THE INDUCTION AND PROPERTIES OF THE ENZYME δ-AMINOLAEVULINIC ACID SYNTHETASE IN MAMMALIAN AND AVIAN LIVER

by

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Thesis presented for the degree of

Doctor of Philosophy

at the University of Glasgow, July, 1973

ABBREVIATIONS

These are, in general, as recommended in the Instructions to Authors of the Biochemical Journal, 1972, with the following additions :-

AIA	2-allyl-2-isopropylacetamide
ALA	δ-aminolaevulinic acid
ALA-S	δ-aminolaevulinic acid synthetase
AA	aminoacetone
BSS	balanced saline solution
Bisacrylamide	NN'-methylene bisacrylamide
CMF	Ca^{++} and Mg^{++} free balanced saline solution + glucose
dpm	disintegrations/minute
EC	Eagle's minimal essential medium
RNase	ribonuclease
SDS	sodium dodecyl sulphate

ACKNOWLEDGEMENTS

I would like to express my sincere thanks to the following people for their contribution towards my research :-

Professor R.M.S. Smellie for providing research facilities.

Dr. W.S. Stevely for his friendly supervision throughout the project.

The many members of the department who have helped me by their constructive discussions and advice.

My fiancee for her patience, help and understanding throughout the period of this research.

Miss B.A. Stanage for illustration of diagrams, Mrs. P. Martin and Mr. D. Mease for technical assistance, and the staff of the animal unit for providing many animals within such short notice.

Mrs. A. Strachan for typing this thesis.

CONTENTS

Page

Page

LIST OF FIGURES

Page

LIST OF TABLES

INTRODUCTION

1. Porphyrin Metabolism in Human and Experimental Porphyrias.

1. 1. Classification of Human Porphyrias and Relationship to Experimental Porphyrias.

The hepatic porphyrias are a group of disorders characterized by excessive levels of porphyrins and their precursors in the liver. Certain types of porphyrias are genetically transmitted diseases, often precipitated by therapeutic dosages of drugs of diverse structure. Classification of the porphyrias based on the mode of inheritance, the pattern of haem precursor accumulation and excretion have been presented (Eales, 1961; Goldberg & Rimington, 1962; Tschudy, 1965; Schmid, 1966). Table 1 summarizes certain human porphyrias which may be simulated experimentally in animals.

1. 2. The Haem Biosynthetic Pathway and Localization of Its Enzymes in the Liver Cell.

The steps of the haem biosynthetic pathway are presented in Fig. 1, including those intermediates of the pathway that are excessively excreted during acute attacks of each of the porphyrias.

The initial step involves the condensation of succinyl-CoA with glycine which has been activated by pyridoxal-5'-phosphate, and results in the formation of δ-aminolaevulinic acid (ALA). This reaction is catalysed by the mitochondrial enzyme, δ-aminolaevulinic acid

Table 1

Classification of Various Types of Human Porphyrias and Possible Resemblance to Clinical Experimental Animal Porphyria

Animal Porphyria	Type of Human Porphyria Resembled Biochemically	Comments
Erythropoietic porphyria in cattle, pigs, and cats (Goldberg & Rimington, 1962; Kaneko & Cornelius, 1970)	Congenital erythropoietic porphyria (erythropoietic uroporphyria)	Inherited as an autosomal recessive trait; uroporphyrin I and coproporphyrin I excreted in urine and faeces: decreased level of uroporphyrinogen III cosynthetase (E_4 in Fig 1
Rabbits given phenylhydrazine, lead, and ultraviolet light (Schwartz et al., 1952)	Congenital erythropoietic porphyria (erythropoietic uroporphyria)	Liver porphyrin content is normal: excess uroporphyrin I excreted in urine by animals; rabbits excreted increased amounts of porphobilinogen which is not observed in human congenital erythropoietic porphyria
Animals given ALA, (rabbits, rats, and mice) (Goldberg & Rimington, 1962)	Acute intermittent porphyria (AIP)	ALA and porphobilinogen excreted during remission as well as during an acute attack; inherited as a Mendelian autosomal dominant trait
Animals given DDC (rabbits and mice) (Nakao et al., 1967)	Porphyria variegata during acute attack (VP)	ALA and porphobilinogen excreted in urine only during an acute attack; protoporphyrin I and coproporphyrin I excreted in faeces
Mice given griseofulvin (De Matteis, 1967)	Porphyria variegata during acute attack (VP)	ALA and porphobilinogen in urine during acute attack; protoporphyrin I and coproporphyrin I excreted in faeces
Rats given hexachlorobenzene (De Matteis, 1967)	Hereditary coproporphyria during acute attack (HCP)	ALA, porphobilinogen, uroporphyrin and coproporphyrin excreted in urine

Table 1 (cont'd)

Animal Porphyria	Type of Human Porphyria Resembled Biochemically	Comments
Rabbits given hexachlorobenzene (De Matteis, 1967)	Porphyria cutanea tarda symptomatica (PCT)	Increased amounts of uroporphyrin and coproporphyrin excreted in rabbit urine; no increase of ALA or porphobilinogen

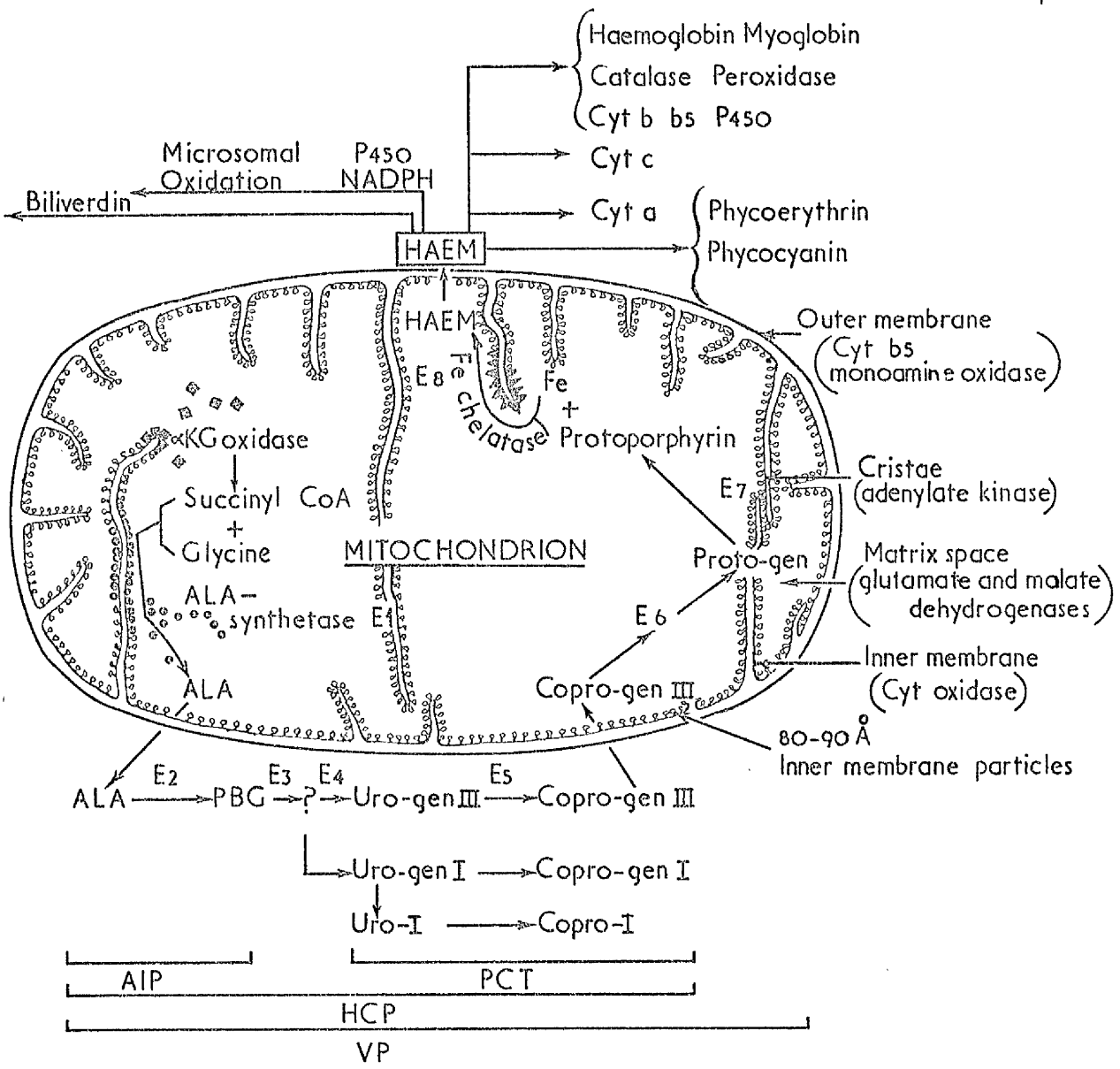


Fig. 1. Distribution of the enzymes of the haem biosynthetic chain between mitochondrion and cytosol. Intermediates of the pathway excessively excreted during acute phases of each of the hepatic porphyrias are within the respective brackets: AIP - acute intermittent porphyria, PCT - porphyria cutanea tarda, HCP - hereditary coproporphyria, VP - variegate porphyria. (ALA - δ-aminolaevulinic acid, PBG - porphobilinogen, Uro-gen - uroporphyrinogen, Copro-gen - coproporphyrinogen, Uro - uroporphyrin, Copro - coproporphyrin, Proto - protoporphyrinogen, E_2 - ALA dehydratase, E_3 - PBG deaminase, E_4 - Uro-gen co-synthetase, E_5 - Uro-gen decarboxylase, E_6 - Copro-gen decarboxylase, E_7 - Proto-gen dehydratase, E_8 - Ferrochelatase, α-KG oxidase - α-ketoglutarate oxidase).

synthetase (succinyl-CoA:glycine succinyltransferase) (ALA-S), the rate-limiting enzyme in the biosynthesis of haem (Granick, 1966). The transfer of ALA out of the mitochondrion may occur rapidly because, at least in a molecular model, ALA can take on a head-to-tail-loop configuration which is electrically neutral. After leaving the mitochondrion, ALA is converted to porphobilinogen (PBG) by δ-aminolaevulinic acid dehydratase (ALA-D; E_2). Porphobilinogen deaminase (uroporphyrinogen I synthetase; E_3) causes polymerization of PBG to an unknown polypyrrole structure. At this point two possible pathways arise. (i) A heat labile enzyme, uroporphyrinogen III co-synthetase (E_4) may cyclize the intermediate polypyrrole to form the uroporphyrinogen III isomer. This is followed by a decarboxylation by uroporphyrinogen decarboxylase (E_5) to yield coproporphyrinogen III. (ii) Alternatively, if uroporphyrinogen III synthetase (E_4) is absent then uroporphyrinogen I synthetase (E_3) converts the polypyrrole to uroporphyrinogen I which in turn is decarboxylated by uroporphyrinogen decarboxylase (E_5) to give coprophyrinogen I. The movement of coproporphyrinogen III from the cytosol back into the mitochondrion must be rather slow if it occurs merely by diffusion and not by an active process, because the molecule has four ionized propionic acid groups. Once coprophyrinogen III has re-entered the mitochondrion, coprophyrinogen oxidative decarboxylase (E_6) catalyzes the decarboxylation of coproporphyrinogen III to protophorphyrinogen III. Six hydrogen atoms are removed from the protoporphyrinogen III by protophorphyrinogen dehydratase (E_7) and the newly formed protoporphyrin chelates ferrous iron to yield haem, the chelation being catalyzed by ferrochelatase (haem synthetase; E_8).

Recent studies have attempted to localize the regions within the mitochondrion where the specific enzymes of the haem biosynthetic chain reside. Mitochondria from animal livers induced with allylisopropylacetamide (AIA), when fractionated, revealed that ALA-S was both in the matrix space and on the cristae membranes because the properties of localization were similar to the marker enzymes glutamate dehydrogenase (for matrix enzyme) and cytochrome oxidase (for cristae membrane) (McKay et al., 1969; Zuyderhoudt, 1969). It is obvious that the localization of ALA-S within the inner mitochondrial membrane, adjacent to the enzyme complex of α-ketoglutarate oxidase (Shnaitman & Greenawalt, 1968), would make it ideal for activity because succinyl-CoA, which is a substrate of ALA-S, is produced by the oxidase action. Succinyl-CoA, once made, must be utilized before it is hydrolyzed by a highly active deacylase or before it is reacted to yield ATP. In the liver the only source of succinyl-CoA is in the mitochondrion, so that only in the mitochondrion can ALA-S function to synthesize ALA from succinyl-CoA and glycine. The iron ferrochelatase enzyme (E_8) is considered to reside on the cristae membranes (Jones & Jones, 1969; Zuyderhoudt, 1969) while the location of the coproporphyrinogen III decarboxylase (E_3) and protoporphyrinogen dehydratase (E_7) is within the matrix space. The four cytosol enzymes, ALA-D (E_2), porphobilinogen deaminase (E_3), uroporphyrinogen III co-synthetase (E_4), and uroporphyrinogen decarboxylase (E_5) are localized in the cytosol possibly as a means of preventing the oxidation of the porphyrinogens via the electron transport system of the mitochondrion.

2. Some Properties of the Inducing System of ALA-S in Liver.

2. 1. Specificity of Tissue Response to Induction.

Response to inducing compounds depends on the tissue. It appears that in drug-induced experimental porphyria, those chemicals which in whole animals induce the formation of ALA-S or increase the porphyrin and haem production are the same ones which induce excessive porphyrin formation in chick embryo liver cells *in vitro* (Granick, 1966). In the whole animal only liver, and no other tissue appears to be induced by porphyrinogenic agents such as the barbiturates or the dihydrocollidines (Granick & Urata, 1963). Similarly, in *in vitro* cultures of various chick embryo tissues (liver, kidney, spleen, and brain tissue) only the liver appears to respond to these chemicals, as determined by fluorescence microscopy (Granick, 1966).

It should be emphasized that there is a spectrum of sensitivity in the various systems employed in the study of induction of porphyria by chemical agents. The chick embryo liver cell system is probably the most sensitive of all. Whether this is related to species differences or the fact that embryonic tissue is employed or whether it is because the cells are bathed in a solution of the compound tested with no excretory mechanism to lower its concentration is not known. It is probably not a high sensitivity of embryonic liver in general to induction of hepatic ALA-S, since no induction could be demonstrated in embryonic rat liver *in vivo* (Song *et al.*, 1968), or *in vitro* (Tschudy, unpublished results, cited from Tschudy & Bonkowsky, 1972). The adult mammalian liver appears to be less sensitive than the chick embryo

liver system and the sensitivity in rat declines as the rat increases in weight from 100 g to 200 g. Furthermore, even among different types of rats there is a variable sensitivity of induction of hepatic ALA-S to compounds like AIA. Buffalo rats, for example, are significantly less sensitive than Sprague-Dawley rats.

2. 2. Advantage of Culture Technique Over Whole Animal Experiments.

The production of porphyrins in chick embryo liver cells grown in primary culture provides a useful technique for studying the mode of action and structural basis for the porphyrogenic action of chemicals, drugs, and steroids. In the whole animal a distinction cannot be drawn between the effect of structural variations at the site of action and on the dynamic phenomena (absorption, distribution, metabolic destruction, and excretion) that control drug concentration at that site. The use of primary chick embryo cultures *in vitro* as contrasted to induction in the whole animal permitted the inference that the inducing drug acted directly on the hepatic cells and not via other stimuli generated from other organs.

The liver cell culture system was originally developed by Granick (1966). Briefly, using certain conditions of culture, liver cells from 16 to 17 day-old chick embryos are grown in vials containing a small cover slip. Following an initial period of incubation during which the liver cells form a monolayer on the cover slip, the cultures are treated with chemicals believed to enhance porphyrin-haem formation. The effect of drug treatment is determined by use of either fluorescence

microscopy or spectrofluorimetry. In cultures with optimal growth the cover slips will be largely covered with confluent colonies of cells of which 80 - 90% are hepatic parenchymal cells (Granick, 1966).

The importance of the in vitro cell system is two fold. First, it has been used to study the factors controlling haem biosynthesis and the effect of numerous compounds on the haem biosynthetic pathway (Granick, 1966; Granick & Kappas, 1967; Levere & Granick, 1967; Levere et al., 1967a; Kappas & Granick, 1968a; Kappas et al., 1968; Sassa & Granick, 1970). Second, the liver cell system may permit the screening of drugs that could prove harmful when administered to patients with hepatic porphyria. Granick has shown that a number of drugs produce significant degrees of porphyria in the chick embryo liver cells. These include bemegride, tolbutamide (Orinase), meprobamate (Miltown), Sulfonal, glutethimide (Doriden), the hydantoin drugs Mesantoin, Celontin, and Milontin, methyprylon (Noludar), chloramphenical (Chloromycetin), sodium phenobarbital, griseofulvin, and methol (Granick, 1966).

The induced response can be prevented or, subsequently promptly terminated by the addition to the cultures of inhibitors of nucleic acid and protein synthesis, such as actinomycin D, puromycin, or cycloheximide. These findings support the inference that increased porphyrin synthesis in the cultures is due to enhanced de novo formation of ALA-S, the rate-limiting enzyme in the pathway (Granick, 1966). For these reasons, the tissue culture system is utilized to examine the effects chemicals, drugs and natural steroids have on porphyrin synthesis in the liver.

3. Properties of ALA-S.

3. 1. Kinetics with Respect to Substrates, Cofactors, and pH.

An absolute requirement for the substrates, glycine and succinyl-CoA, and for the cofactor, pyridoxal-5'-phosphate is exhibited by ALA-S in its purified form (Scholnick et al., 1972a). When any of these substances is omitted from the assay media, no formation of ALA is detectable. Other investigators have attempted to characterize the substrate and cofactor requirements of ALA-S using crude enzyme or partially purified ALA-S (Table 2). These studies have shown that ALA-S required pyridoxal-5'-phosphate as a cofactor and utilized glycine and succinyl-CoA as substrates (Freshney & Paul, 1970; Aoki et al., 1971; Whiting & Elliott, 1972).

The purified enzyme showed a pH optimum between 7.4 and 7.6 when assayed in either Tris-HCl or potassium phosphate (Table 2) (Aoki et al., 1971; Scholnick et al., 1972a; Whiting & Elliott, 1972). The enzyme became unstable at pH values below 6.8 and above 8.0 with irreversible loss of activity (Scholnick et al., 1972a).

The amino acid requirement for enzyme action of purified ALA-S is specific for glycine; neither alanine nor serine forms an aminoketone or is decarboxylated by the enzyme (Scholnick et al., 1972a). The specific acyl-CoA requirements indicated that acetyl-CoA but not propionyl-CoA can be used in addition to succinyl-CoA. However, the rate of this reaction with acetyl-CoA is less than 1% that of succinyl-CoA (Scholnick et al., 1972a).

Table 2

Substrate and Cofactor Requirements for Optimal ALA-S Activity

Source of ALA-S	Drug	Purification (fold)	K_m glycine (10^{-2}M)	K_m succ-CoA (10^{-5}M)	K_m PLP (10^{-6}M)	pH opt.	References
rat liver, cytosol	AIA	150-200	1.1	7.0	3.0	7.5	Scholnick et al., (1972a)
rat liver, mitochond.	DDC	40	1.9	2.0	0.1-1.0	7.4	Whiting & Elliott (1972)
rabbit reticulocyte	PH	4400	1.0	6.0	-	7.6	Aoki et al., (1971)
mouse liver	-	-	0.4	-	-	7.0	Freshney & Paul (1970)

Abbreviations: PH - phenylhydrazine, pH opt. - optimum pH of assay medium, PLP - pyridoxal-5'-phosphate, mitochond. - mitochondria

Table 3

Reported Molecular Weights of Partially Purified ALA-S

	Hayashi et al., (1970)	Scholnick et al., (1972a)	Ohashi & Kikuchi (1972)	Whiting & Elliott (1972)
Source	rat liver	rat liver	cock liver	rat liver
Purification (fold)	-	80	-	40
Drug	AIA	AIA	AIA	DDC
M.W. of cyt. ALA-S	600,000 178,000	300,000 (.3M NaCl) 500,000 (no NaCl)	250,000	178,000
M.W. of mitoch. ALA-S	115,000 113,000		170,000 110,000	77,000
Method for M.W. Determination	gel filtration (G 200) sucrose density centrifugation	gel filtration (G 200)	gel filtration (G 200)	gel filtration (G 150)

Abbreviations: cyt. - cytosol, mitoch. - mitochondrial, M.W. - molecular weight

3. 2. Activation of ALA-S by Cations.

Purified rat liver cytosol ALA-S requires metal cations for maximum activity and stabilization; either monovalent cations at relatively high concentrations (0.25 M) or divalent cations at low concentrations (0.05 M). The latter had a paradoxical effect on ALA-S; that is, they activated the enzyme at low concentrations and inhibited it at high concentrations. The extent of the stimulation is similar regardless of whether monovalent, divalent or both cations were used (Scholnick et al., 1972a). Moreover, the addition of either mercapto-ethanol or dithiothreitol did not enhance the stabilizing effect of these cations. The specific nature of this stabilization and activation effected by cations is not known. Scholnick et al., (1972a, b) suggested the following two possibilities : (i) the activation of the enzyme by metal cations may be a reflection of the ionic strength of the assay mixture and could result from hydration changes in the enzyme, (ii) alternatively, the electrophilic nature of succinyl-CoA could be increased by divalent cations thus rendering it more susceptible to the nucleophilic attack by the glycine-pyridoxal-5'-phosphate complex. This would result in enhancement of the condensation of these substrates.

Recent data has shown that 0.3 M NaCl prevented the conversion of the partially purified enzyme from a highly active, relatively small molecule to a larger, less active form (Scholnick et al., 1972a). Experimental evidence has been presented by Ohashi & Kikuchi (1972) in agreement with this finding of Scholnick et al., (1972b). The large molecular size of ALA-S from the hepatic cytosol of AIA-induced cock

was converted to smaller sizes of the enzyme when treated with 0.4 M NaCl. In addition, several large molecular forms of ALA-S existed in the hepatic cytosol of AIA-treated cock, which when transferred into the mitochondrial matrix, were converted to two smaller molecular sizes (Ohashi & Kikuchi, 1972). This association of a more active ALA-S with a smaller molecular size may be of physiological importance since Hayashi et al., (1970) and Ohashi & Kikuchi (1972) found sufficient evidence suggesting that the enzyme in the mitochondrion, its apparent site of physiological action, is smaller in size than that identified in the cytosol.

3. 3. The Half-Life of ALA-S and Its mRNA.

