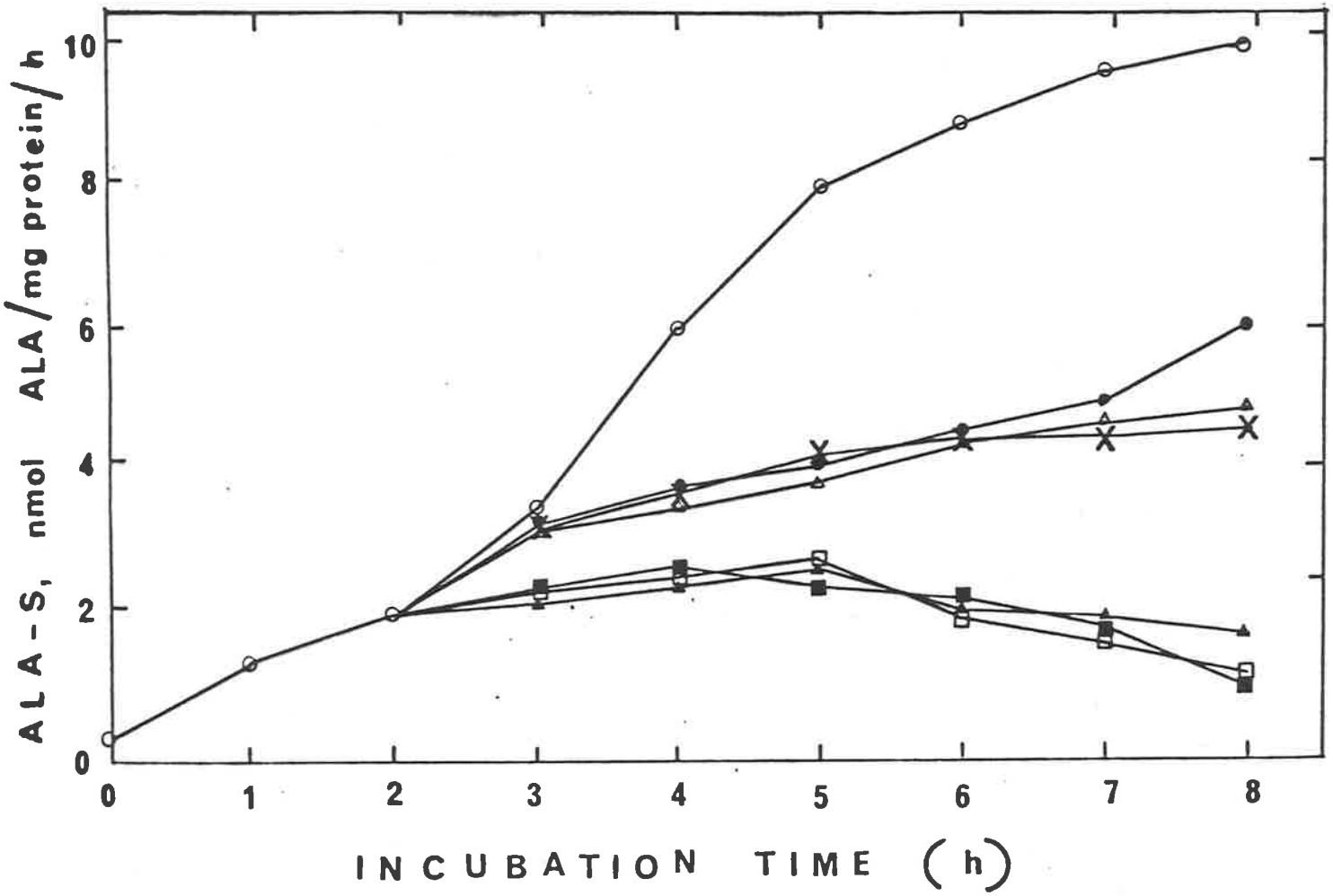

ALA - S, n moles ALA / mg protein / h
0
2
4
6
8
10
12
0
2
4
6
8
10
12
14
16
INCUBATION TIME (h)

FIGURE 7.2.

COMPARISON OF THE EFFECTS OF HEMIN, CYCYLO-HEXIMIDE AND CORDYCEPIN ON THE INDUCTION OF ALA-SYNTHASE

Chick embryo liver cells were incubated in regular media supplemented with AIA (500 µg/ml) for 2 h. The following additions were then made; no addition (○); 50 nM hemin (●); cordycepin (12.5 µg/ml) (△); cordycepin plus 50 nM hemin (▼); cycloheximide (10 µg/ml) (▲); 2 µM hemin (□); and cordycepin plus 2 µM hemin (■). ALA-synthase activity was measured after varying incubation times.