Revell & Hiatt (1964) have suggested that the bulk of rat liver mRNA is stable for at least 40 hours. In contrast, most of the mammalian mitochondrial proteins have been reported to have a half-life of approximately 8 days (Beattie et al., 1967). Rat liver ALA-S has one of the shortest half-lives (20 to 70 minutes; Tschudy et al., 1965; Marver et al., 1966a; Matsuoka et al., 1968; Hayashi et al., 1969), yet reported for any mammalian liver enzyme, even the inducible ones. This suggests that there is a rapid steady rate of synthesis of ALA-S and of its break-down. In contrast, the half-lives of two other mitochondrial enzymes, alanine- and ornithine-aminotransferases, inducible by the cortico-steroid prednisolone acetate were about 17 to 24 hours (Swick et al., 1969).

The methods for estimation of the half-lives of ALA-S are based on the use of inhibitors for blocking RNA or protein synthesis in

the whole animal and in tissue culture. The rat liver mitochondrial ALA-S formed by induction has been shown to be very unstable and turns over very rapidly with a half-life of 60 to 70 minutes (Tschudy et al., 1965a; Marver et al., 1966a; Hayashi et al., 1969; Beattie & Stuchell, 1970). However, the decay of ALA-S in the cytosol of rat liver was much faster than that in the mitochondrial fraction. Its half-life was found to be as short as 20 minutes (Hayashi et al., 1969). Since the half-life of ALA was found to be independent of the level of enzyme, it was suggested that the degradation of the induced enzyme is a first order process (Marver et al., 1966a).

In chick embryo liver in culture, the half-life of the mRNA for the ALA-S is about double that of the enzyme itself (Sassa & Granick, 1970). These workers studied the effect of inducing chemicals on the accumulation of mRNA while protein synthesis was inhibited with cycloheximide, and on the synthesis of the enzyme from accumulated mRNA while RNA was inhibited with actinomycin D. The ALA-S activity in AIA-treated and control tissue culture decayed at a first-order rate with a half-life of 3 hours. Furthermore, inducing chemicals did not act to enhance or decrease the rate of degradation of preformed ALA-S in chick embryo liver cell system in vitro (Sassa & Granick, 1970), and thus, the chemical inducers AIA, DDC, aetiocholanolone, and γ-hexachlorocyclohexane (Lindane) had no effect on the half-life of ALA-S.

3. 4. Distribution of ALA-S in the Liver Cell and Multiple Molecular Forms.

Until recently, ALA-S has been considered generally to exist

exclusively in the mitochondria, but the site of this enzyme has remained unclear.

Hayashi et al., (1969), reported that high activities of ALA-S occurred not only in the mitochondrial fraction but in the soluble fraction of rat liver when the enzyme was induced by AIA. On the other hand, the soluble fraction from control rats without AIA had no activity of ALA-S. As much as 30 - 50% of the total ALA-S activity in AIA treated animals accumulated in the soluble fraction. Studies on the kinetics and effect of inhibitors of protein and nucleic acid synthesis on the induction process indicated that ALA-S in the soluble fraction may be a precursor of that in the mitochondria (Hayashi et al., 1969). These investigators suggested that ALA-S was synthesised originally on the ribosomes of the rough endoplasmic reticulum and modified to some extent either before or after entry into the mitochondria. Beattie & Stuchell (1970) observed substantial inhibition of ALA-S (50 - 60%) by chloramphenicol (as well as cycloheximide) in the mitochondrial fraction but very little effect on ALA-S induction in the postmitochondrial fraction. This finding was not in agreement with the report of Hayashi et al., (1969). They found that chloramphenicol had no inhibitory effect on the AIA-induction of soluble or mitochondrial ALA-S in rat liver.

This observation of ALA-S activity in the soluble fraction of AIA-treated rat liver was ratified by other investigators (Scholnick et al., 1969; Hayashi et al., 1970; Barnes et al., 1971), who also found the substrate and co-factor requirements for the soluble and mitochondrial enzyme to be similar. Subsequent study by Hayashi et al., (1970), using ammonium sulphate and gel filtration showed the molecular

size of the mitochondrial ALA-S to be considerably smaller (115,000) than that of the soluble ALA-S (600,000; in the absence of NaCl) (Table 3), and suggested the possibility of soluble ALA-S as a precursor of the mitochondrial enzyme. Recently Scholnick et al., (1972a, b) have purified the soluble ALA-S from porphyric rat livers 150- to 200-fold. In the presence of NaCl the molecular weight was approximately 300,000 whereas when determined in the absence of NaCl the apparent weight was in excess of 500,000. Ohashi & Kikuchi (1972) added further evidence to the view that cytosol ALA-S may be a precursor of mitochondrial ALA-S. Soluble ALA-S from liver of AIA-induced cock exhibited multiple size heterogeneity. Moreover, as the duration of the induction increased the initial, relatively smaller molecular forms of soluble ALA-S changed and the enzyme of the largest size became predominant. Mitochondrial ALA-S of AIA-treated and untreated cock showed the same two molecular sizes 170,000 and 110,000 when estimated by Sephadex G 200 gel filtration. Cytosol ALA-S isolated from AIA-treated rat liver gave molecular weight of approximately 250,000. In conclusion, Ohashi & Kikuchi (1972) proposed the following hypothesis: (i) ALA-S is synthesized originally in the cytoribosomal protein synthesis system, (ii) the large molecular size of cytosol ALA-S is due to aggregation of smaller sizes of the enzyme, (iii) this aggregate is disrupted by some means to yield intermediate size enzymes, (iv) the enzyme which has been made small enough in the cytosol is transferred into the mitochondria, (v) once inside the mitochondria the enzyme is further converted to a smaller enzyme of molecular weight 110,000.

3. 5. Assay Methods for ALA-S Activity.

Two assay procedures are used depending on the source and amount of tissue available and also the level of ALA-S activity. One is colourimetric. ALA-S activity is assayed by determining the amount of ALA formed. Two different procedures may be used. The first involves separation of ALA from AA by resin chromatography, its conversion to a pyrrole by condensation with acetylacetone and the production of coloured derivative of the pyrrole with Ehrlichs reagent containing Hg^{++} to remove interfering SH groups (Eales, 1961; Goldberg & Rimington, 1962). The alternative procedure involves the separation of the ALA-pyrrole from AA-pyrrole by chromatography and the concentration of ALA-pyrrole by chromatography (Marver et al., 1966b, c). To avoid chromatography which limits sensitivity, Granick (1966) developed a method for the separation of ALA-pyrrole from AA-pyrrole by extraction of the latter from an aqueous neutral mixture using ethyl ether. This method is capable of detecting ALA in the range of 10^{-8} M. The use of semi-microcuvettes of 50 mm length by Levere et al., (1970), has made possible the colourimetric determination of ALA in the range of 10^{-10} mole.

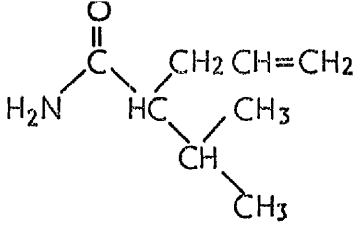
The other general method for determination of ALA-S is by the formation and determination of isotopically labelled ALA. Irving & Elliott (1969) have described a sensitive tracer method based on the incorporation of $[1,4\text{-}^{14}C]$ succinate into ALA. To prevent the incorporation of the $[1,4\text{-}^{14}C]$ succinate into other products that might move with ALA on the chromatographic column inhibitors of the citric acid cycle and the respiratory chain were included in the reaction mixture. A simpler procedure involved a diluted aliquot of whole homogenate as a

source of the enzyme, and labelled succinate or α-ketoglutarate as a substrate (Ebert et al., 1970). A method of successive washing on Dowex 50 (H^+) column permitted the isolation of ALA uncontaminated with other labelled metabolic products. Freshney & Paul (1970) used an isotopic method for estimation of ALA-S which involved [2-^{14}C] glycine as a precursor, unfractionated homogenate as the source of enzyme and electrophoresis to separate ALA, AA and glycine. These methods are sensitive in the range of 10^{-10} mole ALA.

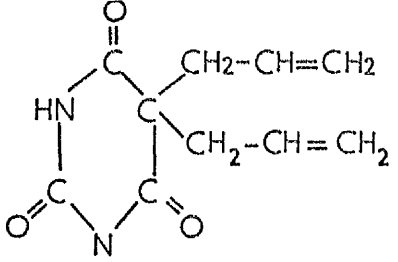
4. Induction of ALA-S by Chemicals, Drugs, and Steroids.

Certain chemicals are capable of inducing clinical and experimental manifestations (in animals and chick embryo liver cells in culture) similar to those of human hepatic porphyria. These inducers consist of a wide variety of organic compounds that differ in physiological activity. They include, for example, the central nervous system depressants such as the barbiturates, sulfonmethanes and glutethimides; the anticonvulsant methsuximide; the central nervous stimulant bemegride; the fungicides griseofulvin and hexachlorobenzene; some chlorinated insecticides; and the isoprenoids, terpenes and steroids (Granick, 1963; De Matteis, 1967; Felsher & Redeker, 1967; Marks, 1969; Racz & Marks, 1972b). The inducing compounds may be divided into four classes on the basis of their subsequent groups - barbiturate, collidine, steroid, and miscellaneous (Granick, 1966) (Fig. 2).

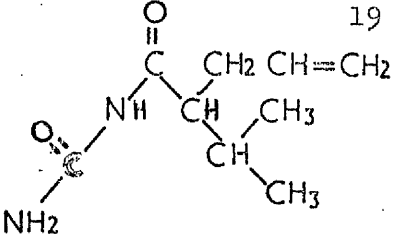
Numerous studies have confirmed the fact that AIA (Marver et al., 1966a; Narisawa & Kikushi, 1966; Nakao et al., 1967;



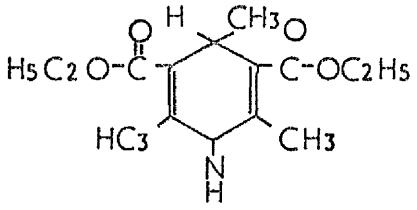
2-allyl-2-isopropylacetamide (AIA)



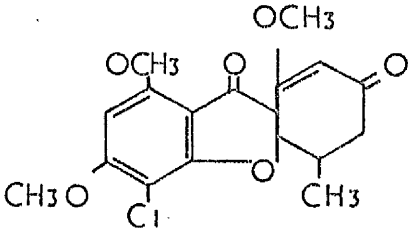
Diallylbarbituric acid



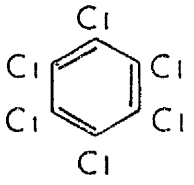
Sedormid



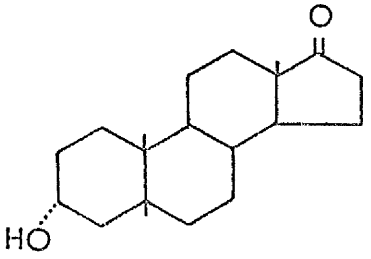
3,5-Dicarbethoxy-1,4-Dihydrocollidine (DDC)



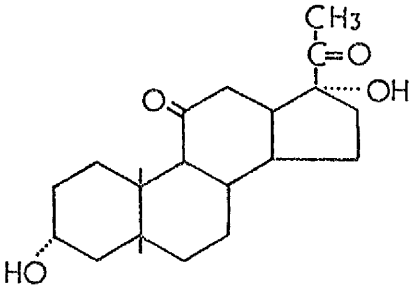
Griseofulvin



Hexachlorobenzene



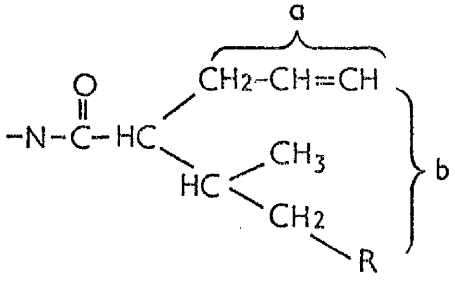
Aetiocholanolone



5β-pregnane-3α,17α-diol,11,20-dione

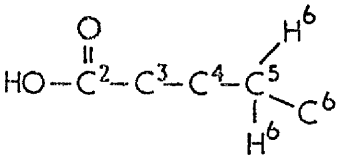
Fig. 2a. Some chemicals that induce porphyrin formation and increase ALA-S activity in primary cultures of chick embryonic liver cells and intact animals.

Fig. 2b. Hypothetical molecular structure required to produce porphyria.



Dialkyl-substituted acetamide

I



Example of the empirical rule of six

II

Matsuoka et al., 1968; Hayashi et al., 1969; Beattie & Stuchell, 1970; De Matteis, 1970; Hayashi et al., 1970; Kaufman et al., 1970; Kurishima et al., 1970; Abbritti & De Matteis, 1971/1972), DDC (Granick & Urata, 1963; De Matteis, 1964; De Matteis, 1967; Nakao et al., 1967; Hutton & Gross, 1970; De Matteis & Gibbs, 1972) griseofulvin (Nakao et al., 1967; De Matteis, 1972), γ-hexachlorobenzene (De Matteis, 1964; Nakao et al., 1967), steroid hormones (Watson et al., 1962; Welland et al., 1964a; Marver et al., 1966d; Granick & Kappas, 1967; Kappas & Granick, 1968a, 1968b) and alterations in diet (Rose et al., 1961; De Matteis, 1964; Tschudy et al., 1964; Welland et al., 1964b; Lottsfeldt & Labbe, 1965; Felsher & Redeker, 1967), cause marked increases in the level of hepatic ALA-S in intact animals and isolated cell systems. AIA and DDC are the compounds most frequently used to produce experimental hepatic porphyria, hence most of the present discussion will relate to studies of the effects of these compounds.

4. 1. Allylisopropylacetamide (AIA), a Member of the Barbiturates, and Induction of ALA-S.

The induction of experimental porphyria by AIA is accompanied by a sequence of complex changes. Livers of starved rats treated with AIA for three days showed a marked hepatomegaly when compared to those of starved controls (Lottsfeldt & Labbe, 1965). Microscopic examination of these enlarged livers showed primarily cellular hypertrophy and to a lesser extent cellular hyperplasia (Lottsfeldt & Labbe, 1965). Chemical analysis found the RNA content per cell significantly increased (44%) in the porphyric liver, but RNA per unit wet weight was similar in both

porphyric and control animals (Lottsfeldt & Labbe, 1965). The DNA content per cell remained unchanged although DNA per gram of wet weight of liver decreased by 36% (Lottsfeldt & Labbe, 1965). Marver et al., (1966a) reported that twenty four hours after the second of two daily injections to fasted rats, the liver size and liver protein are both increased by 65%. The protein concentrations of the various subcellular fractions were not altered significantly, with the possible exception of 15% increase in the mitochondrial fraction. Electron microscopy of the livers from fed rats given a single dose of AIA (400 mg/kg) revealed a progressive increase in number and size of lipid droplets, which were evident as early as 90 minutes after AIA administration, reached a peak between 12 to 24 hours and decreased to normal by 72 hours (Biempica et al., 1967). Other investigators reported that twelve hours after AIA administration to starved rats, there was an increase in cytoplasmic, nuclear and nucleolar volumes of 34%, 21%, and 122% respectively when compared with the liver cells of starved control rats (Moses et al., 1970). In addition to this, electron microscopy showed tubular hypertrophy of the smooth endoplasmic reticulum (Stein et al., 1970).

Administration of AIA to animals appeared to have a marked effect on certain hepatic enzymes and metabolic pathways : increases of tyrosine aminotransferase (Moses et al., 1970; Stein et al., 1970; Wetterberg et al., 1970), NADP-cytochrome c reductase (Narisawa & Kikuchi, 1966), and glucose-6-phosphate dehydrogenase (Narisawa & Kikuchi, 1966); changes in hemoproteins including decreases of liver catalase (Price et al., 1962; Tschudy et al., 1962), increases of tryptophan pyrrolase (Fiegelson & Greengard, 1961; Marver et al., 1966e), and hepatic mitochondrial cytochromes a-a_3, b, c-c_1, (Beattie & Stuchell, 1970), and changes in

microsomal cytochrome P-450 (Wadda et al., 1968; Meyer & Marver, 1971). AIA (as well as DDC-) -treated rabbits developed increased serum levels of total lipids and phospholipids (Taddeini et al., 1964). In mice given AIA, cholesterol synthesis is increased whereas DDC, decreases cholesterol synthesis.

Several studies have been made of the relationship between the chemical structure and porphyria-inducing activities of a variety of drugs related to AIA. These drugs were injected into the yolk sac of 8-day old chick embryos, and the porphyrin concentration of the allantoic fluid measured (Tallman et al., 1957). The molecular structure required to produce porphyria in these chick embryos was identified as a dialkyl-substituted acetamide or acetamide derivative (as illustrated in Fig. 2-I) where one substituent is an alkyl group (denoted a), and the other contains at least three carbon atoms, preferably in a branched chain (denoted b) (Tallman et al., 1957).

The studies of Hirsch et al., (1966) have shown that the allyl group is not essential for porphyria-induced activity in the chick embryo liver cell system and can be replaced by a propyl group. Hirsch et al., (1967b) suggested that the underlying critical feature for activity in the AIA is an amide group which is sterically hindered from hydrolysis. In an attempt to predict the porphyria-inducing activity of a drug, Schneck & Marks (1972) employed the empirical rule of six. This hypothesis, developed by Newman (Newman, 1956), enabled research workers to estimate the degree of steric hindrance of an amide or ester to chemical hydrolysis by a base or acid. 'The rule of six states that in reactions involving addition to an unsaturated function containing a

double bond, the greater the number of atoms in the six position the greater will be the steric effect'. The hydrolysis of a series of aliphatic amides by chick embryo liver amidases was studied by Schneck & Marks (1972) and it was found that the aliphatic amide with a high six number (i.e. AIA's six number = 8; demonstrated in Fig. 2-II) had greater resistance to hydrolysis by chick embryo liver amidase and, as well, a parallel larger porphyrinogen effect in cultured chick embryo liver cells than those drugs with a low six number. They concluded that the degree of steric hindrance to enzyme hydrolysis as assessed in the series of aliphatic amides appeared to parallel the degree of steric hindrance to chemical hydrolysis as assessed by the six-number. It has been shown that for a chemical to induce porphyria it must remain in contact with liver cells for a period of at least several hours in order to induce and maintain high levels of ALA-S (Racz & Marks, 1972b). Drugs that may be metabolized by a hydrolytic mechanism do not give rise to increased haem production in liver cells (Hirsch et al., 1967b). However, where hydrolysis is prevented by steric factors (as mentioned above), the drugs are oxidatively metabolized and increased haem and porphyrin formation takes place (Hirsch et al., 1967b). Thus, the potency of AIA as a porphyria-inducing drug may be partially explained on the basis of its resistance to hydrolysis to the inactive free acid by liver amidase (Schneck & Marks, 1972).

4. 2. 3,5-Dicarbethoxy-1,4-dihydrocollidine (DDC), a Member of the Collidines, and Induction of ALA-S.

Granick & Urata (1963) showed that acute poisoning of guinea pigs with DDC caused an increase of ALA-S (> 40 fold) in the liver

parenchymal cells. In addition, the synthesis of protoporphyrin was greatly enhanced, although there was no major change in any of the enzymatic activities of the porphyrin biosynthetic chain. Histologically, the livers of acutely poisoned animals did not appear abnormal. Electron microscope studies indicated that during the first two days of DDC treatment, the mitochondria increased in diameter approximately 15% and the cristae appeared to increase in area.

Investigators have reported that administration of DDC (or AIA or griseofulvin) to mice, rabbits and rats caused a reduction in cytochrome P450 and haem content, coincidental with the rise in activity of ALA-S (Wada et al., 1968; Waterfield et al., 1969; De Matteis, 1970; Meyer & Marver, 1971; De Matteis & Gibbs, 1972). In addition, DDC and the other porphyrogenic drugs mentioned above have all been reported to lower the activity of liver catalase (De Matteis, 1967; Abbritti & De Matteis, 1971/72), a finding which may indicate that they decrease the concentration of more than one pool of haem in the liver. Tephly et al., (1971) have described an inhibition of the ferrochelatase in the liver of rats 24 hours after a single dose of DDC. As early as one hour after administration of DDC to rats there was an inhibition of mitochondrial ferrochelatase although the ALA-S activity was still normal or just starting to rise (De Matteis & Gibbs, 1972).

Marks and co-workers have investigated the relationship between chemical structure of DDC (and its analogues) and porphyria inducing activity in cultured primary cells of chick embryo liver (Marks et al., 1965; Hirsch et al., 1967b; Schneck et al., 1968; Racz & Marks, 1972a; Racz & Marks, 1972b; Schneck & Marks, 1972). The

following are essential for optimal porphyrogenic activity (a) A study of the Fisher-Hirschfelder-Taylor models of DDC and its analogues indicated that the 2-, 4-, and 6-methyl substituents cause a twisting of the 3- and 5-ethoxycarbonyl substituents out of the plane of the ring. This nonplanar relationship between the ethoxycarbonyl substituents and the pyridine or dihydropyridine ring appears to be necessary for activity (Marks et al., 1965). (b) An ethoxycarbonyl function on a pyridine, dihydropyridine or benzene ring with two ortho - alkyl substituents, in which one of the alkyl groups must be in the 4 position of the ring is required. In addition, a second ethoxycarbonyl group in the molecule with two ortho - methyl substituents reinforces the inducing power (Hirsch et al., 1967b). (c) These ortho - alkyl groups sterically hindered the two ethoxycarbonyls from enzymic hydrolysis (Hirsch et al., 1967b). (d) Replacement of the 3- and 5-ethoxycarbonyls functions of DDC with acetyl substituents led to loss of inducing power (Schneck et al., 1968). (e) In aromatic (and aliphatic) esters, a sterically hindered ester group is a requirement for porphyria inducing activity (Schneck et al., 1968).

4. 3. Steroids and Induction of ALA-S.

Intensive investigations of steroids as possible porphyrogenic agents in liver cells have been performed for two reasons: (a) of all inducing chemicals studied, only the steroids are derived from physiological sources, and (b) a number of clinical and experimental observations (Goldberg & Rimington, 1962; Welland et al., 1964a; Zimmerman et al., 1966; Kottra & Kappas, 1967), suggested that endocrine

secretions - particularly steroids - represent one class of natural agents which, in appropriate circumstances, may exacerbate the chemical and symptomatic abnormalities of hepatic porphyria. Certain clinical observations suggested that during puberty, pregnancy, and menstruation - that is, when steroid production is altered markedly - the symptoms of hepatic porphyria in patients with this disease were exacerbated or diminished (De Matteis, 1967).

When triiodothyronine or hydrocortisone were administered to rats simultaneously with AIA, there was a marked increase of mitochondrial ALA-S activity (Matsuoka et al., 1968). In contrast, the enzyme level was not increased when the rats were treated by the hormone alone. There appeared to be a marked synergistic action of AIA and these hormones to bring about the stimulation of the induction. However, AIA appeared to play the primary role in induction while the function of hormones was only auxiliary. It was also suggested that administration of AIA caused an initial intensive induction phase which was followed by an unreactive or refractory phase. In this period, when enzyme synthesis appeared to be extremely small as judged by the very rapid decline in enzyme activity, the induction machinery of the rat liver does not respond to either AIA or hormones. The cause of this refractory phase is possibly due to the treatment with these hormones. It was suggested that intensive synthesis of ALA-S may subsequently stimulate the synthesis of an inhibitor which interferes with the synthesis or function of mRNA for ALA-S (Matsuoka et al., 1968).

Certain natural steroids, like drugs and foreign chemicals, markedly enhanced porphyrin formation in (i) cultured chick embryo liver

tissue as determined by fluorescence microscopy (Granick, 1966; Granick & Kappas, 1967; Kappas & Granick, 1968a), (ii) the de novo formation of ALA-S in the intact chick embryo, detected directly by the increase of ALA-S activity (Kappas et al., 1968), and (iii) haemoglobin synthesis in the blood islands of the cultured chick blastoderm (Levere & Granick, 1965; Levere et al., 1967a). The same steroids, mainly pregnana lone, pregnandiol, 17α-hydroxypregnanolone, pregnandione, 11 - ketopregnanolone, aetiocholanolone, aetiocholandione, aetiocholanolone-17β, which induced enhanced porphyrin synthesis in chick embryo liver cells in vitro and in vivo also induced a more rapid synthesis of haemoglobin in the erythroblasts of the chick blastoderm (Levere et al., 1967a). In all three experimental designs, the increased porphyrin formation or ALA-S activity can be prevented or terminated by addition of inhibitors of nucleic acid and protein synthesis, such as actinomycin D, mitomycin, puromycin or cycloheximide. This suggests that the mechanism of steroid action involves the formation of ALA-S, the rate-limiting enzyme in the haem biosynthetic pathway. However, intensive administration of these steroids to guinea pigs or rats failed to stimulate ALA-S activity, although the enzyme in these animals is readily induced by drugs and foreign chemicals (Granick & Urata, 1963; Marver et al., 1966a). This lack of response in these animals to the 5β-H steroid induction of ALA-S may indicate the presence of an additional mechanism for controlling haem formation which may be a property of only the mammalian liver (Kappas et al., 1968c). In contrast, foreign chemicals, such as AIA and DDC, which are potent inducers of hepatic porphyrin production in chick embryo liver cells in vitro and in vivo (Granick, 1966; Kappas & Granick, 1968b) and in animals (Granick, 1966), failed to increase haemoglobin synthesis

in the erythroblasts of the chick blastoderm (Levere et al., 1967a). Levere et al., (1967a) proposed that regulation of haem synthesis in these erythroid cells, as compared to that of the liver cells, may reside in a more restricted control mechanism which is responsive only to certain types of physiological substances such as these 5β-H steroids. The structural requirements for potent inducing activity by the steroids have been summarized as follows: (i) a 5β-H configuration, (ii) a fully saturated steroid of the C-19 or C-21 series, (iii) alcohol and carbonyl substituents at C-3, C-17, C-20, and possibly C-11. In addition, the steroids induce porphyrogenesis in concentrations at least as low as 10^{-6} to 10^{-8} M (Sassa & Kappas, 1967). The structure of some representative ALA-S inducing steroids are illustrated in Fig. 2.

Evidence has been presented that steroid induction of porphyrogenesis in liver requires synthesis of mRNA for ALA-S and that haem and other metalloporphyrins inhibited this steroid-induction process (Kappas & Granick, 1968b). This suggested that natural steroids and foreign chemicals or drugs acted at the same cellular site, that is, they competed for a binding site on a repressor protein, as has been stated for AIA and DDC (Granick, 1966).

Once the steroid is inside the cell, it may exist in the free, active form or be converted to the inactive glucuronic conjugate by uridine diphosphate glucuronic acid (UDP-glucuronic acid). In the reaction involving the inactivation of steroids, UDP-glucuronic acid donates glucuronic acid with the aid of the enzyme, UDP-glucuronyl-transferase. When the intermediates of the glucose-glucuronic acid pathway were tested for possible inhibitory effect on steroid induction

in cultured cells, only UDP-glucuronic acid showed significant inhibition (Kappas & Granick, 1968b). Chick embryo liver synthesizes small amounts of UDP-glucuronic acid and has detectable levels of UDP-glucuronyltransferase activity (Skea & Nemeth, 1969).

Kappas et al., (1968b) suggested that inducing steroids control the synthesis of ALA-S at the transcriptional level in the same fashion as the barbiturates or dihydrocollidines (Fig. 3). However, the concentration of active steroid inducers in the hepatic cell would depend on the rate of their conversion to the inactive glucuronides by UDP-glucuronyltransferase and on the rate of hydrolysis of the glucuronide back to the active free steroids by β-glucuronidase.

The administration of glucose to animals is known to prevent the induction of porphyria by certain chemicals (De Matteis, 1964; Tschudy et al., 1964). This has been referred to as the "glucose effect" and is thought to have no relation to the phenomenon of "catabolite repression", that is, the ability of glucose in bacteria to repress formation of certain inducible enzymes. The glucose repression may result from the metabolism of glucose to yield high levels of UDP-glucuronic acid. This would result in glucuronidation of the active steroids and, possibly, other inducing substances thus converting them to the inactive form.

5. Possible Mechanism for the Induction of ALA-S in Liver.

5.1. Transcriptional and Translational Levels.

Two general mechanisms for the control of the first enzyme,

ALA-S, and haem synthesis in the liver cell have been proposed (Granick, 1966; Sassa & Granick, 1970; Tyrrell & Marks, 1972).

Granick (1966) hypothesised that the inducing action of chemicals which caused increased ALA-S, interfered with a repressor mechanism in which a specific repressor (consisting of a protein, aporepressor, to which is attached the haem, corepressor) is considered to block a specific DNA region (operator gene). The function of an operator gene is to control the transcription of the operon (a short length of DNA) into mRNA. When the operator gene is blocked, no mRNA will form that can be translated into the polypeptide chain of ALA-S. A summary of this hypothesis on induction of chemical porphyria in the liver cell is presented in Fig. 3. There appears to be only one site to which the inducing chemicals can attach (i.e. the corepressor site). This inducing site or corepressor site is relatively large, has different regions, each more or less specific for one of the five inducing groups (Granick, 1966). These groups can attach reversibly to the site and thus block the attachment of haem. In Fig. 4, a hypothetical corepressor site is pictured which illustrates how the corepressor site might be occupied by a barbiturate or collidine type molecule. According to this hypothesis, all inducing drugs would act indirectly at the transcriptional level (Granick, 1966).

In the human metabolic disease of acute intermittent hepatic porphyria, which is inherited as a Mendelian dominant trait, the defective gene is probably an operator gene (Op VII, Fig. 3) which is mutated so that it is repressed with difficulty by the repressor. Thus, small amounts of drugs, such as barbiturates, which would cause no

detectable porphyria in normal individuals, brings about a hepatic porphyria in individuals with this defective gene.

Granick has subsequently revised his theory on the mechanism of the induction of ALA-S in liver (Sassa & Granick, 1970). While naturally occurring 5β-H steroids (i.e. aetiocholanolone) and some inducers such as DDC may act at the transcriptional level as already described (Fig. 3; Fig. 5, Reaction 1), in contrast, inducing chemicals like AIA and γ-hexachlorocyclohexane appear to act primarily at the translational level (Fig. 5, Reaction 2). At this level a haemoprotein as repressor is assumed, with haem serving as corepressor. This haemoprotein would prevent the mRNA of ALA-S from being activated or translated. Inducers like AIA or γ-hexachlorocyclohexane would act indirectly to derepress the haemoprotein, perhaps by causing the destruction of the haem. Thus, the "silent" mRNA, conceived as a complex of the haemoprotein repressor with the specific mRNA, might be converted to active RNA (Fig. 5, Reaction 2). Tyrrell & Marks (1972) reported that treatment of chick embryo liver cell cultures with DDC and AIA resulted in the increased levels of "induction-specific" RNA for ALA-S. This induction of the enzyme in chick embryo liver cells in vitro by these two porphyrin-inducing compounds is divided into two phases; a transcriptional phase independent of translation, and a translation phase independent of transcription (these induction phases are described in more detail in INTRODUCTION, 5. 3).

Fig. 3. Hypothetical schema for the detoxification of chemical inducers in liver by derepression of the repressor control on the synthesis of ALA-S. The control of haem biosynthesis in the liver is pictured as a competition between haem and a chemical inducer, such as a barbiturate, for a site on the aporepressor that governs the synthesis of ALA-S. The effect of haem is then used for oxygenase reactions to "detoxify" the chemical.

The schema shows the interaction of the nucleus, mitochondrion, and endoplasmic reticulum in this control. In the nucleus at I, there is a structural gene (S.G.) that codes for ALA-S. This code is transcribed into an mRNA at II. The mRNA is translated into the polypeptide chain of ALA-S which comes to reside in the mitochondrion at III.

The ALA-S is the limiting enzyme in haem biosynthesis. When it is increased, more ALA is formed. The ALA is converted to haem by enzymes of the biosynthetic chain. The haem thus formed can enter the endoplasmic reticulum to become part of the oxygenase enzyme (IX) that detoxicate chemical inducers with the aid of NADPH and O_2, or the haem may be converted to bile pigment (V), or the haem may enter the nucleus to form part of the repressor (VI) that controls the synthesis of ALA-S. When haem sits on the aporepressor, then the operator gene (Op) (VII) is inactive and no mRNA can be formed. When the chemical inducer (VIII) displaces haem from the aporepressor (VI), the operator (VII) becomes active, ALA-S can now be made, and porphyrins and haem are formed.

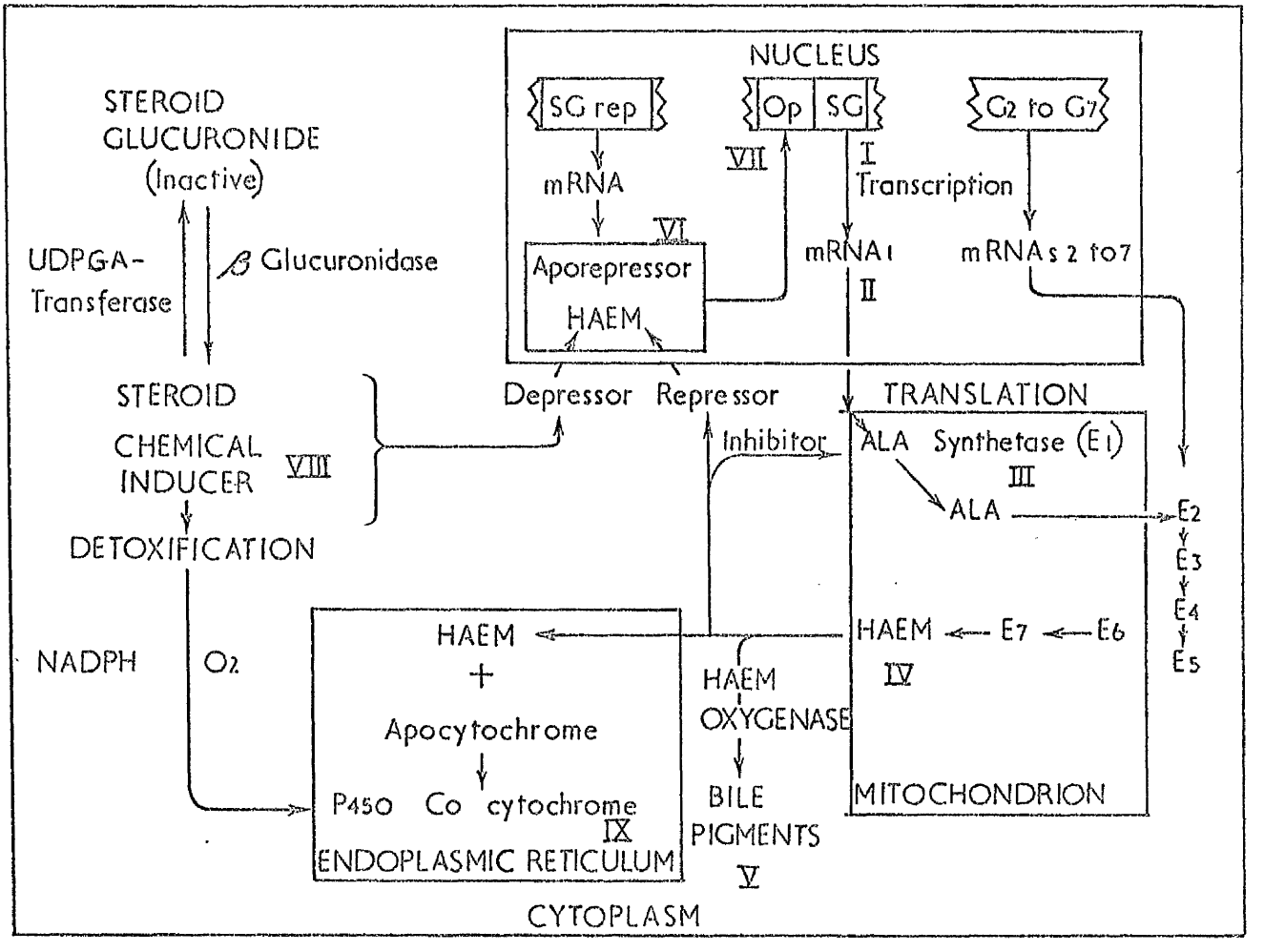


Fig. 3. Schematic representation of negative feedback and repression mechanism thought to control hepatic ALA-S levels (Granick, 1966).

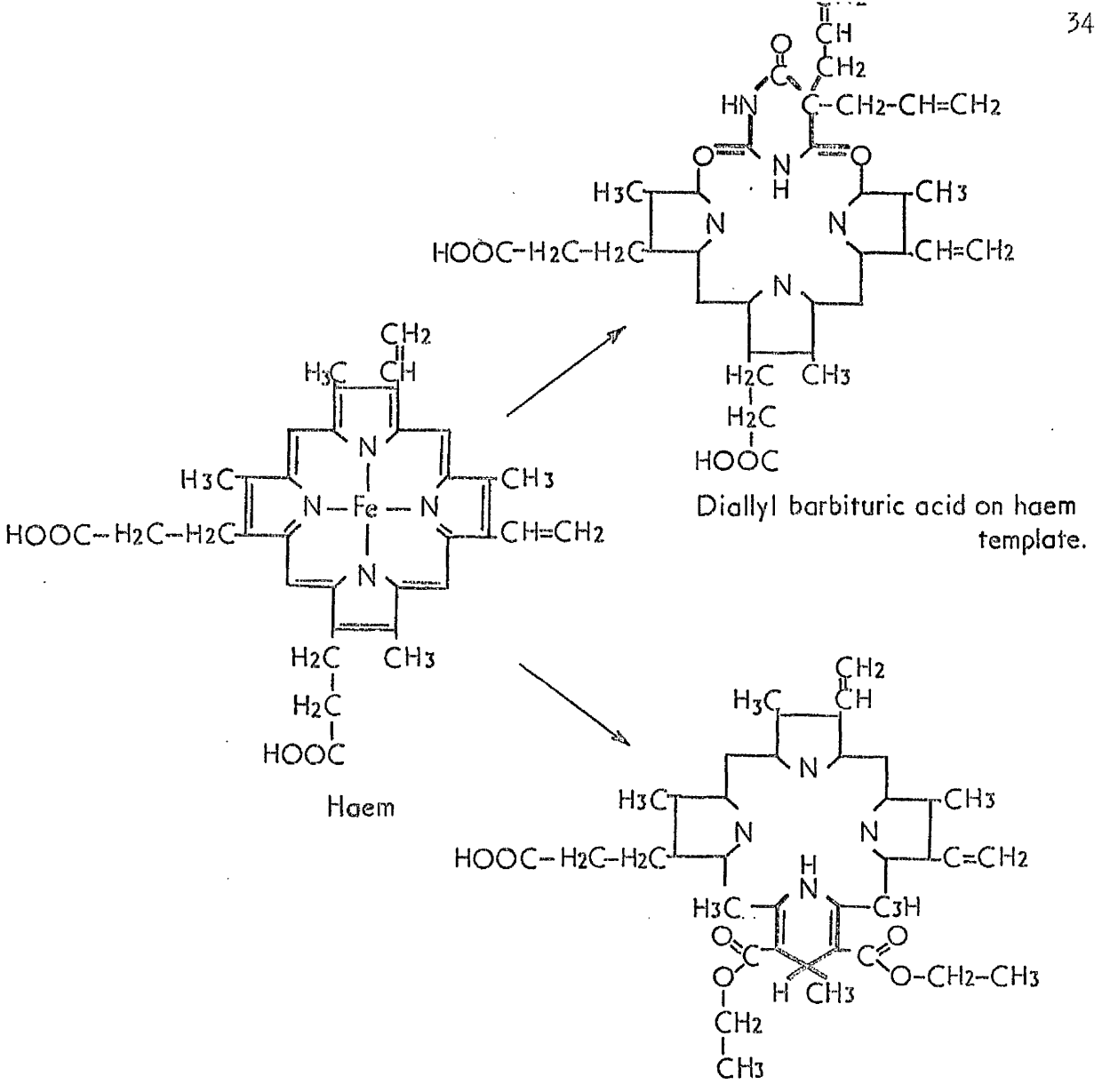


Fig. 4. Hypothesis on the competition of the inducing chemicals DDC and diallyl barbiturate for a portion of a site on the aporepressor (VI in Fig. 3) normally occupied by haem, the corepressor (Granick, 1966).

Fig. 5. Control on induction of ALA-S as proposed by Sassa & Granick (1970).

$$\text{(Operator–repressor)} \xrightarrow[\text{5β-H steroid, DDC}]{\text{derepressor}} \text{free operator} \xrightarrow[\text{structural gene}]{\text{transcription of}} \text{mRNA for ALA-S} \quad (1)$$

$$\text{(mRNA–haemoprotein repressor)} \xrightarrow[\text{AIA, Lindane}]{\text{derepression by haem destruction}} \text{active mRNA} \xrightarrow[\text{of mRNA}]{\text{translation}} \text{ALA-S} \quad (2)$$

5. 2. Role of Ribonucleic Acid in Experimental Porphyria.

Hickman et al., (1967, 1968) have provided direct evidence supporting the theory that chemical induction of ALA-S results from an increase of mRNA for ALA-S. RNA was isolated from the livers of AIA-treated and control rats by the method of Parish & Kirby (1966). The RNA fraction contained ribosomal RNA and rapidly labelled components as indicated by the sedimentation profile in a linear sucrose gradient. The RNA was added to cultured medium containing chick embryo liver fragments and after a period of incubation (usually 16 to 18 hours) the cultured liver tissue was assayed for production of porphyrins (uro-porphyrins and coproporphyrins). The hepatic RNA from livers of porphyric rats as compared to RNA from control rats, caused a 1.5- to 13-fold increase in porphyrin content. A negative diphenylamine reaction, the absence of contaminating protein, the negative experiments with added AIA to control rat-liver preparations and the abolition of the ALA-S inducing effect by purified pancreatic ribonuclease indicated that RNA was responsible for the induction and not DNA, or ALA-S or AIA that may have been carried over by the extraction procedure. Evidence that exogenous RNA may be incorporated by chick embryo liver cells in tissue culture has been presented by Amos & Kearns (1963) and Amos et al., (1964).

Previous studies have shown that the increase of porphyrin precursors in acute intermittent porphyria is reciprocally related to carbohydrate intake in the diet (Welland et al., 1964c), and that this increase results from markedly elevated levels of ALA-S (Tschudy et al., 1965b). Marver et al., (1966a) and Hickman et al., (1968) found that

carbohydrate (in particular, glucose) and actinomycin D inhibited the induction of hepatic ALA-S by AIA and suggested that these compounds may affect the induction process by a similar mechanism, i.e. this effect might be related to the degradation of ALA-S and its mRNA. Hickman et al., (1968) have tested this hypothesis. Porphyrin synthesis was greatly increased when RNA, extracted from livers of rats made porphyric with AIA, was added to embryonic chick liver cells in tissue culture. In contrast, when RNA extracted from the livers of animals given AIA and fed on glucose was added to the liver cells in tissue culture, the increase in porphyrin production was significantly smaller.

Other investigators found the induction of ALA-S activity in cultured chick embryo liver cells to be ribonucleic acid dependent (Skea et al., 1970). Porphyric-rat liver was fractionated on Sephadex G-100 into three fractions of which the first contained 90% of the RNA excluded from the column (28S and 18S ribosomal RNA) and the other two fractions contained smaller molecular weight RNA. Although all three RNA fractions showed significant inducing activity, the greatest effect was shown by the two lower molecular weight fractions. Similar fractions of normal rat liver RNA showed no significant increase in enzyme activity.

5. 3. Mechanisms of Regulation by Haemin of the Level of ALA-S in Mammalian and Avian Liver Cells.

Theoretically, the end product of a biosynthetic chain may control the first enzyme either by feedback inhibition (i.e. haem inhibiting the activity of ALA-S) or by repression (i.e. haem inhibiting the synthesis of ALA-S).

Utilizing primary cultures of embryonic avian hepatocytes as a system for porphyrin biosynthesis in experimental porphyria it was reported that increased synthesis of ALA-S by AIA could be overcome by addition of protohaemin (Granick, 1966). Granick suggested that haemin might function at the transcriptional level (Fig. 3).

There are several conflicting reports as to the mechanism of regulation by haemin of the level of ALA-S in mammalian liver cells.

Schneck et al., (1971) reported that protohaemin exerted an inhibitory effect on porphyrin biosynthesis similar to that observed with actinomycin D and cycloheximide when added at the same time as AIA. However, when added 9 hours after the porphyrin inducing drug, protohaemin exerted no inhibitory effect. As a result of these experiments they were unable to demonstrate protohaemin inhibition of transcription. In addition, protohaemin inhibition did not exert an effect similar to that of cycloheximide and thus it was not considered to inhibit at the translation level.