ALA-S, nmol ALA/mg protein/h
0
2
4
6
8
10
0
1
2
3
4
5
6
7
8
INCUBATION TIME (h)

similar plateau was also observed when cordycepin was added instead of 50 nM hemin.

There was no additional inhibition when 50 nM hemin was added to the cordycepin treated cells. The response of the cells to 2 μM hemin was similar to that of cycloheximide; an immediate inhibition of ALA-synthase induction (Fig. 7.2). When 2 μM hemin was added to cordycepin treated cells an immediate inhibition of ALA-synthase induction also occurred, mimicking the effect of cycloheximide (Fig. 7.2). Similar results were obtained when total porphyrin levels were measured instead of ALA-synthase activity.

These results suggest that hemin may have at least two concentration dependent inhibitory effects within the cells, at low hemin concentrations (50 nM) to inhibit the transcription of ALA-synthase mRNA; at high concentrations (2 μM) to inhibit translation of ALA-synthase mRNA. These results conflict with those of Tyrrell and Marks (1972) and Sassa and Granick (1970), who showed no apparent transcriptional effect of exogenous heme on the induction of ALA-synthase synthesis in cultures of chick embryo liver cells.

7.2.3 Effect of hemin on DES maintained ALA-synthase synthesis following the removal of inducing drug

It has been shown in Chapter Five that following the initiation of ALA-synthase synthesis by AIA or DDC, the inducer can be removed from the cells without inhibiting ALA-synthase synthesis provided that intracellular

heme synthesis is prevented. To examine whether DES maintained synthesis is also sensitive to inhibition by exogenous hemin, the following experiments were carried out.

ALA-synthase induction was initiated by DDC and after cell washing, maintained with DES. The effect of hemin on ALA-synthase synthesis was examined. 12.5 ml aliquots from 50 ml of cell suspension were taken at various time points and ALA-synthase levels were determined. Fig. 7.3 shows that in the presence of 20 nM hemin, the DES maintained induction of ALA-synthase was inhibited by 95% over a 10 h period. However, the DDC maintained induction was inhibited by only 63% over the same time period. The experiment was repeated with AIA treated cells. Fig. 7.4 shows the effect of 50 nM hemin on ALA-synthase synthesis after cells were pre-induced with AIA for 3 h and the drug was then removed by washing. In the control experiment no additions were made subsequent to washing and the increase in ALA-synthase presumably reflects translation of pre-existing mRNA; addition of 50 nM hemin did not effect this. However, in cells re-incubated with DES plus hemin or AIA plus hemin, increase in enzyme synthesis did occur. This was unexpected, since, as shown in Chapter Six, 50 nM hemin added at 0 h inhibits ALA-synthase synthesis completely for 5 h in the presence of AIA, and therefore the curves in Fig. 7.4 would be expected to be coincident with the control in which induction is inhibited. A similar effect was observed when hemin was replaced by its precursor, ALA. At present there is no explanation for this effect, although diminished ALA or

FIGURE 7.3

EFFECT OF HEMIN ON DES MAINTAINED SYNTHESIS OF ALA-SYNTHASE FOLLOWING THE REMOVAL OF DDC BY WASHING THE CELLS

Isolated chick embryo liver cells were incubated in medium supplemented with DDC (25 µg/ml) for 8 h, washed with fresh media as described in Fig. 5.2, and re-incubated in fresh media containing the following additons: DDC (25 µg/ml) (●); DES (1 mg/ml) (○); DDC (25 µg/ml) and hemin (20 nM) (□); DES (1 mg/ml) and hemin (20 nM) (■); and hemin (20 nM) (▲). ALA-synthase activity was determined after various incubation times. Values are the mean of four separate determinations.