Contrary to this, evidence has been presented that protohaemin inhibits ALA-S induction at the level of translation on the basis of the following experiment in which primary cultures of chick embryo liver cells were utilized (Sassa & Granick, 1970). The half-life of AIA-induced ALA-S activity in the presence of actinomycin D is 5.2 hours and 3 hours for cycloheximide. The half-life of the enzyme decreased to 3.6 hours upon addition of haemin and actinomycin D, and remained unchanged, i.e. 3 hours for cycloheximide plus haemin. Tyrrell & Marks (1972) have interpreted these results as follows. Granick's assay procedure (1966)

measures both mitochondrial and cytosol ALA-S activity. When the half-life of the enzyme decreased from 5.2 hours to 3.6 hours for actinomycin D and actinomycin D plus haemin respectively, haemin may have interfered with the movement of newly synthesised ALA-S in the cytosol into the mitochondria as has been suggested by Kurashima et al., (1970). If protohaemin inhibits ALA-S movement into the mitochondria, formation of ALA-S and consequently of porphyrins would decrease in the absence of the mitochondrial succinyl-CoA generating system and pyridoxal phosphate. This would account for the decrease in the half-life of the enzyme in the presence of haemin.

Using a slightly different experimental design from the method of Sassa and Granick (1970), Tyrrell and Marks (1972) found the induction process of ALA-S activity in primary cultures of chick embryo liver cells by the porphyrin inducing compounds, DDC and AIA, to be divided into two phases. Phase one involved the accumulation of induction-specific RNA in the presence of AIA or DDC, and was classified as a transcriptional phase, independent of translation. Then, the cells were washed, and the increased ALA-S activity normally observed after washing was inhibited by cycloheximide but not actinomycin D. It was concluded that this second translation phase was independent of transcription. Granick's model for haem control of porphyrin biosynthesis would result in haem repression of ALA-S synthesis (i.e. transcription). That is, if cells were treated with AIA or DDC plus protohaemin for 5 hours (transcriptional phase), washed and fresh medium added (translational phase), the ALA-S activity should not increase because the protohaemin would repress formation of mRNA for ALA-S synthesis. Thus, haem should

cause repression of the initial phase of induction (transcription) and bear no interference on the second phase of induction (translation). However, these investigators found that when cells, treated with AIA or DDC plus cycloheximide for 5 hours (i.e. transcriptional phase), were washed, and reincubated with fresh medium plus protohaemin (translational phase), no increase in ALA-S activity was observed (Tyrrell & Marks, 1972). This indicated that protohaemin inhibited at the translational level. In another experiment cells were treated with AIA or DDC plus (i) cycloheximide or (ii) cycloheximide and protohaemin for 5 hours (transcriptional phase), washed, and reincubated with fresh medium (translational phase). The increase in ALA-S activity for (i) and (ii) was identical. It was concluded that the inhibitory effect of protohaemin resembled that exerted by cycloheximide, and protohaemin appeared to act at the translational level. Tyrrell and Marks (1972) were unable to rule out the possibility that protohaemin inhibits the transcriptional process because of the following finding. When cells were exposed to protohaemin for 5 hours, washed, and reincubated with DDC, the protohaemin pretreatment appeared to decrease the ability of cells to produce increased ALA-S when exposed to DDC. It was suggested that protohaemin was not completely removed by washing as some of the protohaemin may be irreversibly bound to protein in the cytosol of the liver cell.

Scholnick et al., (1969, 1972b) were able to demonstrate that protohaemin (5×10^{-5} M) inhibited the activity of partially purified ALA-S (150 - 200-fold) from male Wistar rats by 67% (Table 3) but did not inhibit crude preparations of this enzyme at this concentration. Both rat and human albumin and cell sap from a normal rat liver prevented

the haem inhibition of partially purified ALA-S, probably by binding haem and thus rendering it less effective as an inhibitor. This may partially explain why unpurified preparations of mitochondrial ALA-S are not inhibited by haemin. Moreover, there may be incomplete penetration of the mitochondrial membranes by haemin due to the fact that haem possibly binds cytosol proteins, including ALA-S (Scholnick et al., 1969, 1972b). Kurashima et al., (1970) have found that haemin caused inhibition of the conversion of the soluble form of ALA-S into mitochondrial ALA-S. They observed in rats that when haem was administered at 2, 4 and 10 hours after AIA, a marked reduction in mitochondrial ALA-S was balanced by an increase in activity in the soluble fraction.

RESULTS

1. Introduction.

Two general assay methods are used for measuring δ-aminolaevulinic acid synthetase (ALA-S) activity. One is the conventional colourimetric technique (Marver et al., 1966c; Dowdle et al., 1967), and the other involves the formation and isolation of isotopically labelled δ-aminolaevulinic acid (ALA) (Irving & Elliott, 1969; Ebert et al., 1970; Freshney & Paul, 1970).

My initial investigation involved an attempt to isolate mRNA for ALA-S from porphyric rat livers and test its biological activity in cultured chick embryo liver cells. Although the results from the colourimetric assay indicated some evidence for induction of ALA-S by RNA, the actual optical density difference for RNA-treated cultured cells was extremely low (i.e. maximum = 0.05) and thus the colourimetric assay method was found to be inadequate. In addition, 2 x 10 cm petri dishes with approximately 150 to 180 mg wet weight of cultured liver tissue per dish were required to obtain a reasonable homogenate strength for the colourimetric assay method. This meant that 2.0 mg RNA (since the concentration used by Skea et al., (1970) was 0.2 mg RNA per ml Eagles medium, 10 ml of Eagles per dish) was essential for each experiment. In order to conserve on time and amount of tissue required for each experiment I decided that a more sensitive assay for measurement of ALA-S activity which would utilize smaller amounts of RNA and cultured liver tissue was imperative.

1. 1. Colourimetric Assay of ALA-S Activity in Liver Homogenates.

Many studies on ALA-S have used the colourimetric assay method since the measurement has been made on induced levels of enzyme at which the procedure proves adequate. However, levels of this enzyme in normal tissues are extremely low and the amount of material available in chick embryo culture systems (Granick, 1966) and blood cells (Takaku et al., 1968) is limited. This is of importance in assessing the work of Hickman et al., (1967, 1968) and Skea et al., (1970) who reasoned that since the ALA-S activity increased in livers of rats rendered porphyric by allylisopropylacetamide (AIA), there would be a corresponding rise in synthesis of mRNA for this enzyme. Both research groups isolated RNA from porphyric rat livers by the method of Parish & Kirby (1966) and incubated the RNA with cultured chick embryo liver cells, a culture system in which the ALA-S activity is negligible when measured by the conventional colourimetric assay method (Granick, 1966).

ALA-S activity is measured by converting the ALA to 2-methyl-3-acetyl-4-propionic acid pyrrole by condensation with acetylacetone, the ALA-pyrrole then being measured colourimetrically by the use of Ehrlich reagent. Unfortunately, the assay is complicated by the fact that the liver synthesizes aminoacetone (AA). AA, like ALA, is an aminoketone of physiological importance. It serves as a source of 1-carbon fragments (Levere & Granick, 1965). The AA reacts with acetylacetone to give 2,4-dimethyl-3-acetyl pyrrole which in turn combines with Ehrlich reagent and thus interferes with the quantitative estimation of ALA. However, ALA and AA are usually

separated on resin columns before (Urata & Granick, 1963) or after (Marver et al., 1966b; Narisawa & Kikuchi, 1966; Hayashi et al., 1969) conversion to pyrrole, a technique that is not readily adapted to small volumes. Furthermore, ALA-dehydratase (ALA-D) in liver homogenates converts ALA to porphobilinogen (PBG). Therefore, the estimate of ALA-S may be in error by the amount of ALA converted to PBG during the course of incubation. Marver et al., (1966c) found that EDTA (10 mM) enhanced ALA production and reduced AA synthesis by about 70% or 90% in porphyric liver homogenate containing 75 mM Tris-HCl (pH 7.2) or 75 mM sodium potassium phosphate (pH 7.0) as buffer, respectively. This increase of ALA production appears to be the result of noncompetitive inhibition of ALA-D by EDTA (Gibson et al., 1955; Granick & Mauzerall, 1958).

The method of Dowdle et al., (1967), slightly modified, was used for the determination of enzymatically formed ALA. Except where otherwise stated, the incubation mixture contained in μmoles per ml: glycine, 100; sodium citrate, 50 (pH 7.4); Tris-HCl, 75 (pH 7.2); EDTA, 10; the total incubation volume being 200 μl. Incubations were carried out in polyethylene microfuge tubes (46 mm x 4.5 mm) for one hour in a shaking water bath at 37°C and the reaction stopped by addition of 100 μl of 15% trichloroacetic acid. The ALA generated during the one hour incubation was measured colourimetrically as described in MATERIALS AND METHODS.

1. 2. Radiochemical Assay Method for ALA-S Activity in Liver Homogenates.

Three radiochemical procedures for measurement of ALA-S activity have been described (Irving & Elliott, 1969; Ebert et al., 1970; Freshney & Paul, 1970). These methods permit measurement of the enzyme in minute quantities of normal tissue. The isotopic method for estimation of ALA-S activity, reported by Freshney & Paul (1970) utilized unfractionated homogenate in a small incubation volume (30 µl as compared to 200 µl for the colourimetric assay). An attempt to reproduce their assay procedure was unsuccessful. When a porphyric rat liver homogenate was used as a source of ALA-S activity, the colourimetric assay method of Dowdle et al., (1967) gave a satisfactory optical density difference. On the other hand, using the procedure of Freshney & Paul (1970) only background dpm of $[2\text{-}^{14}C]$ glycine were found in ALA isolated by electrophoresis from the trichloroacetic acid treated supernatant of the incubation mixture. Subsequent alteration of the various media to those described for the colourimetric assay resulted in a large increase in dpm of $[2\text{-}^{14}C]$ glycine incorporated into ALA. Electrophoresis of the deproteinized supernatant on silica gel thin-layer sheets (Malinkrodt "Chromar 1000" glass fibre mat, impregnated with silica gel; total thickness 1 mm; Camlab, Cambridge) was difficult to perform. The thin-layer sheets, when moistened with 0.05 M phthalate buffer, tore very easily and thus had to be handled with extreme delicacy. Elution of the incubation generated $[^{14}C]$ ALA from the silica gel thin-layer sheets with either 1 N HCl or hyamine hydroxide (scintillation grade) yielded inconsistent results and proved cumbersome and very time consuming.

The radiochemical procedures of Irving & Elliott (1969) and Ebert et al., (1970) utilized larger incubation volumes (2 ml) and at least 4 to 5 times as much protein as the amount used by Freshney & Paul (1970). In addition, it appeared from their report that a Dowex 50 (H^+) column per assay was used to isolate the newly synthesized [^{14}C]-ALA from the incubation reaction mixture. This meant that numerous Dowex 50 (H^+) columns would be required along with several buffers of various pH to elute the labelled ALA from the columns. As a result, these two techniques did not appear to fulfill the requirements for assay of pmole quantities of ALA in the chick embryo culture system. Therefore I developed a sensitive radiochemical assay method, independent of the three existing procedures, which permits investigation of control mechanisms in minute amounts of normal tissues. A comparison of this radiochemical assay with the conventional colourimetric method of Dowdle et al., (1967) has been made.

In the radiochemical assay estimation of ALA-S activity, [2-^{14}C] glycine acts as a precursor of ALA and unfractionated homogenate as the source of enzyme.

Several advantages of my radiochemical assay when compared to the modified colourimetric assay procedure of Dowdle et al., (1967) are as follows: (i) only 50 mg wet weight of cultured chick embryo liver per 5 cm petri dish is used as opposed to 2 x 10 cm dishes containing at least 75 mg wet weight of tissue per dish for the colourimetric assay: (ii) 10 µl of homogenate is required compared with 400 µl for the colourimetric assay: (iii) as many as 100 assays may be performed in

one day whereas any more than 30 assays using the colourimetric technique provided problems: (iv) the polyester thin-layer sheets, coated with silica gel F1500 (Camag) are much easier to handle than the silica gel thin-layer sheets used by Freshney & Paul (1970): (v) once the trichloroacetic acid supernatant of the reaction mixture is spotted and allowed to dry on the polyester thin-layer sheets the [^{14}C] ALA that was generated in the reaction does not appear to degrade with time: (vi) the radiochemical assay requires less time for completion than any of the previous isotopic assay procedures (Irving & Elliott, 1969; Ebert et al., 1970; Freshney & Paul, 1970): (vii) this extremely sensitive assay is capable of detecting ALA produced in pmoles quantities. The ALA-S activity in cultured chick embryo liver system, when assayed by the radiochemical procedure, is negligible. Upon addition of the porphyrinogenic chemical, AIA, the increase in ALA-S is easily observed from the large increase of dpm of ^{14}C in ALA and may be measured in pmoles ALA per µg of protein.

The incubation mixture for the radiochemical assay contained the following, unless otherwise stated, in µmoles per ml: glycine, 10; 1 µC [2-^{14}C] glycine (specific activity 52 mC/mmol); sodium citrate, 50 (pH 7.4); Tris-HCl, 75 (pH 7.2); EDTA, 10; in a total volume of 20 µl. The polyethylene tubes (46 mm x 4.5 mm) were incubated at 37°C in a shaking water bath for one hour. The reaction was stopped by addition of 5 µl of 30% trichloroacetic acid. The [^{14}C] ALA produced during the incubation is separated by electrophoresis and estimated by scintillation counting, as described in MATERIALS AND METHODS.

1. 3. Resolution of [2-^{14}C] glycine and [3,5-^{3}H] ALA by Electrophoresis on Silica Gel Polyester Supported Thin-Layer Sheets.

The three existing radiochemical procedures for measuring ALA-S activity involve either [1,4-^{14}C] succinate (Irving & Elliott, 1969; Ebert et al., 1970) or [2-^{14}C] glycine (Freshney & Paul, 1970) incorporation into ALA generated by liver and spleen homogenates. However, the methods for separation of ^{14}C-substrates from the newly formed [^{14}C] ALA differ for each of the three micro assays.

Irving & Elliott (1969) studied [1,4-^{14}C] succinate incorporation into ALA by mitochondrial ALA-S from DDC-treated guinea pig liver. Trichloroacetic acid supernatants of incubation mixtures were applied to Dowex 50 (H^+) columns and the newly generated [^{14}C] ALA was eluted by 2 M pyridine acetate buffer, pH 6.0. The eluate was dried, dissolved in water and counted on glass fibre discs by liquid scintillation. Alternatively, the [^{14}C] ALA in the eluate from the Dowex column was converted to [^{14}C] ALA-pyrrole by the method of Urata & Granick (1963) and chromatographed on thin-layer plates coated with Kieselgel G7731. This separated [^{14}C] ALA-pyrrole from any possible [^{14}C] AA-pyrrole contamination. The [^{14}C] ALA-pyrrole was then eluted and counted. To ensure that [1,4-^{14}C] succinate was incorporated solely into ALA, antimycin A and malonate were added to inhibit succinic dehydrogenase activity while arsenite inhibited the α-oxoglutarate oxidase system thus preventing succinyl-CoA formation from α-oxoglutarate. The latter would be unlabelled and thus compete with [^{14}C] succinyl-CoA.

Ebert et al., (1970) reported a similar micro method for direct determination of [^{14}C] ALA production in mouse spleen or liver homogenates. [^{14}C] ALA generated in the reaction by homogenate of spleen liver incubated with α-keto [^{14}C] glutarate or [1,4-^{14}C] succinate substrates respectively, is selectively adsorbed on to a Dowex 50 (H^+) column run at pH 3.9. Moreover, labelled amino acids and unreacted substrates are not retained by the column at this pH and thus contamination of [^{14}C] ALA by these substances did not occur.

Freshney et al., (1970) established a micro assay procedure that involved electrophoretic separation of [^{14}C] ALA from [2-^{14}C] glycine on silica gel thin-layer sheets. The distribution of radioactivity corresponded to the absorbance of glycine and added marker ALA, and thus indicated no contamination by other metabolites which would interfere with the specificity of the assay.

Fig. 6 shows the separation of [2-^{14}C] glycine and [3,5-^{3}H] - ALA by high voltage electrophoresis on silica gel F1500 polyester supported thin-layer sheets. A spray of the chromatogram with 0.1% ninhydrin in absolute alcohol revealed only two spots: yellow for added unlabelled ALA and purple for glycine. When the thin-layer sheet was cut up into sections (1.5 x 2 cm) and counted by liquid scintillation, the distribution of radioactivity corresponded to the ninhydrin stained carrier ALA and glycine. Only background counts of [^{14}C] (i.e. below 50 dpm) from [2-^{14}C] glycine were found in the ALA spot. However, a small amount of tailing by [3,5-^{3}H] ALA was observed in the glycine spot, possibly due to overlap of ^{3}H counts in the ^{14}C channel. When this electrophoretic method was used to isolate [^{14}C] ALA generated in a liver

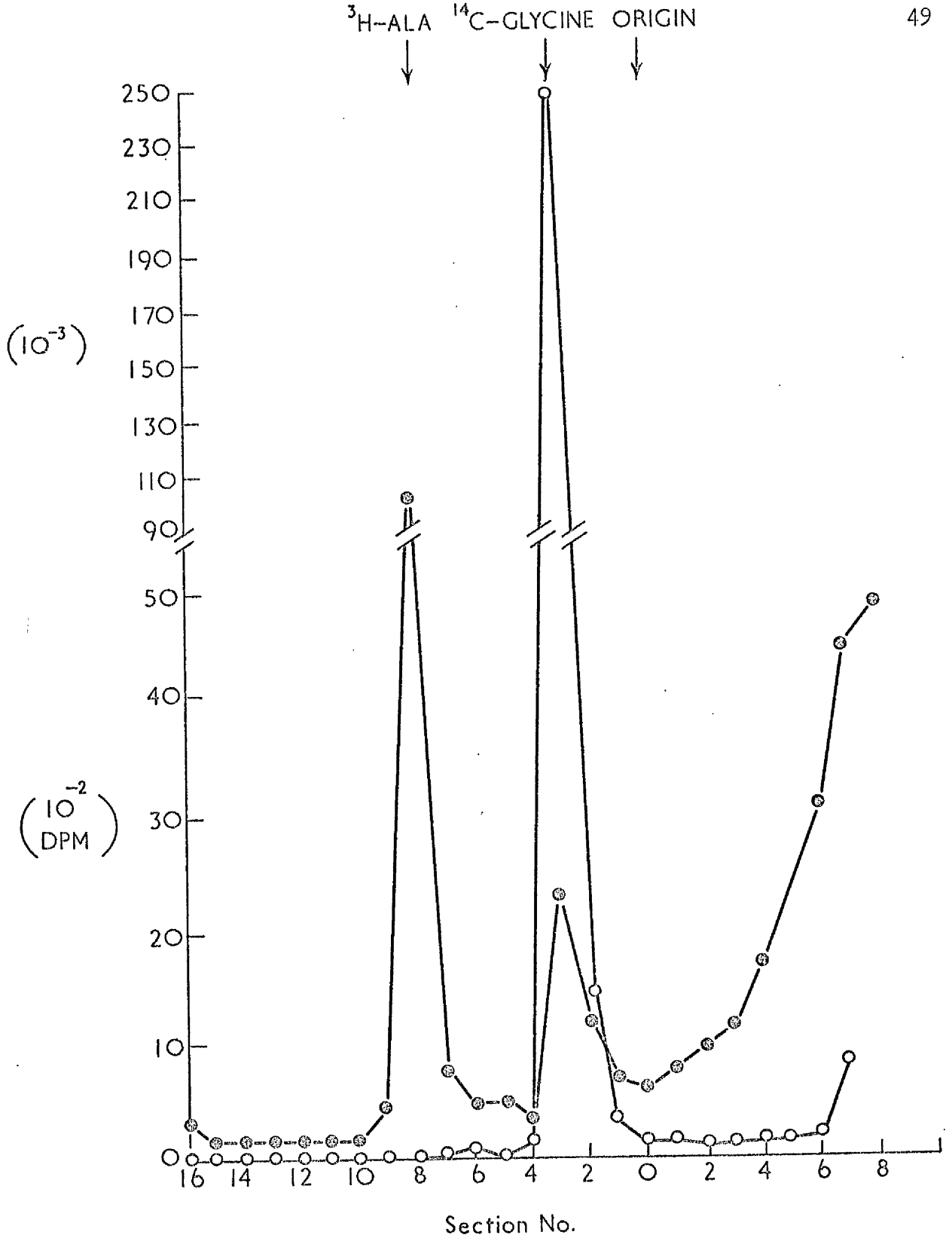


Fig. 6. Identification of products by electrophoretic separation of [3,5-^{3}H]δ-aminolaevulinic acid (ALA) and [2-^{14}C] glycine on polyester sheets coated with silica gel. 5 µl of the incubation mixture was spotted on the silica gel thin-layer sheet, electrophoresed for 30 minutes at 130 - 195 mA and 3 KV using 0.05 M phthalate buffer containing 0.01 M EDTA. Sections (1.5 x 2.0 cm) were cut out and counted as described in MATERIALS AND METHODS. The incubation contained 10 µl of 150 mM Tris-HCl in 20 mM EDTA, pH 7.2, 10 µl of 20 mM glycine in 100 mM sodium citrated, pH 7.4, 1 µC [2-^{14}C] glycine (52 mCi/mmol), 1 µC [3,5-^{3}H] ALA (2 mCi/mmol), 5 µl of 30% trichloroacetic acid, and 5 µl of 15 mM unlabelled ALA.

homogenate, AA, synthesis of which is low in the presence of EDTA (Marver et al., 1966c), appeared to migrate just in advance of glycine and thus did not contaminate ALA. This AA spot was not always visible when the ninhydrin was applied. Moreover, the distribution of radio-activity in this experiment corresponded precisely to the ninhydrin stained ALA and glycine with negligible tailing between the product and substrate respectively. This indicated the absence of other metabolites which would possibly alter the specificity of the assay. However, if greater than 1 μC of $[2\text{-}^{14}C]$ glycine per assay tube were used, then severe tailing of counts occurred between the two separated components.

1. 4. Determination of Optimum Substrate Concentration for the Colourimetric and Radiochemical Assay of ALA-S Activity in Crude Liver Homogenates.

Marver et al., (1966c), using the colourimetric assay procedure, reported maximal ALA synthesis and minimal AA synthesis with 100 mM glycine for porphyric liver homogenate. The K_m values for ALA-S and AA-S in the assay system were approximately 5 mM and greater than 150 mM, respectively.

Optimal substrate condition for ALA-S activity in crude AIA-liver homogenates were determined with the colourimetric assay method in order to devise a suitable incubation system for the radio-chemical assay method. Omission of citrate and/or glycine resulted in negligible ALA synthesis in porphyric or normal liver homogenate. ALA formation in crude liver homogenate was maximal with 100 to 200 mM glycine in 50 mM sodium citrate (pH 7.4). Repetition of this experiment using both

assay procedures on the same liver homogenate is illustrated in Fig. 7. Although the rate of ALA synthesis is highest with 100 mM unlabelled glycine for the radiochemical assay, the total dpm of $[2\text{-}^{14}C]$ glycine converted to $[^{14}C]$ ALA was greatest in the absence of unlabelled glycine. It was decided that in future experiments the colourimetric assay procedure would contain 100 mM glycine and 10 mM unlabelled glycine for the radiochemical assay technique in order to ensure that substrate concentration did not become limiting. The final numerical value of nmoles ALA produced per g protein per minute differed between the two methods when measuring enzyme activity of the same homogenate because of the initial glycine concentrations. The radiochemical technique, however, was more sensitive since a slight increase in ALA-S activity caused a more noticeable increment of dpm in the incubation generated $[^{14}C]$ ALA. Unfortunately, increased specific activity of glycine caused the assay to be more sensitive but led to other problems such as cascading of the scintillation counter, cost of the isotope, and poor resolution by electrophoresis due to trailing of counts.

1. 5. Effect of EDTA Concentration of ALA Production in Crude Liver Homogenates.

Marver et al., (1966c) described a colourimetric method for measurement of ALA-S activity in crude liver homogenates of normal and porphyric animals. EDTA was included in the incubations to minimize ALA-dehydratase activity and also to reduce AA synthesis. The effect of EDTA concentration upon ALA production is shown in Fig. 8. The lowest production of ALA occurred in the absence of EDTA. ALA accumulation

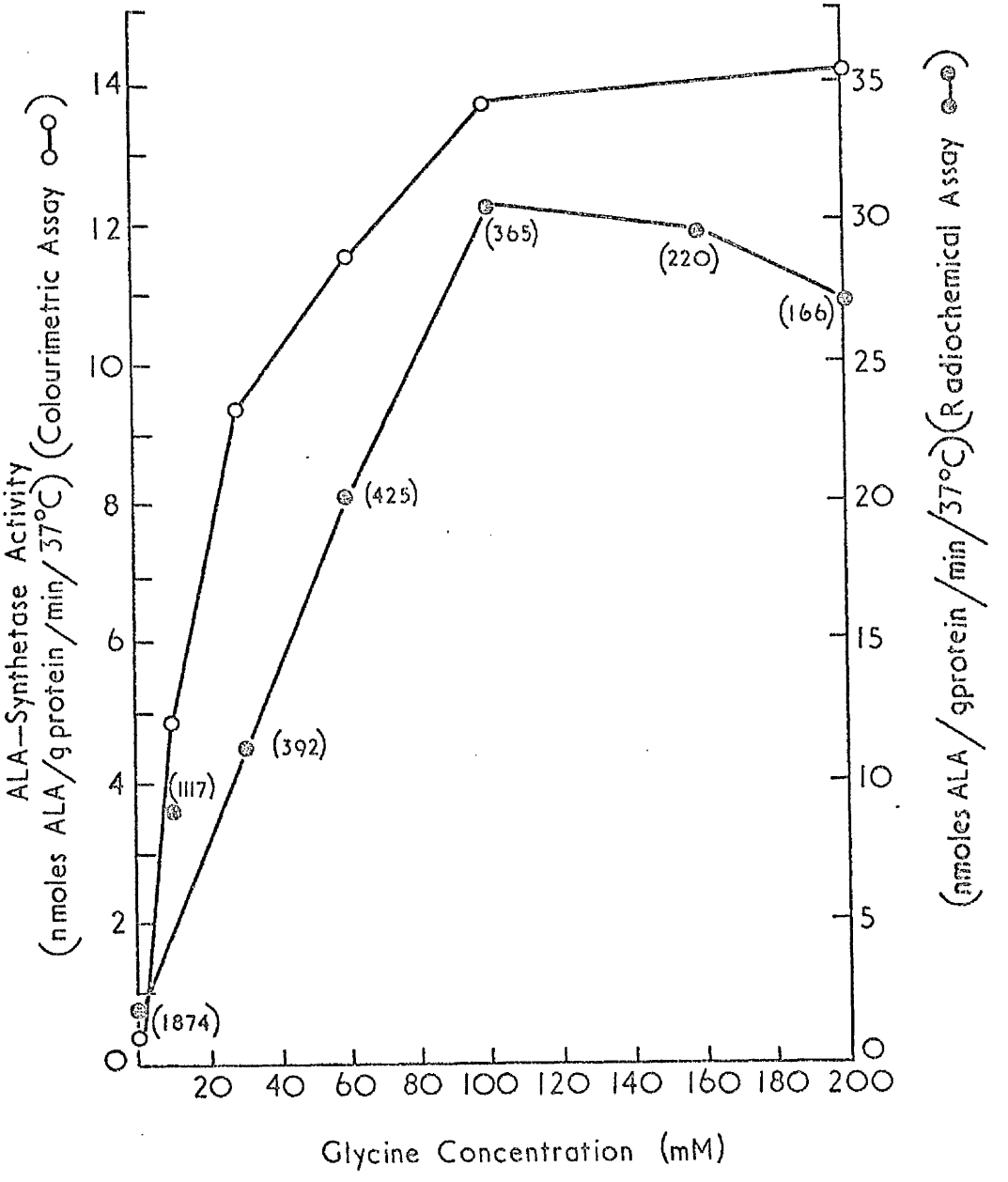


Fig. 7. Comparison of radiochemical and colourimetric assay methods for δ-aminolaevulinic acid (ALA) production in porphyric rat liver homogenate as a function of glycine concentrations. A fasted rat received 2 doses of 400 mg AIA per kg body weight at 24 hour intervals and was sacrificed 3 hours after the last dose. The liver was homogenised in 2 volumes of 150 mM Tris-HCl and 20 mM EDTA, pH 7.2. 100 µl and 10 µl samples of homogenate were transferred to microfuge tubes containing 100 µl and 10 µl of 100 mM sodium citrate and the appropriate glycine concentration for the colourimetric and radiochemical assay, respectively. Procedures for measurement of ALA generated during the one hour incubation at 37°C by the colourimetric and radiochemical assays are described in MATERIALS AND METHODS. Numbers within the square brackets indicate dpm of [2-^{14}C] glycine incorporated into ALA formed during the incubation. The colourimetric assay method measured ALA production in a porphyric rat liver homogenate different from the homogenate used in the radiochemical assay procedure.

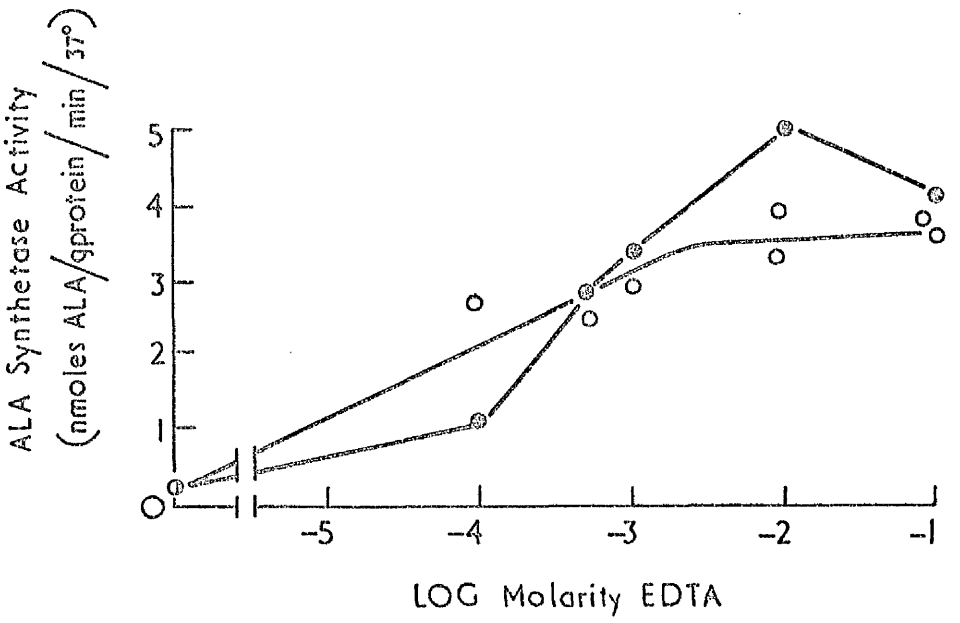


Fig. 8. ALA production in porphyric rat liver homogenate as a function of EDTA concentration. 30% homogenate was incubated in the presence of 100 mM glycine in 50 mM sodium citrate, pH 7.4, and 75 mM Tris-HCl, pH 7.2, for the colourimetric assay (a), and in 10 mM glycine, 1 μC [2-^{14}C] glycine (52 mCi/mmol) in 50 mM sodium citrate, pH 7.4, and 75 mM Tris-HCl, pH 7.2, for the radiochemical (b), and varying concentrations of EDTA as indicated. The total volume for (a) was 200 μl and (b) 20 μl. The reaction mixtures were incubated for 40 minutes with shaking at 37°C and the reaction stopped by addition of 100 μl of 15% trichloroacetic acid for (a) and 5 μl of 30% trichloroacetic acid for (b). The generated ALA was isolated and determined as described in MATERIALS AND METHODS section.

(o—o , colourimetric assay; ⊛—⊛ , radiochemical assay)

was maximal at EDTA concentration of 10 mM which is in agreement with the data reported by Marver et al., (1966c). Although no experiment was performed to detect AA production at 10 mM EDTA, it has been shown, using a similar colourimetric assay procedure to the one used in this laboratory, that AA synthesis in liver homogenates is inhibited by about 70% and that 10 mM EDTA almost completely blocked the conversion of ALA to porphobilinogen (Marver et al., 1966c). Thus, future experiments included EDTA at a final concentration of 10 mM to yield optimal ALA synthesis.

1. 6. Effect of Incubation Time on ALA synthesis in Crude Liver Homogenates.

Previous investigations (Marver et al., 1966c; Dowdle et al., 1967) have shown that ALA synthesis was linear up to one hour as measured by the colourimetric method of Marver et al., (1966c). Using their radiochemical assay method, Freshney & Paul (1970) found the rate of $[^{14}C]$ ALA synthesis in normal mouse liver homogenate linear up to four hours while Ebert et al., (1970) reported a curvilinear response. In the latter case, a plateau of $[^{14}C]$ ALA production was observed for incubation greater than 90 minutes.

The response of ALA production in crude AIA-treated liver homogenate is shown in Fig. 9. The results from the colourimetric assay method showed a linear response of ALA synthesis up to 60 minutes. ALA formation determined by both assays, plateaued for incubations above 60 minutes. The dpm of $[2\text{-}^{14}C]$ glycine incorporated into newly synthesized

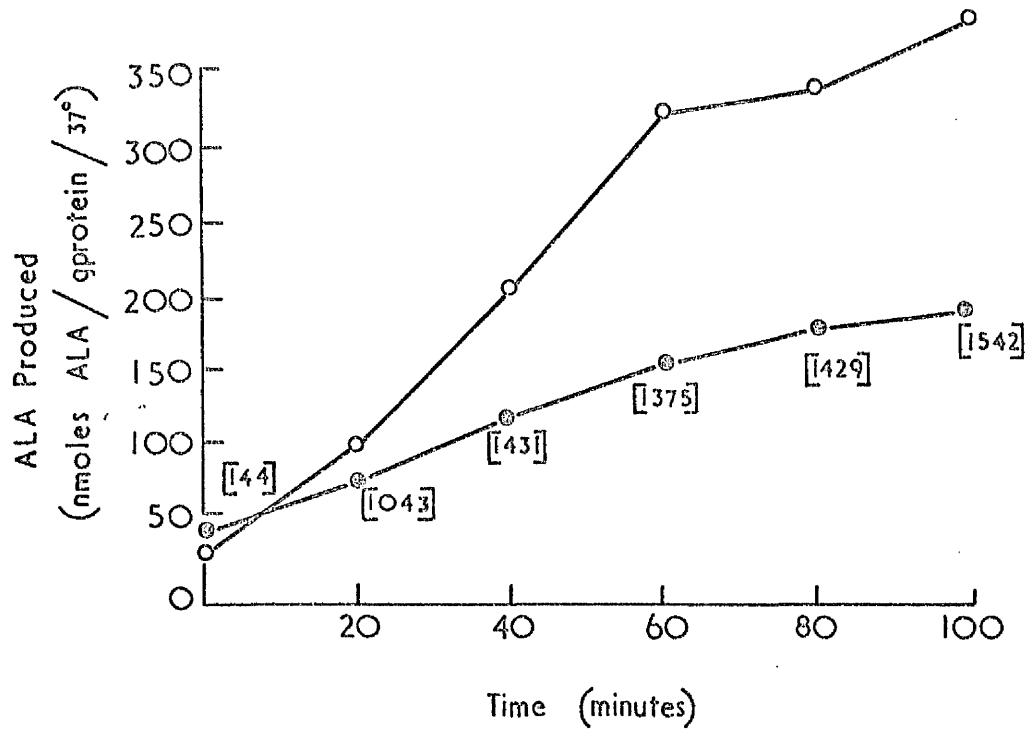


Fig. 9. ALA production in crude liver homogenate from an AIA-treated rat as a function of time. Incubation conditions and procedures for the colourimetric and radiochemical assay methods are described in MATERIALS AND METHODS with the incubation time varied as indicated. Values in square brackets indicate dpm of $[2\text{-}^{14}C]$ glycine incorporated into ALA.

(o———o , colourimetric assay; ●———● , radiochemical assay)

[^{14}C] ALA during the incubation reached a maximum after 40 minutes while the absorbance of the ALA-pyrrole - Ehrlich complex at 552 nm appeared to level off after 60 minutes of incubation (Fig. 9). As a result of this experiment it was decided that incubation time for the colourimetric and radiochemical assay methods would be 60 and 40 minutes, respectively.

1. 7. Comparison of Colourimetric and Radiochemical Assay Methods for Measurement of ALA as a Function of Crude Liver Homogenates.

Previous reports using either the colourimetric assay (Marver et al., 1966c) or the radiochemical assay (Irving & Elliott, 1969; Ebert et al., 1970; Freshney & Paul, 1970) have shown that the rate of ALA synthesis is proportional to the amount of protein added to the incubation mixture.

Fig. 10 shows the effect of varying liver homogenate concentration upon ALA production. A linear increase of ALA synthesis with enzyme concentration was observed over the range of 3 mg to 12 mg protein per assay for the colourimetric method and linear from 70 µg to 270 µg protein per assay tube for the radiochemical technique.

Marver et al., (1966c) used large amounts of homogenate so that satisfactory optical densities were obtained for the ALA-pyrrole-Ehrlich colour complex. The present results show that the radiochemical method has the advantage of permitting use of much smaller amounts of tissue. From Fig. 10 it is observed that only 70 to 100 µg protein were

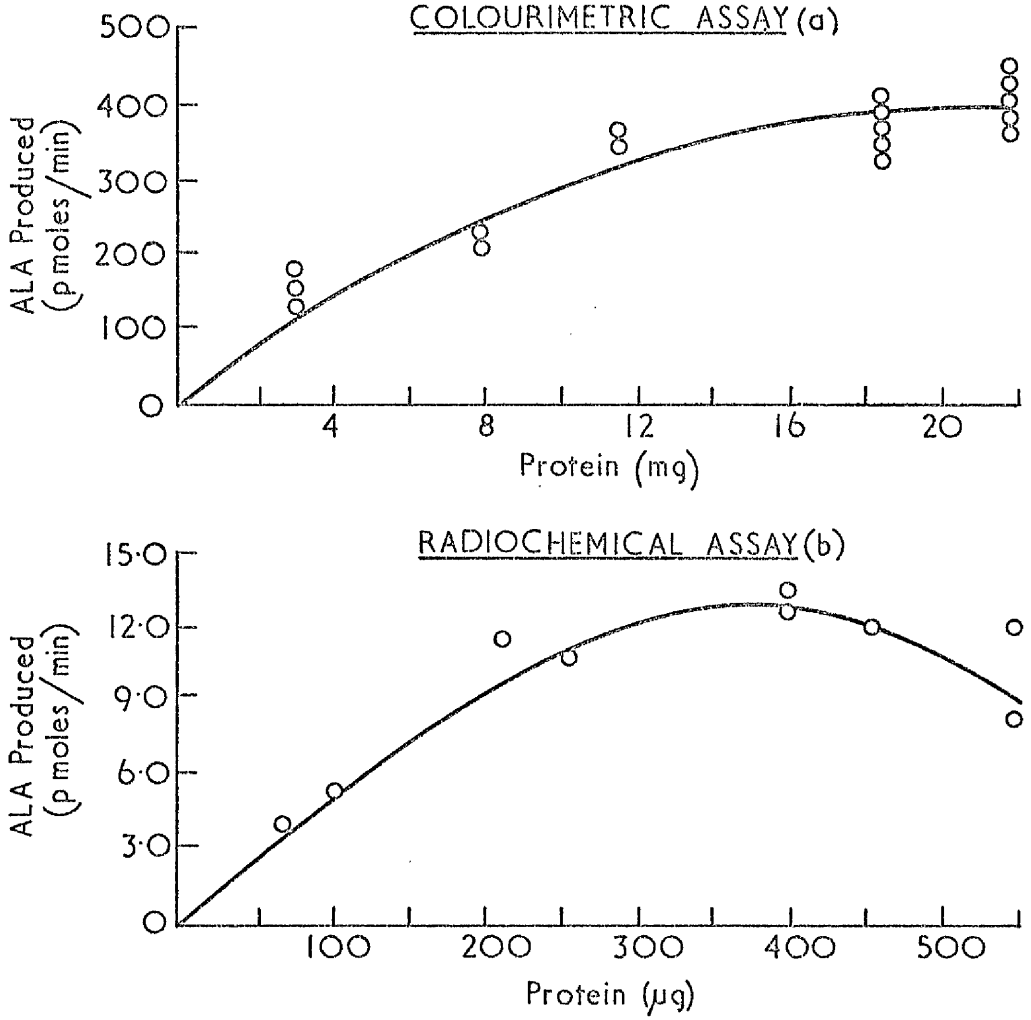


Fig. 10. Rate of δ-aminolaevulinic acid (ALA) production by different concentrations of porphyric rat liver homogenate as measured by the colourimetric (a), and radiochemical (b) assay methods. Varying concentrations of liver homogenate were incubated in 75 mM Tris-HCl, and 10 mM EDTA, pH 7.2, with the substrates 200 mM glycine and 100 mM sodium citrate (pH 7.4), in a total volume of 200 µl for the colourimetric assay (a), or 20 mM glycine, 1 µC [2-^{14}C] glycine (52 mCi/mmol) and 100 mM sodium citrate (pH 7.4), in a total volume of 20 µl for the radiochemical assay (b). The 40 minute incubation at 37°C in a shaking water bath was stopped by addition of 100 µl of 15% trichloroacetic acid to (a), or 5 µl of 30% trichloroacetic acid to (b). The ALA generated in the reaction mixture was isolated and determined according to procedures described in MATERIALS AND METHODS. Total pmoles ALA produced per tube are plotted as a function of total protein in the reaction mixture. Each point is a single observation. Both assay methods were performed on the same source of porphyric liver tissue.

necessary in order to measure ALA synthesis in pmoles per minute. In contrast, at least 2 mg protein is required for the colourimetric assay in order to obtain an optical density difference of 0.10. When both assay methods were performed on the same porphyric liver homogenate 10^4 dpm were obtained in ALA by the ^{14}C method per mg of protein while the colourimetric method gave an observed optical density difference of 0.053 for the same amount of protein. In normal chick embryo liver and cultured chick embryo liver the actual optical density differences are so small (i.e. maximum absorbance of colour complex is 0.03) that it is impossible to place significance on the colourimetric assay results.

1. 8. Conclusion.

A new sensitive method for measuring ALA-S activity has been developed, based on the incorporation of $[2\text{-}^{14}C]$ glycine into ALA and using crude homogenate as the source of enzyme. Electrophoresis of the trichloroacetic acid supernatants of the incubation mixture on silica gel thin-layer sheets separates the $[^{14}C]$ ALA from $[2\text{-}^{14}C]$ glycine.

2. Physical Properties of ALA-S in Different Tissues.

Sassa & Granick (1970) have proposed that DDC induction of ALA-S in cultured chick embryo liver system occurs at the transcriptional level and AIA induction at the translational level. It is possible that formation of different isozymes of ALA-S may arise depending on which drugs are used to induce the enzyme. Thus, heterogenous molecular

forms of the enzyme (as have been reported to exist for AIA-induced ALA-S in cock liver by Ohashi & Kikuchi, 1972) induced by AIA may have certain physical characteristics (i.e. metal cation requirements, pH optimum, and heat stabilities) which differ from those of the DDC-induced isozymes.

AIA-induced ALA-S, purified 150- to 200-fold from hepatic cytosol of rat liver, requires metal cations from maximum activity and stabilization (Scholnick et al., 1972a, b) (described in more detail in INTRODUCTION, section 3.2). NaCl (0.3 M - 0.4 M), results in conversion of the larger, but less active form of the AIA-induced ALA-S to a smaller, more active molecule (Ohashi & Kikuchi, 1972; Scholnick et al., 1972a, b). In comparison, Whiting & Elliott (1972) found that a high salt concentration (0.8 M) and a reducing agent dithioerythritol (1 mM) was required to convert the aggregated form of hepatic mitochondrial ALA from DDC-induced rats to its soluble form (molecular weight of 77,000) of greater activity. Treatment of the cytosol enzyme (molecular weight of 178,000) using the same conditions did not convert its molecular weight to that obtained for the solubilized mitochondrial enzyme.

The nature and physiological significance, if any, of NaCl conversion of the hepatic cytosol AIA-induced ALA-S (Ohashi & Kikuchi, 1972; Scholnick et al., 1972a, b), but not the cytosol DDC-induced, is not known. The different effect of NaCl may be due to an artefact produced during the isolation procedures, but it is feasible that ALA-S induced by AIA may have different metal cation requirements than the enzyme induced by DDC.

2. 1. Effect of Cations on ALA-S Activity.

ALA-S in crude homogenates from AIA and DDC-induced chick embryo livers appears to require similar Na^{+} cation concentrations (0.1 M) (Fig. 11). In contrast, addition of Mg^{++} to the incubation mixtures decreased the enzyme activity for both drug induced enzymes but between 0.02 M and 0.1 M Mg^{++} there was a significant increase of the DDC-induced ALA-S but not for the AIA-induced enzyme. It is impossible to compare these results with those of other investigators since the latter have studied cation requirements of the partially purified enzyme. It is of interest, however, that both radiochemical assays devised by Irving and Elliott (1969) and Freshney & Paul (1970) included 20 mM Mg^{++} in the assay incubation medium.

2. 2. Comparison of Heat Stability of AIA- and DDC-Induced ALA-S in Crude Homogenates of Rat and Chick Embryo Liver and Cultured Liver Cells.