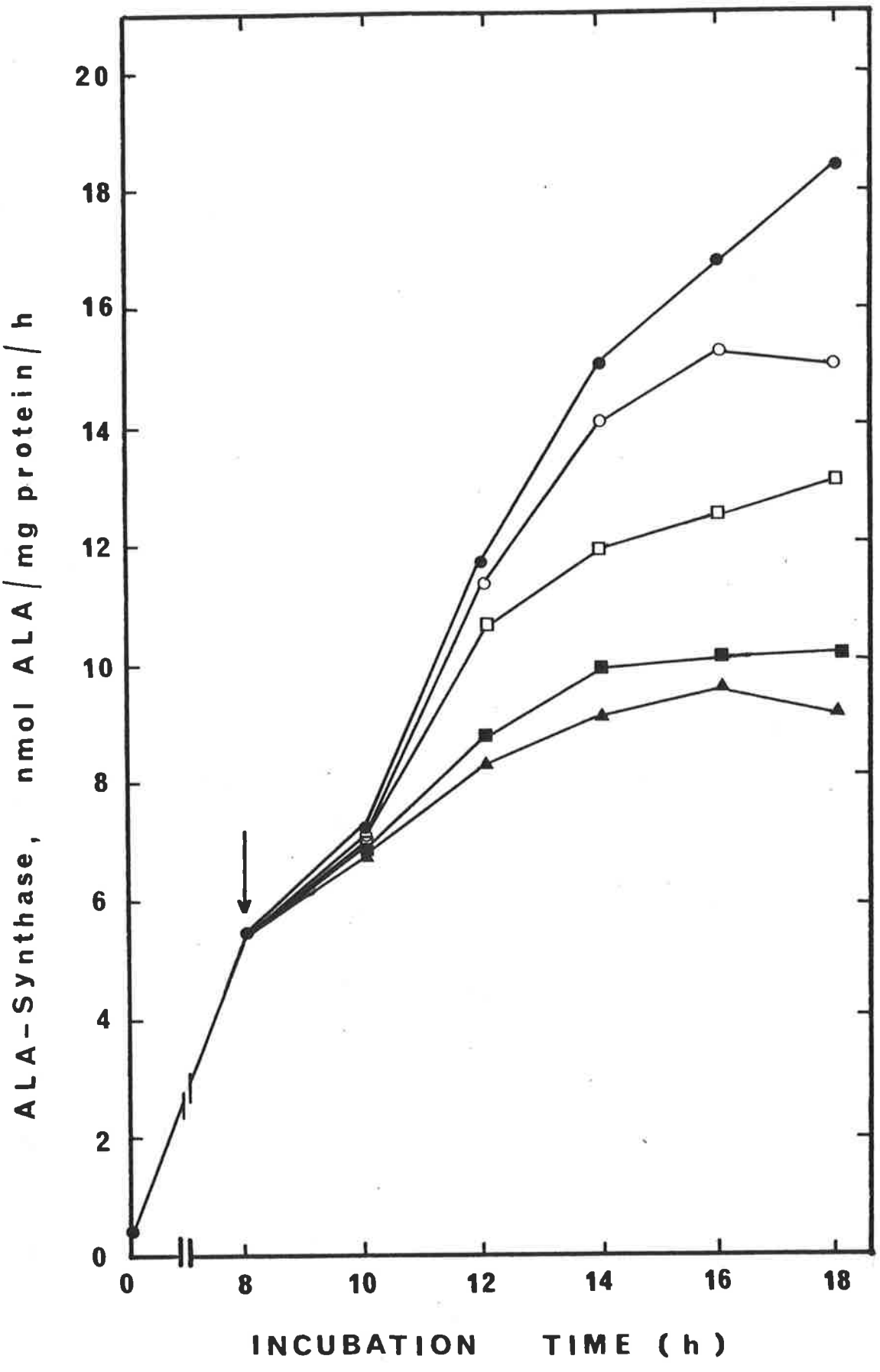
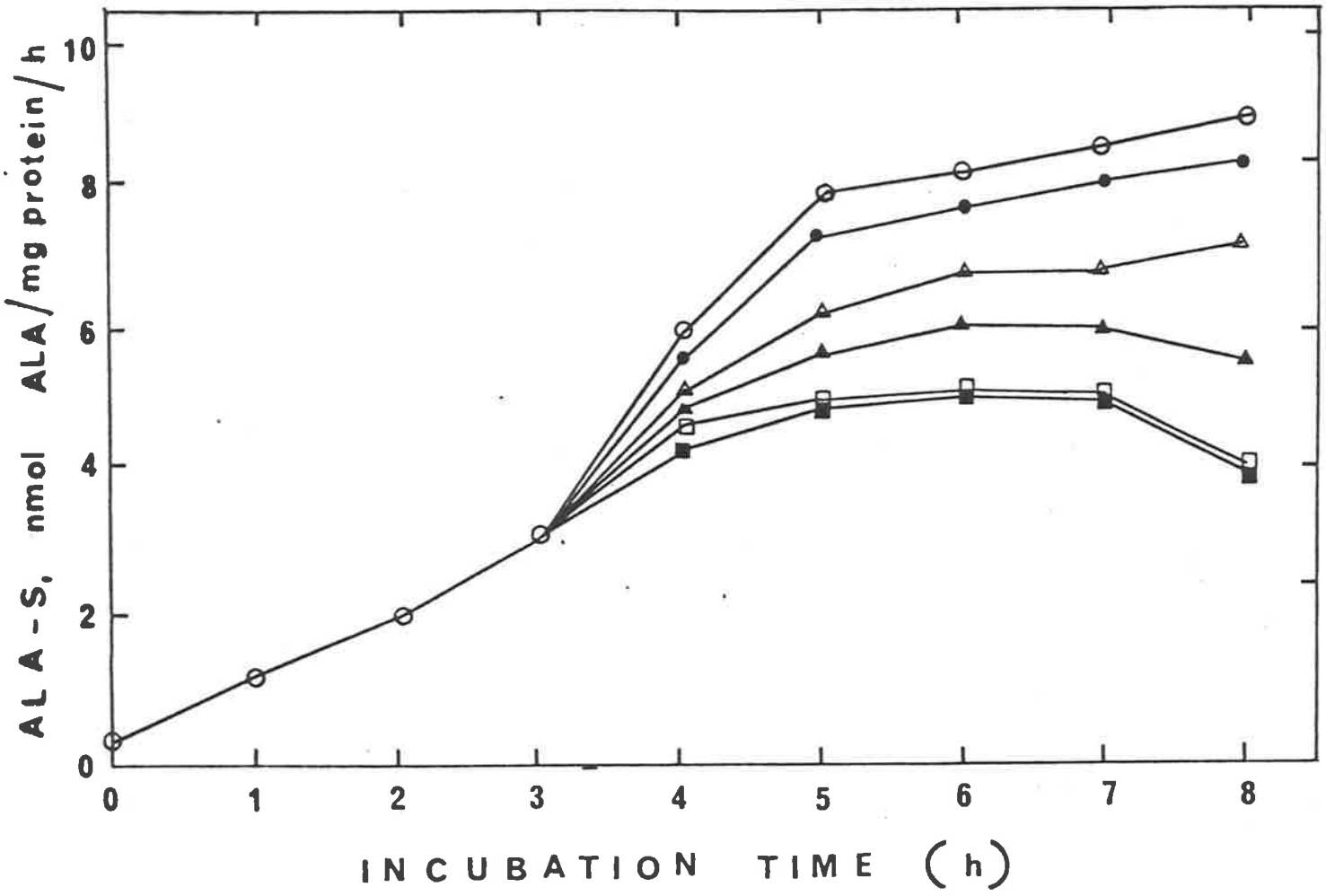

ALA-Synthase, nmol ALA / mg protein / h
0
2
4
6
8
10
12
14
16
18
20
0
8
10
12
14
16
18
INCUBATION TIME (h)

FIGURE 7.4

EFFECT OF HEMIN ON DES MAINTAINED SYNTHESIS OF ALA-SYNTHASE FOLLOWING THE REMOVAL OF AIA BY WASHING THE CELLS

Isolated chick embryo liver cells were incubated in normal media supplemented with AIA (500 µg/ml) for 3 h, washed with fresh media as described in Fig. 5.1, and re-incuabted in fresh media containing the following additions: AIA (500 µg/ml) (○); DES (500 µg/ml) (●); AIA (500 µg/ml) and hemin (50 nM) (△); DES (500 µg/ml) and hemin (50 nM) (▲); no addition (□); and hemin (50 nM) (■). ALA-synthase activity was determined after varying incubation times.


ALA-S, nmol ALA/mg protein/h
0
2
4
6
8
10
0
1
2
3
4
5
6
7
8
INCUBATION TIME (h)

hemin uptake by these washed cells is a possibility.

7.3 DISCUSSION

The experiments described in this chapter show that following addition of 50 nM hemin to AIA-induced cells, the time course of the hemin effect closely parallels the effect of cordycepin. This concentration of hemin was shown to completely prevent induction when added at zero time. Furthermore, 50 nM hemin did not have any additive effect when added together with cordycepin. The plateau observed after the addition of 50 nM hemin or cordycepin was perhaps a combined effect of enzyme synthesis and breakdown i.e., a steady state. These results suggest that 50 nM hemin inhibits ALA-synthase induction by preventing the synthesis of ALA-synthase mRNA. However, it should be noted that these results do not necessarily mean that hemin and cordycepin both effect mRNA synthesis directly. It is equally conceivable that hemin may affect the processing or transport of ALA-synthase mRNA. Hemin does not inhibit total RNA synthesis in these cells (results not shown). These data also indicate that this level of hemin (50 nM) probably does not affect translation of ALA-synthase mRNA (at least directly). Addition of 2 μM hemin to cordycepin treated cells showed a synergistic effect, and the response was similar to that of cycloheximide.

These data indicate that, at low concentrations, heme is a very effective regulator of ALA-synthase synthesis

and suggest that at the expected physiological concentrations of 10 - 100 nM (Sinclair *et al.*, 1975) it may act at the level of transcription. Only concentrations of hemin above 1 μM appear to have any post-transcriptional effects. Hemin concentrations upto 50 μM had no effect on general protein synthesis.