Heat inactivation experiments performed on the partially purified mitochondrial and soluble ALA-S of porphyric rat liver resulted in similar behaviour of both enzyme fractions (Hayashi *et al.*, 1969).

A comparison of heat stability patterns at 45°C was made on AIA- and DDC-induced ALA-S in rat and chick embryo liver and cultured monolayer liver cells (Fig. 12). There was no apparent difference in the inactivation pattern for either AIA- or DDC-induced enzyme in rat liver (Fig. 12, a & b). Similar findings were observed for chick embryo

liver and the cultured liver cells (Fig. 12, b & c). However, the denaturation of induced enzyme activity for rat and chick embryo liver differed from that of the cultured liver cells. After 10 minutes of homogenate incubation at 45°C, approximately 80% of the initial enzyme activity had disappeared for rat and chick embryo liver whereas there was less than 75% of original activity for cultured liver cells. After 20 minutes of incubation at 45°C had elapsed, there was negligible enzyme activity for both rat and chick embryo liver while even after 30 minutes at 45°C 10% of original activity still remained in the homogenate of the cultured liver cells.

2. 3. Comparison of pH Optima of AIA- and DDC-Induced ALA-S in Crude Homogenates of Rat and Chick Embryo Liver.

Recent studies on the dependence of the activity of the four partially purified forms of hepatic tyrosine aminotransferase on pH have shown that form I has a higher pH optimum (pH 8.4) than the other three forms (II, III, IV) (pH 7.7) (Iwasaki et al., 1973).

Up to the present time there have been no published reports which compare the pH optima of AIA- and DDC-induced ALA-S in crude homogenates of rat and chick embryo liver.

The curves of the dependence of the AIA- and DDC-induced enzyme activities on the pH of the incubation medium are illustrated in Fig. 13. In chick embryo liver, there was only one pH optimum for the DDC-induced enzyme, while the AIA-induced ALA-S appeared to have two pH optima : (i) one at pH 7.7, which was identical to that of the DDC-induced

enzyme, and (ii) the other at a lower pH of 6.7. In rat liver, however, there was only one pH optimum for both AIA- and DDC-induced ALA-S. Noteworthy was (i) the absence of a pH optimum at 7.7 for the AIA-induced enzyme in rat liver, and (ii) the difference between the observed pH optimum of the DDC-induced enzyme for rat liver (pH 6.7) and chick embryo liver (pH 7.7). These experiments were repeatable and the importance of these observations is analysed in the DISCUSSION.

3. Kinetics of AIA-Induced Increase of ALA-S in Rat Liver Mitochondria and Cytosol.

Since attempts were to be made to isolate the hepatic mRNA for ALA-S, it was necessary to find the experimental conditions that furnished optimal levels of AIA-induced enzyme activity.

There are several reports on the kinetics of AIA-induced formation of ALA-S in rat liver mitochondrial and cytosol (Tschudy *et al.*, 1966c; Marver *et al.*, 1966a; Narisawa & Kikuchi, 1966; Hayashi *et al.*, 1968; Matsuoka *et al.*, 1968; Hayashi *et al.*, 1969; Beattie & Stuchell, 1970; Stein *et al.*, 1970). The general procedure has been to administer AIA (multiple doses were usually 12 hours apart) to fasted male rats, sacrifice the animals at different periods of time, isolate the hepatic mitochondria and/or cytosol, and measure the ALA-S activity using the conventional colourimetric assay. Tschudy *et al.*, (1965c), Marver *et al.*, (1966a), and Stein *et al.*, (1970) reported a pronounced rise (1.5- to

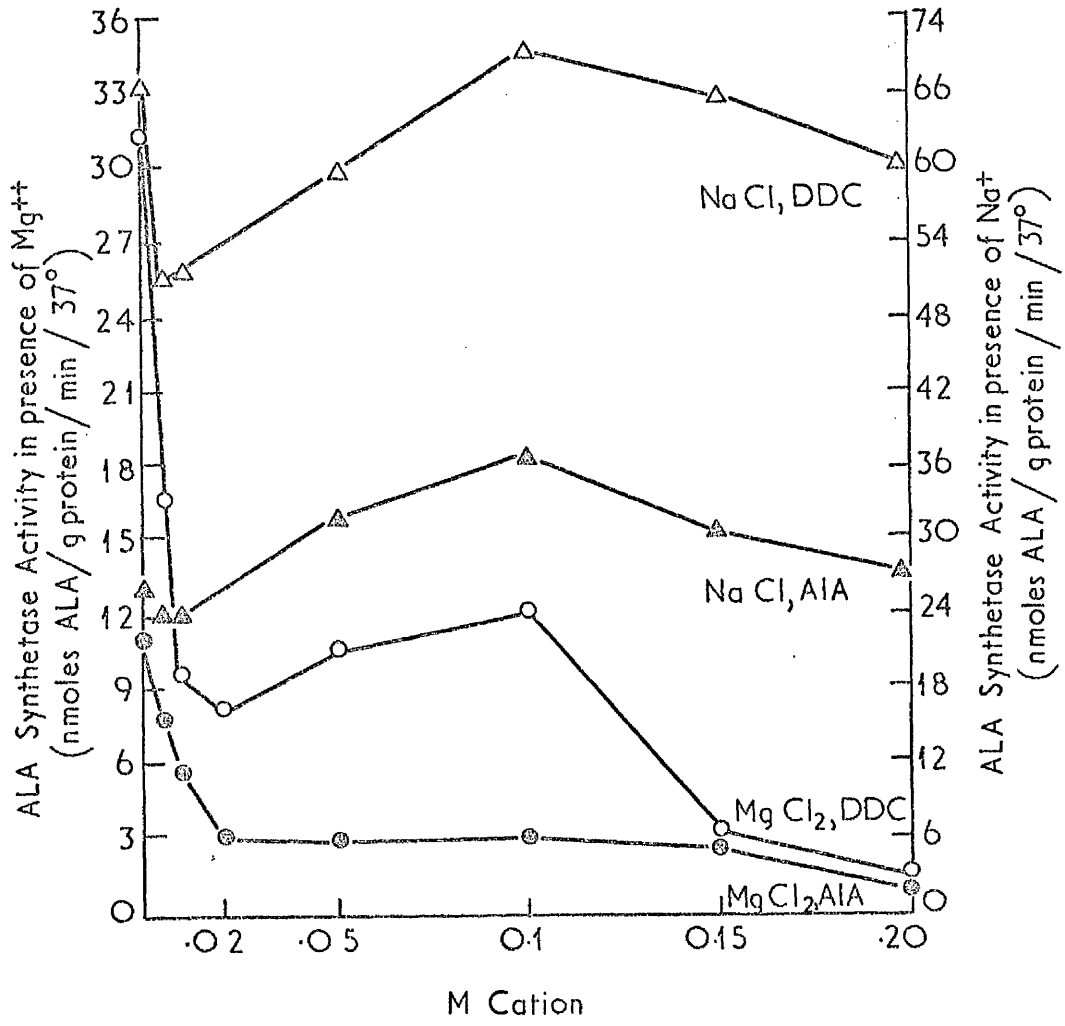
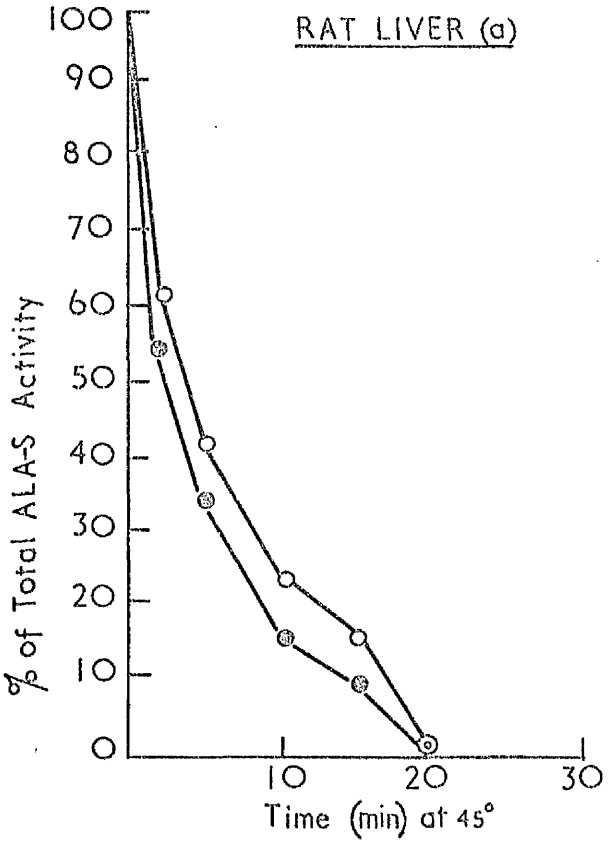
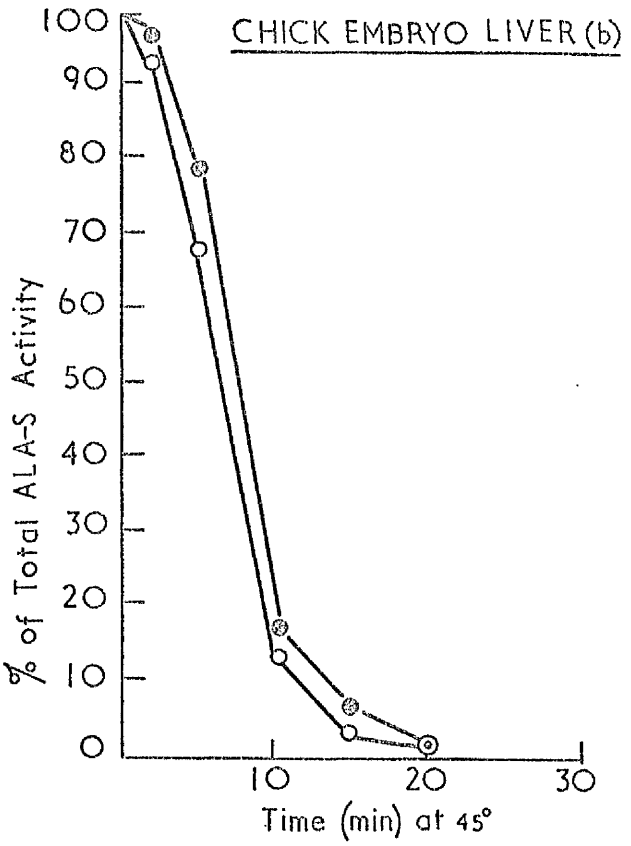


Fig. 11. Effect of cations on AIA- and DDC-induced δ-aminolaevulinic acid synthetase (ALA-S) activity from chick embryo liver. Assays of ALA-S were performed by the radiochemical assay as described in the MATERIALS AND METHODS section with the exception that the cation concentration varied as indicated. AIA-treated liver homogenate, ●——● Mg^{++}, ▲——▲ Na^{+}; DDC-treated liver homogenate, ○——○ Mg^{++}, △——△ Na^{+}. 14 day-old chick embryo received 10 mg AIA or 4 mg DDC and were sacrificed 9 hours later. Experiments involving Mg^{++} were performed separately from the Na^{+} experiments.

Fig. 12. Heat stability of AIA- and DDC-induced δ-aminolaevulinic acid synthetase (ALA-S) in crude homogenates of rat and chick embryo liver and cultured chick embryo liver cells. 30% liver homogenate containing 75 mM Tris-HCl and 10 mM EDTA, pH 7.2, (80 µl total volume) were incubated at 45°C in a shaking water bath. 10 µl aliquots were removed at the times indicated and the ALA-S activity determined by the radiochemical assay procedure as described in MATERIALS AND METHODS. A 250 g fasted rat and a 14 day-old chick embryo were rendered porphyric by an injection of AIA (450 mg per kg body weight or 10 mg per egg, respectively) or DDC (200 mg DDC per kg body weight or 4 mg per egg, respectively) for 9 hours. Cultured liver cells from 14 day-old chick embryos were incubated with 200 µg AIA/ml Eagles or 100 µg DDC/ml Eagles for 22 hours at 37°C. Experimental results are expressed as percentage of ALA-S activity at time 0 at 45°C (o——o AIA-induced ALA-S; @——@ DDC-induced ALA-S).


RAT LIVER (a)
% of Total ALA-S Activity
100
90
80
70
60
50
40
30
20
10
0
10
20
30
Time (min) at 45°


CHICK EMBRYO LIVER (b)
% of Total ALA-S Activity
100
90
80
70
60
50
40
30
20
10
0
10
20
30
Time (min) at 45°

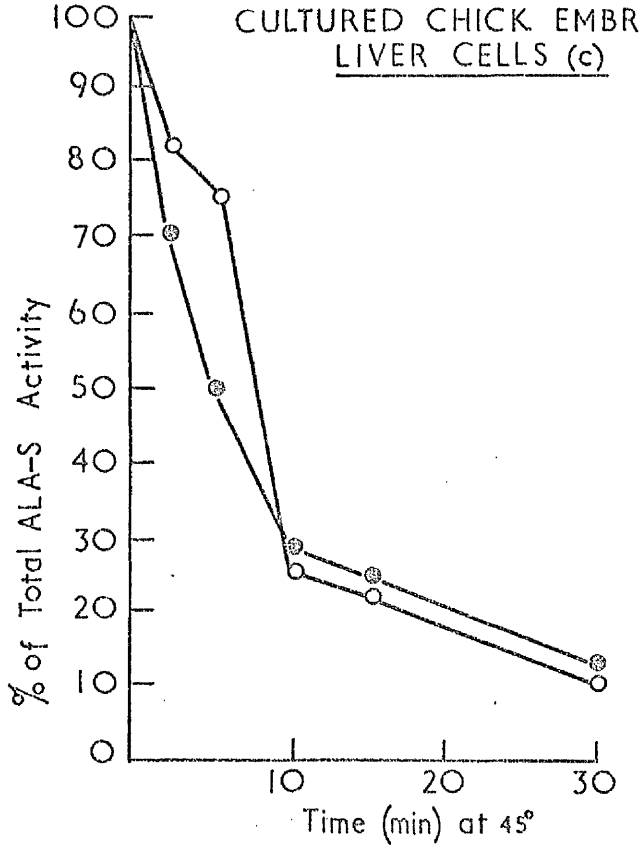
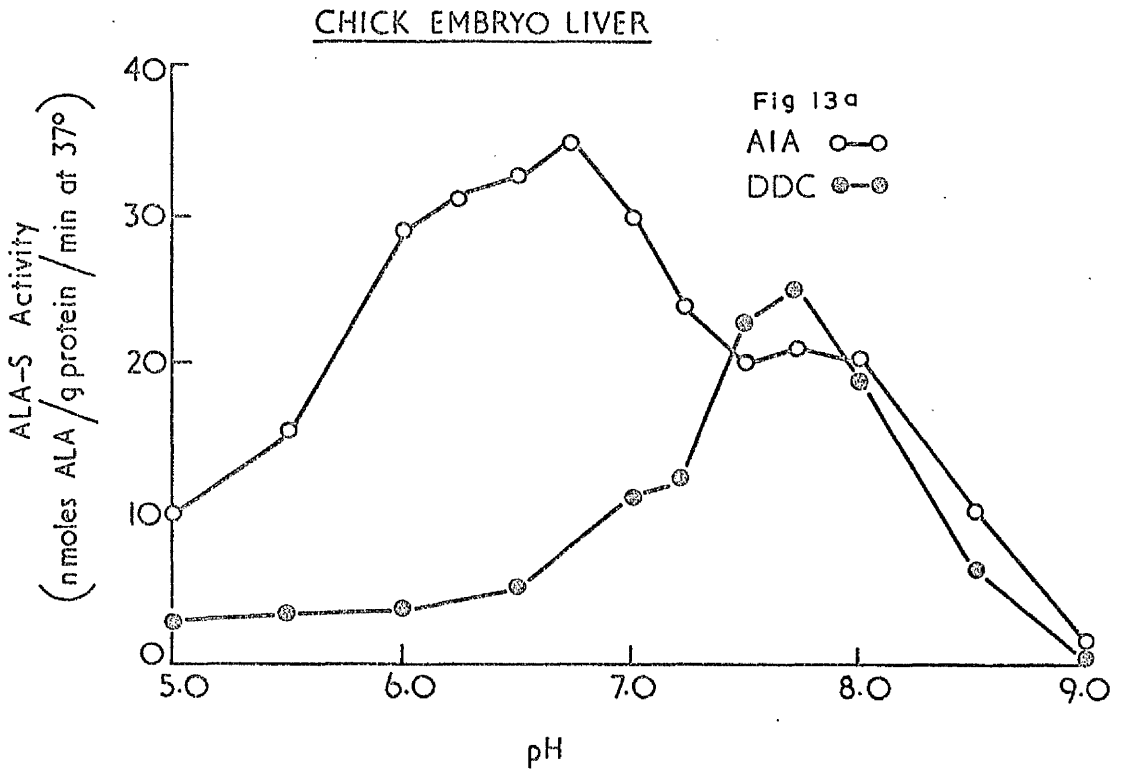
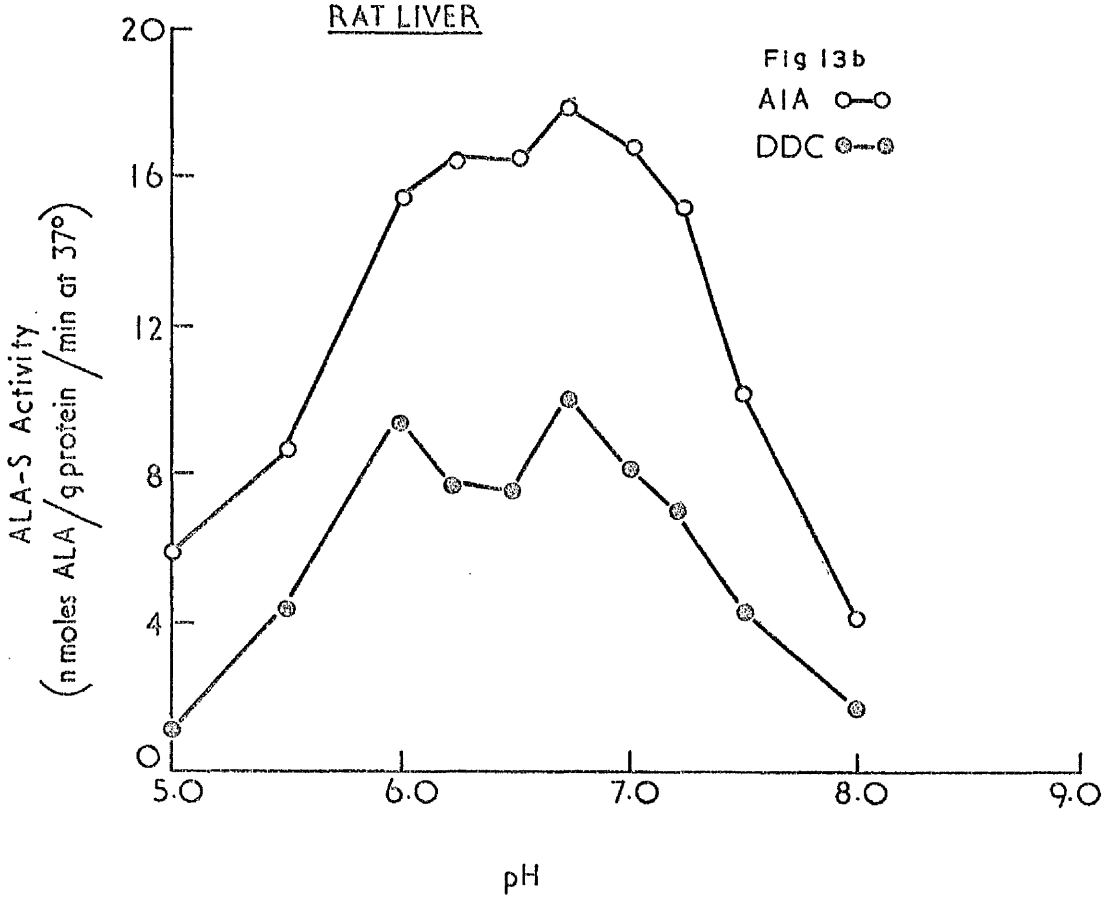

CULTURED CHICK EMBRYO
LIVER CELLS (c)
% of Total ALA-S Activity
100
90
80
70
60
50
40
30
20
10
0
10
20
30
Time (min) at 45°

Fig. 13. Comparison of pH optima for the AIA- and DDC-induced ALA-S activity in crude liver homogenate from rat and chick embryo liver. ALA-S activity was induced in rat liver by AIA (400 mg/kg) DDC (200 mg/kg) and in chick embryo liver by AIA (10 mg/egg) or DDC (4 mg/egg). In general, the liver was homogenized in an equal volume of normal saline. This homogenate was further diluted with an equal volume of 300 mM Tris-HCl containing 40 mM EDTA (pH was varied as indicated). 100 µl of this homogenate was transferred to a polyethylene microfuge tube (46 mm x 4.5 mm) containing 100 µl of 200 mM glycine in 100 mM sodium citrate (pH varied as indicated). The ALA produced during the one hour incubation at 37°C was determined by the colourimetric assay procedure described in MATERIALS AND METHODS.

(SEE pp 136-137 FOR MORE DETAIL OF Fig 13 a,b)


CHICK EMBRYO LIVER
Fig 13a
AIA
DDC
ALA-S Activity
(n moles ALA/g protein/min at 37°)
40
30
20
10
0
5.0
6.0
7.0
8.0
9.0
pH


RAT LIVER
Fig 13b
AIA
DDC
ALA-S Activity
(n moles ALA/g protein/min at 37°)
20
16
12
8
4
0
5.0
6.0
7.0
8.0
9.0
pH

3.0-fold greater than control levels) of ALA-S in unfractionated liver homogenates one hour following a single injection of AIA (400 mg per kg). Optimal enzyme activity, approximately 5.0- to 6.0-fold above control values, occurred between 8 to 16 hours after this single administration of AIA and the enzyme activity returned to control levels within 48 hours.

Further studies of the induction process of ALA-S in rat liver mitochondria have shown that sequential doses of AIA give increasing levels of enzyme activity (Narisawa & Kikuchi, 1966; Hayashi et al., 1969). The enzyme level increased immediately after the first dose of AIA and reached maximum activity (3.0- to 4.0-fold above control values) within 6 hours. The second administration of this drug, 12 hours later, gave rise to another rapid increase of enzyme activity. This second induction phase took place earlier as larger doses of the drug (up to 250 mg per kg) were administered to the animals. It was also reported that as the number of doses of AIA administered to rats increased, each 12 hours apart, so did the ALA-S activity in the unfractionated liver homogenate (Narisawa & Kikuchi, 1966), in rat liver mitochondria (Hayashi et al., 1968) and in the soluble and mitochondrial fraction of rat liver (Hayashi et al., 1969).