These above results conflict with those of Tyrrell and Marks (1972) and Sassa and Granick (1970), who showed no apparent transcriptional effect of exogenous hemin on the induction of ALA-synthase synthesis in monolayer cultures of chick embryo liver cells using 3 μM and 1.0 μM of hemin respectively. A possible reason for this conflict may be indicated by the present finding that after washing of AIA pre-treated cells, hemin caused only a partial inhibition of ALA-synthase synthesis compared with complete inhibition in unwashed cells. A similar effect was observed when hemin was replaced with its precursor, ALA. Possibly, the prolonged cell culture used by the above authors or the extensive washing of chick embryo liver cells in the experiments described in this chapter may have decreased the ability of these cells to absorb exogenous hemin or ALA. However, it should be emphasized that the washed cells in the experiments described in this chapter were still capable of responding to endogenously synthesized heme, since after washing, ALA-synthase synthesis ceased in the absence of DES or AIA. In general however, the results indicate that hemin may have at least two sites of action, transcription and a post-transcriptional process. Heme, at higher concentrations, may also exert an effect by

controlling the transport of cytoplasmically synthesized ALA-synthase into the mitochondria. However, this is less likely since heme does not effect ALA-synthase transport in embryonic chick embryo liver cells in suspension (results not shown). But clearly, conclusions based solely on the results of inhibitor experiments must be regarded as tentative, and confirmation by direct measurement of ALA-synthase mRNA is required before it can be fully accepted that heme is in fact a transcriptional regulator.

CHAPTER EIGHT

CONCLUSIONS AND FINAL DISCUSSION

The problem of how drugs induce ALA-synthase is a complex one and this thesis does not provide any final answer as to the mechanism. The main contribution of the work is that it does appear to narrow down the possible roles of drugs. If one can obtain induction of ALA-synthase in the absence of drug as suggested by the heme oxygenase work, then any hypothesis which demands the presence of a drug for a specific role in gene activation is difficult to sustain. The fact that induction initiated by drugs can be maintained by DES after washing away the inducer points to the same conclusion. Linked to this is the demonstration that heme represses ALA-synthase at remarkably low levels.

The objections to these conclusions are of a general nature: the intact cell system is complex, no direct measurements of cellular heme have been made and one must also assume a very low rate of heme pool turnover.

The best way to the future would appear to be via a molecular biological approach in which mRNA levels can be directly measured by hybridisation and, ideally, transcription of the gene for ALA-synthase studied in an *in vitro* system where controlling factors can be directly studied. Work in this laboratory is aimed at this. The decision on whether heme acts both transcriptionally and translationally would seem to require such a system.

If for the moment and as a working hypothesis it is accepted that heme destruction is the trigger for induction of ALA-synthase, there still remains the major problem of how drugs achieve this; whether this involves the chemical destruction of heme as evidenced by De Matteis and co-workers, or the induction of apo-cytochrome P-450 which takes up

cellular heme as the main mechanism remains to be elucidated. There may well be different mechanisms for different drugs or one drug may exert several effects. If induction of cytochrome P-450 apo-protein is a mechanism then we are left with the equally interesting and perplexing problem of how such a variety of drugs induce a variety of apo-proteins. It seems that a large amount of work remains to be done before we can arrive at any reasonably certain answers in this field of research.

REFERENCES

Abbritti, G., De Matteis, F. (1971) *Chem. Biol. Interact.* 4, 281.

Anderson, N.G. (1953) *Science* 117, 627.

Barnes, R., Jones, M.S., Jones, O.T.G..and Porra, R.J. (1971) *Biochem. J.* 124, 633.

Beale, S.I., Gaugh, S.P. and Granick, S. (1975) *Proc. Nat. Acad. Sci. (Wash).* 72, 2719.

Beavo, J.A., Rogers, N.L., Crofford, O.B., Hardman, J.G., Sutherland, E.W. and Nellman, E.V. (1970) *Mol. Pharmacol.* 6, 597.

Berry, M.N. and Friend, D.S. (1969) *J. Cell Biol.* 43, 506.

Beuzard, Y., Rodvieu, R. and London, I.M. (1973) *Proc. Nat. Acad. Sci.* 70, 1022.

Bishops, D.F. and Woods, W.A. (1977) *Anal. Biochem.* 80, 466.

Bissell, D.M. and Hammahes, L.E. (1976) *Arch. Biochem. Biophys.* 176, 103.

Bock, K.W., Krauss, E. and Fröhling (1971) *Europ. J. Biochem.* 23, 266.

Bonkowsky, H.L. and Pomeroy, J.S. (1978) *Anal. Biochem.* 91, 82.

Branster, M.V. and Morton, R.K. (1957) *Nature* 180, 1283.

Briggs, D.W., Condie, L.W., Sedman, R.M. and Tephly, T.R. (1976) *J. Biol. Chem.* 251, 4996.

Brooker, J., May, B. and Elliott, W. (1980) *Europ. J. Biochem.* 106, 17.

Condie, L.W. and Tephly, T.R. (1980) In *'Methods in Enzymology'* (S.P. Colowick and N.O. Kaplan, Eds.) 52, 350. Academic Press: New York.

Correia, M.A. and Meyer, U.A. (1975) *Proc. Nat. Acad. Sci.* 72, 400.

Correia, M.A., Farrell, G.C., Schmid, R., Ortiz de Montellano, P.R., Yost, G.S. and Mico, B.A. (1979) *J. Biol. Chem.* 254, 15.

Creighton, J.M. and Marks, G.S. (1975) *Canad. J. Physiol. Pharmacol.* 58, 485.

De Matteis, F. (1967) *Pharmacol. Rev.* 19, 523.

De Matteis, F. (1970) *F.E.B.S. Letters* 6, 343.

De Matteis, F. (1971a) *Biochem. J.* 124, 767.

De Matteis, F. (1971b) *S. Afr. J. Lab. Clin. Med.* 17, 126.

De Matteis, F. and Gibbs, A. (1972) *Biochem. J.* 126, 1149.

De Matteis, F. (1973b) *Drug. Met. Disp.* 1, 267.

De Matteis, F. (1975a) In *'Enzyme Induction'* (D.V. Parke, Ed.) 185. London, New York; Plenum Press

De Matteis, F. and Gibbs, A.H. (1975b) *Biochem. J.* 146, 285.

De Matteis, F. and Aldridge, W.N. (1978) In *'Heme and Hemoproteins'* (F. De Matteis and W.N. Alderige, Eds.) Spring-Verlag, Berlin-Heidelberg, New York.

De Matteis, F., Gibbs, A.H. and Tephyl, T.R. (1980) *Biochem. J.* 188, 145.

Doss, M. (1969) *Z. Klin. Chem. U. Klin. Biochem.* 2, 133.

Druyan, R. and Kelly, A. (1972) *Biochem. J.* 129, 1095.

Druyan, R., Jakovcic, S. and Rabinowitz, M. (1973) *Biochem. J.* 134, 377.

Ebert, P.S. Tschudy, T.S., Choudry, J.N. and Chirigos, M.A. (1970) *Biochim. Biophys. Acta.* 208, 236.

Edwards, A.M. and Elliott, W.H. (1974) *J. Biol. Chem.* 249, 851.

Elder, G.H. (1981) In *'Iron in Biochemistry and Medicine'* (A. Jacob and M. Worwood, Eds.) 2nd Ed. 245. Academic Press, New York.

Estabrook, R.W., Shigematsu, A. and Schenkman, J.B. (1970) *Advan. Enzyme Regul.* 8, 121.

Farrell, G. and Schmid, R. (1979) *Biochem. Biophys, Res. Commun.* 89, 456.

Fischer, P.W.F., Morgan, R.O. Krupa, V. and Marks, G.S. (1976) *Biochem. Pharmacol.* 25, 287.

Gonzales-Cadavid, N.F., Weiksler, M. and Bravo, M. (1972) *F.E.B.S. Letters* 7, 248.

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

Granick, S. and Beals, S.I. (1978) In *'Advances in Enzymology'* (A. Meister, Ed.) 46, 33. John Wiley and Sons

Granick, S. and Sassa, S. (1971) In *'Metabolic Pathways'* (H.J. Vogel, Ed.) 3rd ed., 5, 77. New York - London, Academic Press.

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

Granick, S. and Urata, G. (1963) *J. Biol. Chem.* 238, 821.

Gross, M. and Rabinowitz, M. (1972) *Proc. Nat. Acad. Sci.* 69, 1565.

Hayashi, N., Kurashima, Y. and Kikuchi, G. (1972) *Arch. Biochim. Biophys.* 148, 10.

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

Honn, K.V., Singley, J.A. and Chavin, W. (1975) *Proc. Soc. Exp. Biol. Med.* 149, 344.

Howard, R.B. and Pesch, L.A. (1968) *J. Biol. Chem.* 243, 3105.

Irving, E.A. and Elliott, W.H. (1969) *J. Biol. Chem.* 244, 60.

Kaufman, S. (1955) In *'Methods in Enzymology'* (S.P. Colowick

and N.O. Kaplan, Eds.) 1, 714.