A comparison between the ALA-S activity in rat liver mitochondrial and soluble fractions established that the level of enzyme activity changed in close parallel and increased biphasically after AIA administration (Hayashi et al., 1969; Beattie & Stuchell, 1970). In the early stages of induction after the first dose of AIA, maximum enzyme activity was reached within 4 hours and the majority of ALA-S activity was located in the mitochondrial fraction. However, after

another dose of AIA, the enzyme activity was equally distributed between the two isolated tissue fractions.

3. 1. Time Course of the AIA-Induced Increase of ALA-S Activity in Unfractionated Rat Liver.

The data presented by Bock et al., (1971) showed that in AIA-treated rats enzyme induction is reduced in fed as compared with starved animals. This has been confirmed in this laboratory. In addition, my results are in general agreement with those of Marver et al., (1966a) and Hayashi et al., (1969), who reported the dose response of hepatic ALA-S activity following a single injection of AIA and showed that maximum levels are attained with 300 to 450 mg per kg body weight. Therefore, 450 mg AIA per kg was the dosage used in fasted animals that received only a single injection. If 2 or more drug administrations were made, then it became necessary to lower the AIA level to 400 mg per kg otherwise the fatality rate was high, the animals generally became unconscious after the third dose of AIA, and also appeared to be suffering while in their induced state of porphyria.

Studies on the AIA-induced increase in levels of ALA-S in rat liver demonstrated the occurrence of distinct phases which commenced after each AIA treatment (Fig. 14). Fasted rats received intraperitoneal injection of 400 mg AIA per kg body weight at 12 and 24 hour intervals for different periods of time. As shown, the maximum enzyme activity was obtained about 3 hours following the first AIA dose, after which time the activity decreased slightly but was maintained at a fairly elevated

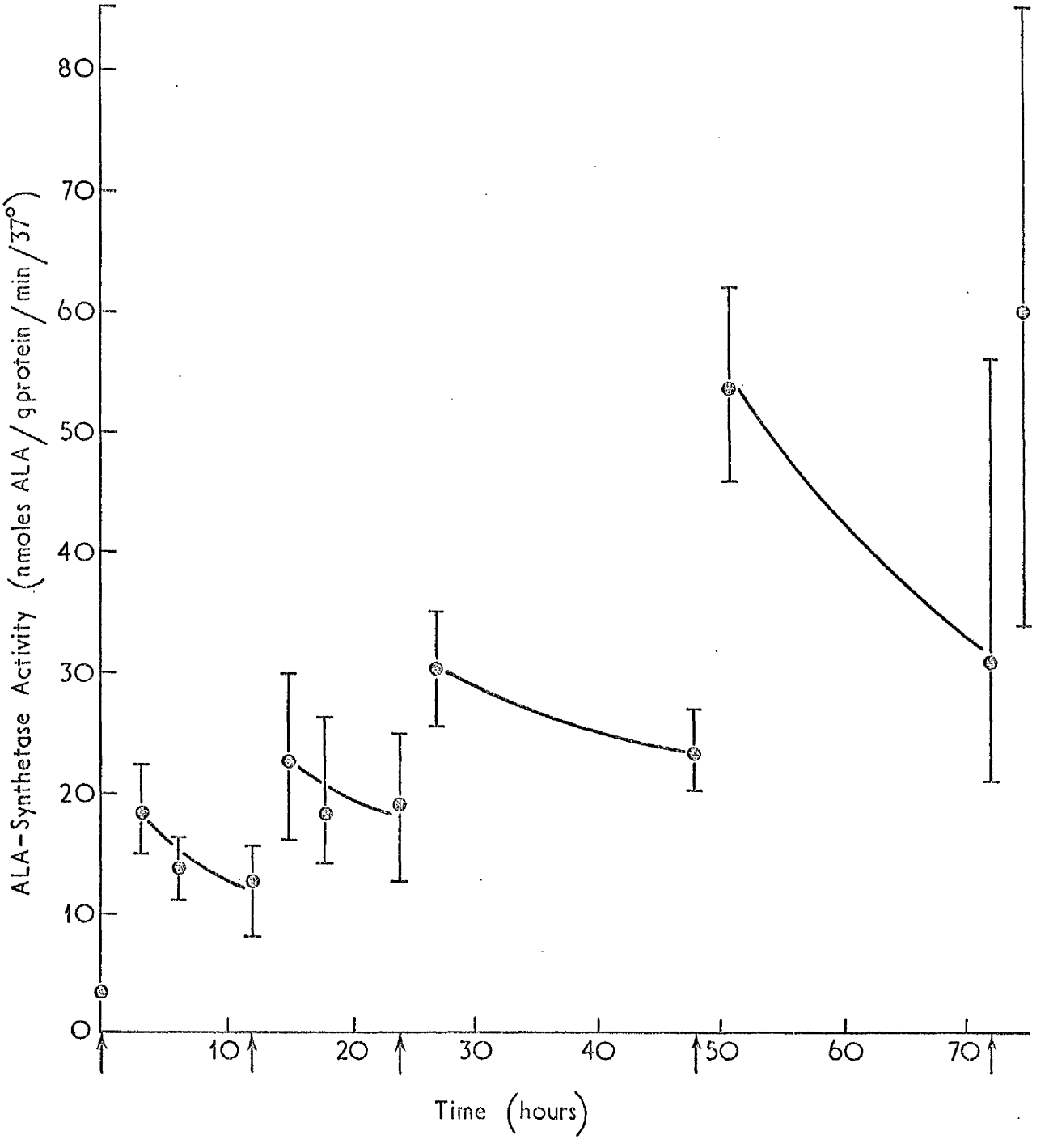


Fig. 14. Time courses of AIA-induced increase of ALA-S activity in unfractionated rat liver homogenate. AIA (40 mg per 100 g body weight) was injected at the times indicated by the arrows. Each point represents one experimental animal. The colourimetric assay method was used to measure the ALA-S activity.

level above control values. A second injection of AIA at 12 hours after the first resulted in a second induction phase of ALA-S. This is in good agreement with the data of Hayashi et al., (1968), Hayashi et al., (1969) and Beattie & Stuchell (1970).

Following each administration of AIA at 0, 12, 24, 48, and 72 hours, there was always a sharp increase in activity with maximum levels reached at approximately 3 hours after each dose (Fig. 14). However, the level of AIA-induced ALA-S in the experimental animals varied drastically from one animal to the next, and this variation increased with the number of drug administrations.

Several reports have indicated that in rat liver the half-lives of mRNA for ALA-S and the enzyme itself may be about one hour, (Tschudy et al., 1965c; Marver et al., 1966a; Matsuoka et al., 1968; Hayashi et al., 1969) and about 5 and 3 hours in chick embryo liver cells in culture, respectively (Sassa & Granick, 1970). It should be emphasized that these reports are based on the assumption that at the dosage used puromycin specifically blocks the ability of mRNA to stimulate protein synthesis and actinomycin D specifically inhibits the synthesis of mRNA. Three hours after the fourth administration of AIA (i.e. 75 hours of AIA exposure to the experimental animals, as shown in Fig. 14), the ALA-S activity in rat liver was approximately 10- to 12-fold above normal rat levels as opposed to a 3- to 4-fold increase observed 3 hours after the first injection of AIA. Therefore, taking into consideration that the life span of mRNA for ALA-S in rat liver has been reported to be about 3 hours (Tschudy et al., 1965c; Marver et al., 1966a; Matsuoka et al., 1968; Beattie & Stuchell, 1970), the higher

levels of induced enzyme activity after four doses of AIA as opposed to a single administration, and the sharp increase in AIA-induced ALA-S activity up to 3 hours after each drug treatment, I decided that synthesis of mRNA for ALA-S may be maximal 3 hours after an AIA injection since the peak of induced enzyme activity occurred at this time. Thus, RNA was isolated from rat livers 3 hours after either a single or multiple doses of AIA.

4. Role of Ribonucleic Acid in Experimental Porphyria.

Previous investigations have shown that AIA induction of ALA-S in rat liver is mediated by nucleic acids (Tschudy et al., 1965c; Marver et al., 1966a; Matsuoka et al., 1968; Stein et al., 1969). Furthermore RNA, isolated from porphyric rat livers by the Parish & Kirby (1966) procedure, was reported to stimulate porphyrin formation (Hickman et al., 1967, 1968) or ALA-S activity in embryonic chick liver cells in tissue culture. These latter investigators utilized rat livers for the source of mRNA for ALA-S and cultured chick embryo liver cells to test for mRNA translation of ALA-S for the following main reasons: (i) the kinetics of the AIA-induced increase of ALA-S activity in rat liver is well documented (Tschudy et al., 1965c; Marver et al., 1966a; Narisawa & Kikuchi, 1966; Matsuoka et al., 1968; Hayashi et al., 1969; Beattie & Stuchell, 1970; Stein et al., 1970), (ii) compounds which inhibit the synthesis or biological function of nucleic acids also inhibit the induction of ALA-S in liver (Granick, 1966; Marver et al., 1966a; Narisawa & Kikuchi, 1966), (iii) a large yield of RNA can be obtained due to the bulky size of rat liver,

(iv) evidence that exogenous RNA may be incorporated into chick embryo liver cells in tissue culture has been presented by Amos & co-workers (Amos & Kearns, 1963; Amos & Moore, 1963; Amos *et al.*, 1964), (v) in animals the regulation of haem biosynthesis appears primarily to be a control on the rate of biosynthesis of the enzyme, ALA-S, (vi) the advantage of studying cells growing in monolayers in culture is that they can be observed with the aid of a phase microscope and thus one may establish whether the effect of an added chemical or RNA is specific or is merely the result of cell injury, (vii) the use of chick embryo liver culture *in vitro* would permit the inference that the inducing chemical or RNA acts directly on the hepatic cells and not via other stimuli generated from other organs as may happen in experiments involving whole animals, (viii) the enzyme activity in cultured chick embryo liver cells is negligible, so any increase in ALA-S activity will be easily detected.

4. 1. General Procedure Used for Isolation and Identification of mRNA for ALA-S.

Rats, fasted after the last two doses of AIA (i.e. approximately 48 hours), were rendered porphyric by four intraperitoneal injections of AIA (400 mg per kg) in propylene glycol at 24 hour periods and killed 3 hours after the last dose (as suggested in RESULTS, 3. 1). Control rats received similar volumes of propylene glycol without AIA. At the time of death, the livers were quickly removed and placed in crushed ice. A small sample (0.1 - 0.2 g) of liver was removed and the ALA-S activity determined using the Dowdle *et al.*, (1967) colourimetric

assay method. RNA was isolated from the remaining liver using the specified procedure.

14- to 18 day-old chick embryo livers were cultured using 10 cm petri dishes (2 dishes per assay) and allowed 18 hours at 37°C to form complete monolayers. The old medium was discarded and the cells were washed three times with warm (37°C) Eagles to remove any calf serum that may contain RNase. Eagle's plus the RNA to be tested was added to 2 dishes and incubated at 37°C for 3.5 hours. Cells were harvested from the 2 dishes and assayed in duplicate for ALA-S activity.

4. 2. Increase of ALA-S Activity in Cultured Chick Embryo Liver Cells by Rat Liver Isolated by Different Methods.

Various cellular RNAs were isolated from porphyric and normal rat livers and were tested for ability to increase ALA-S activity in cultured embryonic chick liver cells.

4. 2. 1. Ribosomal RNA (Parish & Kirby, 1966).

Skea et al., (1970) reported that porphyric rat liver RNA (P-RNA), isolated by the Parish & Kirby method (1966), caused a 2- to 3-fold increase in ALA-S activity in embryonic chick liver cells in culture at a concentration of 0.2 mg RNA per ml Eagles medium. This effect was not observed for cultures incubated with normal rat liver RNA (N-RNA). Moreover, incubation of RNase with this P-RNA prior to its addition to the cultured cells, abolished the induction effect. Fractionation of this P-RNA on Sephadex G-100 by the method of Kay &

Cooper (1969) indicated that the biologically active RNA was of low molecular weight. These studies used the colourimetric assay procedure to measure ALA-S activity.

Isolation of P-RNA in this laboratory by the Parish & Kirby (1966) procedure from the livers of porphyric rats (as described in RESULTS 4. 1) failed to increase ALA-S activity in cultured chick embryo liver cells above control levels. The enzyme activity was determined using the colourimetric assay method of Dowdle et al., (1967). An active P-RNA which Skea et al., (1970) had isolated and was known to cause induction of ALA-S in cultured cells was tested on 7.5% sodium dodecyl sulphate (SDS) polyacrylamide gels for the presence of low molecular weight RNA. When the gels were scanned at A_{260}, only 4S was present in their P-RNA preparation. However, all of my P-RNA preparations, which were isolated by the same procedure, did not contain 4S as indicated by its absence on the 7.5% SDS polyacrylamide gels. Although isolated by the same method (as described by Parish & Kirby, 1966) the difference in my rRNA preparation and that of Skea et al., may be due to one critical step in the RNA preparative procedure. At one stage during the Parish & Kirby (1966) preparation of ribosomal RNA (rRNA) and rapidly labelled RNA from rat liver, the ethanol precipitated total cellular nucleic acids (DNA and RNA) were dissolved in 0.1 M sodium acetate. In order to separate DNA and transfer RNA from the desired rRNA, solid NaCl is added to give a 3 M solution and left at $-5^{\circ}C$ for about 15 hours. This allowed only the rRNA to precipitate leaving the DNA and transfer RNA dissolved in 0.1 M sodium acetate. However, Skea et al., (1970) did not remove all of the transfer RNA whereas I had

completely eliminated all traces of it. This suggested that the active RNA was possibly of low molecular weight, that is, below 18S rRNA.

4. 2. 2. Polyribosomal RNA (Henshaw, 1968).

Rats were rendered porphyric by AIA injections (described in RESULTS, 4. 1) and polyribosomal RNA was isolated from their livers according to the method of Henshaw (1968). Primary cultures of chick embryo livers were incubated with Eagles medium containing the RNA to be tested for its inducibility of ALA-S. As shown in Table 4, only the cultured cells exposed to P-RNA had a 2-fold increase in ALA-S activity above the values obtained for cells incubated with N-RNA or without any RNA. In addition, the ability of P-RNA to increase ALA-S activity in chick embryo liver cells in culture was abolished by previous incubation with purified pancreatic ribonuclease (RNase) (proportion 10 mg RNA + 0.5 mg RNase at 37°C for 12 hours, pH 7.5). This induction effect was repeatable from one preparation to another of porphyric polyribosomal RNA.

4. 2. 3. Other RNA Species.

Other hepatic cellular RNAs, such as nuclear (modified Henshaw method, 1968), transfer RNA (Henshaw method, 1968), whole cytoplasmic (modified Henshaw method, 1968), ribosomal (Parish & Kirby, 1966), and polyribosomal (Henshaw method, 1968) were isolated from pooled liver of 4 rats that had been rendered porphyric by AIA treatment (as described in RESULTS 4. 1) and from livers of normal rats. The AIA-induced increase of ALA-S activity in the rat livers ranged from 20.3 to

Table 4

Increase of ALA-S Activity in Cultured Chick Embryo Liver Cells After Incubation with Polyribosomal RNA

ALA-S activity in rat liver used as source of RNA (nmoles ALA/gprotein/min)	Source of RNA	ALA-S activity in cultured cells after incubation with or without RNA (nmoles ALA/gprotein/min)		
		I*	II*	III*
-	Control (no RNA)	7.4 7.0	10.2 11.1	7.9 -
3.2 6.6 5.7	Polyribosomal RNA from normal rats	8.6 7.6	12.3 -	9.0 -
70.5 88.8 137.0	Polyribosomal RNA from AIA injected rats	18.6 20.6	20.4 -	17.1 -
70.5 88.8 137.0	Polyribosomal RNA from AIA injected rats incubated with RNase	- -	7.3 -	8.8 7.9

I*, II*, III* are three separate experiments.

Polyribosomal RNA was isolated from rat livers by the method described by Henshaw (1968).

The colourimetric assay was used to measure the ALA-S activity.

43.6 nmoles ALA per g protein per minute at 37°C while levels in control rats were 1.2 to 4.3 nmoles ALA per g protein per minute at 37°C. RNA, both P-RNA and N-RNA at 0.2 mg per ml of Eagles, was incubated with the primary cultures at 37°C for 3.5 hours after which the enzyme activity was determined by the colourimetric assay method of Dowdle et al., (1967). As shown in Table 5 the results in column (a) and (b) indicated that the active RNA exists solely in the polyribosomal RNA preparation.

4. 2. 4. ALA-S Activity in Cultured Cells After Incubation with 28S, 18S, and Below 18S Ribosomal RNA Fractions From Biologically Active Polyribosomal RNA.

Hickman et al., (1967) reported that the ribosomal RNA, isolated from the livers of AIA-treated rats by the Parish & Kirby (1966) technique, contained a rapidly labelled component that sedimented slightly above the 18S ribosomal RNA in a linear sucrose gradient. In this laboratory, polyribosomal porphyric RNA (P-RNA), which was previously tested and known to induce ALA-S in cultured cells, was fractionated into 28S, 18S, and below 18S ribosomal RNA fractions using sucrose density centrifugation (see MATERIALS AND METHODS for detail). As illustrated in Table 6, columns 1 and 2, the fraction containing 18S rRNA induced the greatest increase of ALA-S activity in the cultured cells, although some increased activity was observed for the fraction containing the 28S rRNA. Biologically active polyribosomal RNA, which was fractionated and used in experiments 1 and 2 in Table 6, was again fractionated by sucrose density centrifugation. However, the 18S rRNA peak was divided into a left and right-hand fractions. When all fractions, that

Table 5

ALA-S Activity in Embryonic Chick Liver Cells in Culture Incubated with Different Hepatic Cellular RNAs

Treatment of cells with Eagles medium plus:	ALA-S activity in cultured cells after incubation with RNA	
	(a)	(b)
Control (no RNA)	3.0	3.5
N-Ribosomal RNA	2.9	4.0
P-Ribosomal RNA	5.4	3.9
N-Cytoplasmic RNA	5.5	5.3
P-Cytoplasmic RNA	2.9	3.3
N-Transfer RNA	4.5	5.0
P-Transfer RNA	2.2	2.6
N-Nuclear RNA	2.2	1.9
P-Nuclear RNA	2.1	1.8
N-Polyribosomal RNA	2.9	3.8
P-Polyribosomal RNA	8.2	9.1

(a) and (b) are separate incubations using the same RNA preparation. ALA-S activity, measured by the colourimetric assay, is expressed in nmoles ALA/gprotein/min at 37°C.

is 28S, unfractionated 18S, the left- and right-hand fractions of 18S rRNA, were tested in cultured chick embryo liver cells for inducibility of ALA-S, there was no increase in enzyme activity for any fraction except the unfractionated 18S rRNA (Table 6).

4. 2. 5. Comparison of Colourimetric and Radiochemical Assays for Measurement of RNA Mediated Increase of ALA-S Activity in Cultured Chick Embryo Liver Cells.

In the previous section (4. 2. 4), the experiments used the colourimetric assay to measure the ALA-S activity in cultured chick embryo liver cells after incubation with P-RNA and N-RNA of various molecular sizes. Preparation of these RNA fractions, as well as the amount of cultured liver tissue necessary for each experiment, proved very laborious. At least 20 250 g rats (10 rats for normal polyribosomal RNA and 10 for porphyric polyribosomal RNA) were required to provide enough crude polyribosomal RNA in order that after fractionation by sucrose density centrifugation, at least 8 mg of either 28S, 18S, or below 18S rRNA fractions were obtained. This amount of each RNA fraction enabled the experiments to be repeated using three different sets of cultured liver cells. To provide enough of this fractionated RNA for testing in cultured cells, two centrifugations of the crude polyribosomal RNA, each lasting 32 hours in length, were required. Therefore, the radiochemical assay method, described previously, which would require μg quantities of RNA, measure ALA production directly and use 1 x 5 cm petri dish of cultured liver tissue per assay as opposed to 2 x 10 cm dishes, was employed.

Table 6

ALA-S Activity in Cultured Cells after Incubation with 28S, 18S, and below 18S Ribosomal RNA Fractions

Treatment of cells with Eagles medium plus:	ALA-S activity in cells after incubation with RNA		
	(1)	(2)	(3)
Control (no RNA)	2.1	1.1	1.3
N 28S RNA	1.6	1.1	-
P 28S RNA	3.7	4.4	3.0
N 18S RNA	0.8	0.9	-
P 18S RNA	5.9	6.8	6.8
P LHS 18S RNA	-	-	3.2
P RHS 18S RNA	-	-	2.2
N below 18S RNA	1.3	2.4	-
P below 18S RNA	1.6	4.2	2.6
Unfractionated N-RNA	2.3	1.9	-
Unfractionated P-RNA	8.5	8.2	8.4

(1), (2), & (3) are three separate experiments.

ALA-S activity, as measured by the colourimetric assay method, is expressed in nmoles ALA/g protein/min at 37°C.

Orcinol estimations indicated approximately 0.2 mg RNA per ml of Eagles medium except for the unfractionated N-RNA and P-RNA which were 0.36 and 0.29 mg RNA per ml Eagles, respectively.

Polyribosomal RNA was isolated by the method described by Henshaw (1968) from normal (N-RNA) and porphyric (P-RNA) rat livers. RNA (0.2 mg per ml Eagles medium, 5 ml per dish of cultured cells, 2 dishes per assay) was dissolved in Eagles medium and incubated with the cultured liver cells for 3.5 hours at 37°C. The colourimetric and radiochemical assay methods were performed on the same homogenate. As shown in Table 7, only the P-RNA mediated an increase of ALA-S activity over control levels as detected by the colourimetric assay. In contrast, and surprisingly, the results from the radiochemical assay indicated no increase of enzyme activity for cultured cells incubated with P-RNA (over control levels). Due to the outcome of this experiment the colourimetric assay was considered in detail. The actual absorbances from the test and trichloroacetic acid incubation mixtures (described in MATERIALS AND METHODS), obtained from the colourimetric measurement of ALA in homogenates of cultured liver cells incubated with P-RNA, ranged from 0.150 to 0.080 respectively, and for N-RNA, 0.120 to 0.080, respectively. In a personal communication from Skea et al., they indicated that differences in absorbances from the colourimetric assay of test and trichloroacetic acid incubation mixtures obtained were similar to the above values from this laboratory. Moreover, they stated that duplicate determinations by the colourimetric assay using the same homogenate (obtained from cultured liver tissue incubated with P-RNA) rendered indecisive results. For example, a duplicate determination gave absorbances of 0.150 and 0.130 for test incubation mixtures while the trichloroacetic acid values were 0.080 to 0.090. These ambiguities in the absorbance values made it very difficult to determine which ones were a true representation of ALA production.