Kikuchi, G., Kumar, P., Talmage, P. and Shemin, D. (1958) *J. Biol. Chem.* 233, 1214.

Krupa, V., Creighton, J.C., Freeman, M. and Marks, G.S. (1974) *Canad. J. Physiol. Pharmacol.* 52, 891.

Macalpine, I. and Hunter, R. (1969) *Sci. American* 221, 38.

Maines, M.D. and Sinclair, P. (1977) *J. Biol. Chem.* 252, 219.

Marks, G.S. Krupa, V., Rommi, M.W. (1973) *Canad. J. Physiol. Pharmacol.* 51, 863.

Marks, G.S. Stephens, J.K., Fischer, W.F. and Morgan, R.O. (1979) *Mol. Cell. Biochem.* 25, 111.

Marver, H.S. (1969) In *'Microsomes and Drug Oxidations'* (J.R. Gillette, A.H. Conney, C.J. Cosmides, R.W. Estabrook, J.R. Fouts, J.R. and G.J. Mannering. Eds.) 495. Academic Press, New York.

Marver, H.S. and Schmid, R. (1972) In *"The Metabolic Basis of Inherited Diseases'* (J.B. Stanbury, J.B. Wyngaarden, D.S. Fredickson, Eds.) 3rd Ed. 1087. New York, McGraw - Hill.

Marver, H.S., Tschudy, D.P., Perlroth, M.G. and Collins, A. (1966a) *J. Biol. Chem.* 241, 2803.

Marver, H.S., Tschudy, D.P., Perlroth, M.G. and Collins, A. (1966b) *Science* 154, 501.

Marver, H.S., Tschudy, D.P., Perlroth, M.G., Collins, A., and Hunter, G. (1966) *Anal. Biochem.* 14, 53.

Mauzerall, D. and Granick. S. (1956) *J. Biol. Chem.* 219, 435.

McKay, R., Druyan, R., Getz, G.S. and Rabinowitz, M. (19) *Biochem. J.* 114, 455.

Meyer, U.A. and Marver, H.S. (1971) *Science* 171, 64.

Meyer, U.A., Meier, P.J. and Correia, A.M. (1976) In *'Liver, Quantitative aspects of structure and function'* (R. Preisig, J. Bircher, and G. Paumgartner, G. Eds.) 172. Aulendorf, Germany: Editio contor.

Morgan, R.O., Stephens, J.K., Fischer, P.W.F. and Mark, S. (1977) *Biochem. Pharmacol.* 26, 1389.

Ohashi, A. and Kikuchi, G. (1972) *Arch. Biochem. Biophys.* 153, 34.

Ohashi, A. and Sato, S. (1973) *Tohoku, J. Exp. Med.* 111, 297.

Ortiz de Montellano, P.R., Mico, B.A. and Yost, G.S. (1978) *Biochem. Biophys. Res. Comm.* 83, 132.

Paterniti, J.R. and Beattie, D.S. (1979) *J. Biol. Chem.* 254, 6112.

Patton, G.M. and Beattie, D.S. (1973) *J. Biol. Chem.* 248, 4467.

Poland, A. and Glovern E. (1973) *Science* 179, 476.

Rajamanickam, L., Satyanarayana, M.R.S. and Padmanaban, G. (1975) *J. Biol. Chem.* 250, 2305.

Ramaley, R.F., Bridger, W.A., Moyer, R.W. and Boyer, P.D. (1967) *J. Biol. Chem.* 242, 4287.

Rifkind, (1979) *J. Biol. Chem.* 254, 4636.

Sassa, S. and Kappas, A. (1977) *J. Biol. Chem.* 252, 2428.

Sassa, S. and Granick, S. (1970) *Proc. Nat. Acad. Sci.* 67, 517.

Schmid, R. (1960) In *'Metabolic Basis of Inherited Disease'* (J.B. Stanbury, J.B. Wyngaarden, and D.S. Fredrickson, Eds.) 939. McGraw - Hill, New York.

Simpson, D.M. and Beattie, D.S. (1980) *J. Biol. Chem.* 255, 1630.

Sinclair, P.R. and Granick, S. (1974) *Biochem. Biophys.*

Res. Commun. 61, 124.

Sinclair, P.R. and Granick. S. (1975) *Annals New York Academy of Sciences* 224, 509.

Sinclair, P. and Granick, S. (1970) *Anal. Biochem.* 79, 380.

Strand, C.J., Swanson, A.L., Manning, J., Branch, S. and Marver, H.S. (1972) *Anal. Biochem.* 47, 457.

Sweeney, G.D., Janigan, D., Mayman, D. and Lai, H. (1971) *S. Afr. J. Lab. Clin. Med.* 17, 68.

Taub, H., Krupa, V., and Marks, G.S. (1976) *Biochem. Pharmacol.* 25, 511.

Tephly, T.R., Gibbs, A.H. and De Matteis, F. (1979) *Biochem. J.* 180, 241.

Tomita, Y., Ohashi, A. and Kikuchi, G. (1974) *J. Biochem. (Tokyo)* 75, 1007.

Tschudy, D.P. (1974) In *'Duncan's Diseases of Metabolic Genetics and Metabolism'* (P.K. Bundy, L.E. Rosenberg, Eds.) 7th ed. 775. Philadelphia, London, Toronto, W.B. Saunder Co.

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

Wada, O., Yano, Y., Urata, G. and Makao, K. (1968) *Biochem. Pharmacol.* 17, 595.

Waterfield, M.D., Del Favero, A. and Gray, C.H. (1969) *Biochem. Biophys. Acta. (Amst).* 184, 470.

Welch, R.M. (1979) *Pharm. Rev.* 30, 457.

Whiting, M.J. (1976) *Biochem. J.* 158, 391.

Whiting, M.J. and Granick, S. (1976) *J. Biol. Chem.* 251, 1340.

Yamauchi, K., Hayashi, N. and Kikuchi, G. (1980a) *F.E.B.S. Letters* 115, 15.

Yamauchi, K., Hayashi, N. and Kikuchi, G. (1980b) *J. Biol. Chem.* 255, 1746.

Yoda, B., Schacter, B.A. and Israels, L.G. (1975) *Anal. Biochem.* 66, 221.

Zuyderhoudt, F.M.J., Brost, P. and Huijing, J. (1969) *Biochim. Biophys, Acta. (Amst).* 178, 408.

PUBLICATIONS

PAPERS DESCRIBING STUDIES INCLUDED IN THIS THESIS

SRIVASTAVA, G, May, B.K., and Elliott, W.H. (1979) "cAMP-dependent induction of δ-aminolevulinate synthase in isolated embryonic chick livers cells" *Biochem. Biophys. Res. Comm.* 90 (1), 42.

SRIVASTAVA, G., Brooker, J.D., May, B.K., and Elliott, W.H. (1980) "Heme control in experimental porphyria - The effect of hemin on the induction of δ-aminolevulinate synthase in isolated chick embryo liver cells" *Biochem. J.* 188, 781.

LIM, L.K., Srivastava, G., Brooker, J.D., May, B.K., and Elliott, W.H. (1980) "Evidence that in chick embryos destruction of hepatic microsomal cytochrome P-450 heme is a general mechanism of induction of δ-aminolevulinate synthase by porphyria-causing drugs" *Biochem. J.* 190, 519.

SRIVASTAVA, G., Brooker, J.D., May, B.K. and Elliott, W.H. (1980) "Induction of hepatic δ-aminolevulinate synthase by heme depletion and its possible significance in the control of drug metabolism" *Biochem. Int.* 1, 64.

BROOKER, J.D., Srivastava, G., May, B.K. and Elliott, W.H. (1981) "Radiochemical assay for δ-aminolevulinate synthase" (In Press).

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SRIVASTAVA, G., Brooker, J.D., May, B.K., and Elliott, W.H. (1980) "Heme control in experimental porphyria - The effect of hemin on the induction of δ-aminolevulinate synthase in isolated chick embryo liver cells" *Biochem. J.* 188, 781.

LIM, L.K., Srivastava, G., Brooker, J.D., May, B.K., and Elliott, W.H. (1980) "Evidence that in chick embryos destruction of hepatic microsomal cytochrome P-450 heme is a general mechanism of induction of δ-aminolevulinate synthase by porphyria-causing drugs" *Biochem. J.* 190, 519.

SRIVASTAVA, G., Brooker, J.D., May, B.K. and Elliott, W.H. (1980) "Induction of hepatic δ-aminolevulinate synthase by heme depletion and its possible significance in the control of drug metabolism" *Biochem. Int.* 1, 64.

BROOKER, J.D., Srivastava, G., May, B.K. and Elliott, W.H. (1981) "Radiochemical assay for δ-aminolevulinate synthase" (In Press).

PAPERS PRESENTED AT CONFERENCES

SRIVASTAVA, G., and May, B.K. (1979) "A study of induction of δ-aminolevulinate synthatase in a newly designed isolated chick embryo liver cell system" *Proc. Aust. Biochem. Soc.* 12, 85.

SRIVASTAVA, G., Brooker, J.D., and Elliott, W.H. (1980) "Mechanism of control of synthesis of δ-aminolevulinic acid synthase in isolated chick embryo liver cells" *Proc. Aust. Biochem. Soc.* 13, 44.