Table 7

Comparison of Colourimetric and Radiochemical Assays for Measurement of Increased ALA-S Activity in Cultured Chick Embryo Liver Cells by Polyribosomal RNA

Incubation of cultured cells with Eagles medium plus:	ALA-S activity in cultured cells after incubation with RNA	
	Colourimetric assay	Radiochemical assay
Control (no RNA)	2.9	1.4
Normal polyribosomal RNA	2.8	1.6
Porphyric polyribosomal RNA	6.5	1.7
Allylisopropylacetamide (200 µg/ml)	8.4	9.4

Polyribosomal RNA, isolated from the livers of normal and porphyric (4 doses of AIA at 400 mg per kg) by the method described by Henshaw (1968), was incubated with cultured chick embryo liver cells for 3.5 hours at 37°C. ALA-S activity was determined by the colourimetric method (Dowdle et al., 1967) and my radiochemical assay. Orcinol estimations indicated approximately 0.2 mg RNA per ml Eagles. ALA-S activity is expressed as nmoles ALA/gprotein/min at 37°C.

Previously, I reported in the RESULTS, section 4. 2. 4, that only 18S rRNA fraction, as separated from porphyric polyribosomal RNA by sucrose density centrifugation, increased ALA-S activity in cultured cells above control levels. However, in those experiments, the colourimetric assay method was employed to measure the enzyme activity. Since then it has been observed (Table 7, and as mentioned in the above paragraph) that the colourimetric assay gave inconclusive results which were not in agreement with those of the radiochemical assay, and it was decided to test these same RNA fractions for inducibility in cultured chick embryo liver cells and measure the enzyme activity using the radiochemical assay method. As illustrated in Table 8, none of the RNA fractions from P-RNA increased the enzyme activity above values obtained for cells incubated with the N-RNA fractions or without any RNA (control). There were no differences in the number of dpm of $[2\text{-}^{14}C]$ glycine incorporated into the incubation generated $[^{14}C]$ ALA for control, N-RNA, and P-RNA treated cells. In addition, AIA increased ALA-S activity which indicated that induction could occur in these cultured cells.

Because of the discrepancies in absorbances obtained from the colourimetric assay determination in this laboratory, which are in agreement with those of Skea et al., (1970), and due to the failure by the radiochemical assay to detect any RNA mediated increase of enzyme activity, I considered that the previous reports by Hickman et al., (1967, 1968) and Skea et al., (1970), which indicated RNA induced ALA-S activity in cultured liver cells, to be insignificant and invalid.

Table 8

Measurement of ALA-S Activity in Cultured Chick Embryo Liver Cells Incubated with RNA of Various Molecular Sizes

Incubation of cells with Eagles medium plus:	ALA-S activity in cells after incubation with RNA
Control (no RNA)	2.7
N 28S RNA	2.9
P 28S RNA	1.4
N 18S RNA	1.5
P 18S RNA	2.9
N below 18S RNA	1.7
P below 18S RNA	2.3
Unfractionated N RNA	2.7
Unfractionated P RNA	2.5
AIA (200 μg/ml)	8.6

ALA-S activity, as measured by the radiochemical assay method, is expressed in nmoles ALA/g protein/min at 37°C

The polyanion, dextran sulphate, and the cationic diethylaminoethyl dextran (DEAE dextran) have been used in whole animal and cultured cell experiments to prevent degradation of added RNA by extracellular and endogenous ribonucleases (Dianziani et al., 1970; Deckers & Pilch, 1971). Up to the present time, it has been assumed that the bond established between RNA and DEAE dextran occurs along the nucleic acid phosphate backbone and the bases are left free. The result is that when this complex enters the susceptible cells the free bases are able to perform their messenger function (Maes et al., 1967). In this laboratory, addition of either DEAE dextran or dextran sulphate to porphyric polyribosomal RNA just prior to incubation with cultured chick embryo liver cells did not enhance ALA-S activity above control levels, as measured by the radiochemical assay method. The porphyric polyribosomal RNA used in this experiment is from the same RNA preparation which caused an increase of ALA-S activity which was measurable by the colourimetric assay method but not detected by the radiochemical method (RESULTS 4. 2. 5 and Table 7). This experiment involving the use of inhibitors of extracellular and endogenous RNase suggested the two following reasons for no RNA mediated increase in enzyme activity: (i) the translational machinery of the intact chick embryo liver cell in culture is incapable of reading the mRNA for ALA-S, that is, the code is not universal between mammalian and non-mammalian tissues, and (ii) AIA may induce ALA-S at the translational level, which would result in no increased synthesis of the mRNA.

4. 2. 6. Isolation of the Rat Liver mRNA for ALA-S by Binding to Millipore Filters or Oligodeoxythymidylate-Cellulose.

A distinctive characteristic of the mRNAs from eukaryotic or viral origin is the polyadenylic acid segment (poly(A)), approximately 150 to 200 nucleotides long (Darnell et al., 1971; Edmonds et al., 1971; Lee et al., 1971; Adesnik et al., 1972) with the messenger for histones being the only reported exception (Adesnik & Darnell, 1972). RNAs containing this sequence have been separated from total cellular RNAs by their selective adsorption at high ionic strength to millipore filters (nitrocellulose membrane filters) or by binding to polyuridylate (poly (U))or oligodeoxythymidylate (oligo d(T)) covalently linked to cellulose. Isolation of the mRNA for ovalbumin and avidin (Means et al., 1972; Rosenfeld et al., 1972) employed the millipore filter method. Oligo d(T)-cellulose has been used for purification of the mRNA for globin (Aviv & Leder, 1972), mouse light chains (Swan et al., 1972), and mouse myeloma heavy and light immunoglobin chains (Stevens & Williamson, 1972). Chromatography on poly (U)-cellulose columns was successfully employed for the isolation and purification of the globin mRNA (Morrison et al., 1972), while Schutz et al., (1972) have demonstrated that at high ionic strength cellulose (nucleotide free, Sigmacell type 38) selectively retain mRNA for globin and ovalbumin.

Since the mRNAs described above have been reported to contain poly(A) rich regions it seemed likely that this feature may be true for the mRNA for ALA-S.

4. 2. 7. ALA-S Activity in Cultured Chick Embryo Liver Cells After Incubation with RNA Isolated from Total Cellular RNAs by Selective Binding to Millipore Filters.

Henshaw's method (1968) for isolation of polyribosomal RNA involved a phenol extraction of the polyribosomes in the presence of Tris-HCl, pH 7.6 at 4°C. However, under these conditions Brawerman et al., (1972) found that, during phenol extraction of RNA from polyribosomes isolated from mouse carcinoma 180 Ascites cells, nearly all the rRNA is removed in the aqueous phase, but the majority of rapidly labelled non-rRNA remained behind in the nonaqueous residue (interphase). These investigators discovered that the poly(A)-containing RNA molecule bound to denatured proteins in the nonaqueous phase under conditions of phenol extraction (4°C) in the presence of Tris-HCl, pH 7.6. This unusual behaviour of these RNA molecules rich in poly(A) segments was thought to be the result of the effects of the ionic strength and pH of the water-phenol mixtures. Re-extraction of this nonaqueous phase with phenol and Tris buffer, pH 9.0 at 4°C (Brawerman et al., 1972) or sodium acetate buffer, pH 5.1 at 60°C (Edmonds & Caramella, 1969) led to the appearance of the poly(A)-containing molecules almost free of rRNA in the aqueous phase.

In this laboratory RNA was isolated from polyribosomes, that were prepared by the Henshaw method (1968) and obtained from porphyric and normal rat livers, by the methods described by Burdon et al., (1969) or Brawerman et al., (1972). RNA was applied to millipore filters (Millipore Filter Corp., 25 mm (0.22 μ)) using the method described by Lee et al., (1971) or Rosenfeld et al., (1972).

Unfractionated polyribosomal RNA, millipore filter bound and eluted RNA, from N-RNA and P-RNA preparations were incubated with embryonic chick liver cells in culture for 3.5 hours at 37°C. The ALA-S activity, as measured by the sensitive radiochemical assay method, was not increased above control levels, that is, the enzyme activity obtained for cells incubated without RNA. This experimental design was repeated several times using polyribosomal RNA from individual RNA preparations but none of these RNA fractions (either unfractionated, millipore filter bound, or eluted RNA) increased the ALA-S activity in cultured cells above control levels.

4. 2. 8. Isolation of Rat Liver mRNA for ALA-S by Chromatography on Oligodeoxythymidylate-Cellulose.

As has been mentioned in RESULTS 5. 1, oligo d(T), covalently linked to cellulose, has been used to bind poly(A)-rich RNA and thus isolate and purify the mRNA of globin (Aviv & Leder, 1972) and myeloma light chain (Swan et al., 1972). There are several advantages of this method over the millipore filter technique. The oligo d(T) is chemically stable and can be repeatedly used after treatment with alkali. Millipore filters can be used only once. These columns have a relatively high capacity and can be used to bind several mg of poly(A)-mRNA from several mg of crude polysomal RNA as compared to μg quantities adsorbed to the millipore filters. The chromatography on oligo d(T) can be done at room temperature when suitable precautions to avoid nuclease contamination are taken.

Total cellular RNA, isolated by the method described by Burdon et al., (1969) from porphyric (P-RNA) and normal (N-RNA) rat livers, was applied to an oligo d(T)-cellulose column (purchased from Collaborative Research Inc.). Over 96% of the total RNA applied was eluted with application in presence of a high salt (KCl) concentration. When the column was washed with a low salt buffer (no KCl) a small amount of RNA (less than 4%) was released from the column. However, incubation of unfractionated RNA, RNA eluted in the high salt and low salt (in Eagles) with cultured chick embryo liver cells did not increase ALA-S activity (determined by the radiochemical assay method) above control levels (Table 9).

Table 9

ALA-S Activity in Cultured Chick Embryo Liver Cells After Incubation with RNA Fractions Obtained by Chromatography on Oligodeoxythymidylate-Cellulose

Incubation of cultured cells with Eagles medium plus:	ALA-S activity in cultured cells after incubation with RNA
Control (no RNA)	2.3
RNA eluted from column by high salt buffer	2.0
RNA bound to column and eluted by low salt buffer	3.3
Allylisopropylacetamide (200 μg/ml)	9.0
Unfractionated RNA	2.0

ALA-S activity, as measured by the radiochemical assay, is expressed in nmoles ALA/g protein/min at 37°C.
The RNA used in this experiment was isolated from livers of rats rendered porphyric by 4 doses of AIA (400 mg per kg). One 5 cm petrie dish of cultured cells was used per assay.

DISCUSSION

In animals, chick embryo liver *in vivo*, and in culture, the regulation of haem biosynthesis appears to be primarily a control on the rate of biosynthesis of the enzyme, ALA-S. The activity of this enzyme may be stimulated several fold above normal levels by such drugs as barbiturates, dihydrocollidines, and certain 5_{β}-H steroids. Since conflicting reports appear in the literature it was of interest to determine whether or not AIA (a barbiturate) and DDC (a dihydrocollidine) have similar or different modes of inductions of ALA-S. In particular, I explored the possibility that AIA-induced ALA-S activity was the result of increased synthesis of mRNA for this enzyme. Since ALA-S activity was found to be negligible in chick embryo liver cells in culture, this system provided a means for translation of possible mRNA from livers of AIA-treated rats. This assumed that the intact liver cell in culture could ingest the added macromolecules of RNA and that these molecules could function in the host cell for at least a few hours. Direct evidence that exogenous RNA from the liver of AIA-treated rats can be translated by a heterologous species such as the chick embryo liver cells in tissue culture has been presented by Hickman *et al.*, (1967, 1968) and Skea *et al.*, (1970). RNA, isolated from livers of porphyric rats was incubated with cultured chick embryo liver cells. Only the induced RNA, as compared with non-induced RNA, RNase-treated induced-RNA, and non-induced RNA preparations contaminated with AIA prior to purification, caused an increase in porphyrin formation (Hickman *et al.*, 1967, 1968) or ALA-S activity (Skea *et al.*, 1970). Using the same

experimental design as that employed by Skea et al., (1970), I was unable to obtain any increased ALA-S activity (as measured by the (colourimetric) assay method) in cultured chick embryo liver cells incubated with RNA isolated from livers of AIA-treated rats. Even polyribosomal RNA from porphyric rats and its fractionated components (28S, 18S, and low molecular weight RNA fractions) did not increase ALA-S activity (measured by the sensitive radiochemical assay method) in the cultured liver cells. Recent investigations have shown that the mRNAs for globin, avidin, ovalbumin, and the light and heavy chains of immunoglobins contain poly(A) segments which bind to millipore filters, poly (U)-, or oligo d(T)-cellulose at high ionic strength. Attempts were made to purify the mRNA for ALA-S from hepatic polyribosomal and total cellular RNAs of AIA-treated rats. The RNA fractions isolated by the two procedures, however, did not increase ALA-S activity in cultured cells above control levels. The positive results reported by Hickman et al., (1967, 1968) and Skea et al., (1970) were obtained in spite of (a) the presence of extracellular and endogenous RNase activity in cultured cells, (b) the short half-life of the mRNA for ALA-S, (c) the use of a colourimetric assay method (only by Skea et al., 1970) (an analytic procedure which is inadequate for accurate measurement of control and RNA mediated increases in ALA-S activity and is also limited by the small amounts of tissue which can be obtained in experiments involving cultured chick embryo liver cells), and (d) problems of permeability of the intact cell membrane by RNA.

It is possible, therefore, that this porphyri nogenic drug, AIA, does not increase or stimulate synthesis of the mRNA for ALA-S,

and/or the cultured chick embryo liver cells was not a sensitive enough system to detect changes in ALA-S activity due to added mRNA. Currently there are two conflicting theories. Sassa & Granick (1970) and subsequently Strand et al., (1972) have suggested that the control of the enzyme occurs at the translational level if AIA is used and at the transcriptional level for DDC. Yet, Strand et al., (1972), in the same report, failed to discuss the finding that both 2-mercapto-1-(2-(4-pyridyl)-ethyl)benzimadole (a purine analog inhibitor of RNA polymerase) and cordycepin (3'-deoxyadenosine) (an inhibitor of mRNA maturation) prevented AIA induction of ALA-S activity in primary cultures of avian hepatocytes. These observations would give support to the theory proposed by Tyrrell & Marks (1972), who suggested that AIA and DDC caused an accumulation of an induction-specific RNA (mRNA) which appeared to mediate ALA-S activity by translation into either this enzyme or into some other protein involved in ALA-S activation. It is possible to reconcile the results of Tyrrell & Marks (1972) with those of Sassa & Granick (1970) and Strand et al., (1972) in the following manner. DDC stimulates the mRNA for ALA-S by increasing the rate of its transcription. On the other hand, AIA may retard the degradation of the mRNA, or facilitate the mRNA transport through the nuclear membrane to the cytoplasm thus increasing the level of cytoplasmic mRNA. The results described in this thesis indicating the failure of various types of RNA isolated from livers of AIA-induced rats to increase ALA-S activity would however add some support to the original proposal by Sassa & Granick (1970), assuming that any mRNA for ALA-S added to cultured chick liver cells is capable of penetrating the cell membrane and can be translated by the heterologous system.

It was suggested by Skea et al., (1970) that the RNA gained entry by pinocytosis. Contamination with extracellular RNase contributed by the calf serum used in culture procedure and also by cell surfaces probably constitutes a major obstacle to the penetration of intact RNA molecules. In the experiments reported in this thesis involving incubation of normal and porphyric RNA with the cultured cells, the latter were washed 3 times with warm Eagles ($37^{\circ}C$) as a precautionary method to remove most of the calf serum and any dead cells, both of which act as a source of RNase activity. Even the addition of either DEAE-dextran or dextran sulphate to porphyric polyribosomal RNA just prior to incubation with culture cells did not enhance ALA-S activity as measured by the radiochemical assay. This does not appear to support the theory that mRNA for ALA-S accumulates in AIA-induced rat liver.

The translational efficiency of the added rat liver mRNA for ALA-S to the cultured intact liver may be very low if the mRNAs of rat (mammalian) liver are different from those of chick embryo (avian) liver. This consideration seems unlikely because mouse mRNA for globin is translated as well by a non-mammalian (duck) reticulocyte system as it is by the mammalian rabbit system which indicates no evidence for species - specific factors in the reticulocytes for the translation of globin mRNA (Lockard & Lingrel, 1972). On the other hand differences have been reported in translational efficiencies by a cell-free extract of its endogenous mRNA as opposed to added mRNA. The translation efficiency of mouse haemoglobin mRNA in rabbit and duck reticulocyte cell-free systems is lowered compared with the respective endogenous mRNA efficiency (Lockard & Lingrel, 1972). Clearly, this is of importance in assessing the validity of the findings reported here.

The reported differences in molecular sizes of cytosol ALA-S from AIA- and DDC-treated rat livers may be an indication of ALA-S existing in isozymic forms (see INTRODUCTION 3.4, & Table 3). Molecular weight values for the AIA-induced enzyme in the rat liver cytosol ranged from 150,000 to 600,000 as compared to the single observation of 178,000 for the DDC-induced enzyme. These molecular weight values should be considered with extreme caution since there are vast discrepancies between values of the same AIA-induced ALA-S when applied to Sephadex G 200 gel filtration (600,000) and sucrose density centrifugation (178,000) (Hayashi et al., 1970). One may estimate the size of RNA responsible for the cytosol AIA- and DDC-induced enzymes assuming the average weight of an amino acid and a nucleotide to be 120 and 330, respectively. From the molecular weight values obtained from either gel filtration or sucrose density centrifugation of AIA- and DDC-induced ALA-S, assuming a single protein chain and not subunits, the expected RNA size for the AIA-induced enzyme (molecular weight value of 200-fold purified enzyme is 150,000 as determined on sucrose density centrifugation by Scholnick et al., 1970) is approximately 1.2×10^6, while for the DDC-induced enzyme (molecular weight value of the 40-fold purified enzyme is 178,000 as determined by sucrose density centrifugation) is about 1.5×10^6. Both these theoretical molecular weight values of the mRNA for ALA-S are larger than the mammalian cell 18S rRNA (molecular weight of 0.7×10^6) while smaller than 28S rRNA (molecular weight of 1.8×10^6). If the mRNA for AIA-induced ALA-S is about the size of 28S rRNA then the observation by Skea et al., (1970) that the biologically active RNA was of low molecular weight size further casts doubt on the validity of their finding.

During the past decade investigation on the control and synthesis of other hepatic enzymes such as tyrosine aminotransferase, and serine dehydratase have been numerous. It has been demonstrated that serine dehydratase exists in two distinct isozymic forms, each of which is controlled by different hormones, such as cortisone and glucagon (Inoue et al., 1971; Iwasaki & Pitot, 1971; Iwasaki et al., 1973). Similarly, isolatable forms, arbitrarily designated I (found only in kidney, heart, and brain), II, III, IV (all three located in rat liver), of soluble tyrosine aminotransferase have been demonstrated to exist in both crude extracts as well as in the partially purified form (Iwasaki et al., 1973). In view of the possibility that those four enzymes were the result of mechanisms involved in enzyme induction, the four purified forms have been characterized such that forms II, III, IV have similar molecular weights, identical heat stability curves at 60°C, the same K_m and pH optimum (in the incubation medium). In contrast, form I was very heat labile even in the presence of substrate or cofactors, required a higher pH in the incubation mixture and had a significantly different K_m. It was of particular interest that one or more of these closely related multiple forms of soluble tyrosine aminotransferase may be regulated independently by various hormones. That is, administration of hydrocortisone to perfused rat liver resulted in a simultaneous and virtually equal induction of forms II, III, and IV but not I. Stimulation by adenosine-3'-5'-monophosphate (cyclic AMP) was limited to forms II and III as compared to an insulin increased level of form IV only. Experiments described in this thesis in the form of pH optimum and Mg^{++} cation requirements of ALA-S may serve to suggest that one or more multiple forms of ALA-S may be regulated independently by barbiturates as opposed to

dihydrocollidines. Hepatic ALA-S activity from AIA and DDC treated rats and chick embryo liver gave different pH optima. This suggested that those experiments of Tyrrell & Marks (1972) and Sassa & Granick (1970), which compared the mechanism of AIA- and DDC-induction of ALA-S, may be detecting only one of several forms of ALA-S. That is, since the pH of their incubation media was 7.2 - 7.4, then not all of the induced forms of ALA-S may have been measured. Moreover, further evidence for support of multiple inducible forms of ALA-S is that the activation of the AIA- and DDC-induced ALA-S in chick embryo liver occurred at different Mg^{++} concentrations, a finding which has also been observed by Beattie (D.S. Beattie, personal communication).

On the basis of the results described in this thesis it would appear that AIA and DDC possibly induce different forms of the enzyme, ALA-S, even should both drugs act at a transcriptional level, as suggested by Tyrrell & Marks (1972). Indeed, it is clear that failure to take this into account requires a re-assessment of much of the previous work in this field of work on drug induction of ALA-S. While no direct evidence for increased levels of the mRNA for ALA-S in AIA-induced rat liver was obtained the limitations of the cultured chick embryo liver cell system do not allow one to dismiss the possibility that AIA acts at the transcriptional level.

SUMMARY

1. A new sensitive radiochemical assay method has been described for the measurement of pmole quantities of δ-aminolaevulinic acid (ALA) in minute amounts of normal tissues, for which the existing conventional colourimetric assay method is inadequate.

2. This isotopic procedure, based on the incorporation of [2-^{14}C] glycine into ALA, utilized crude homogenate as the source of enzyme.

3. Electrophoresis of the trichloroacetic acid supernatant of the incubation mixture on silica gel thin-layer sheet separates the reaction generated [^{14}C] ALA from glycine and aminoacetone.

4. Maximal [^{14}C] ALA production in liver homogenate was obtained in the presence of glycine, citrate, Tris, and EDTA (pH 7.2-7.4).

5. A comparison of this radiochemical assay with conventional colourimetric assay was made. Several advantages of my radiochemical assay method when compared to the colourimetric assay procedure are as follows :-

(i) Only 70 to 100 μg protein were required in order to measure ALA synthesis in pmoles per min. In contrast, at least 2 mg protein is required for the colourimetric assay in order to obtain an optical density difference of 0.1. When both assay methods were performed on the same porphyric liver homogenate, 10^4 dpm were obtained in ALA by the ^{14}C method per mg of protein while the colourimetric method gave an observed optical density difference of 0.053 for the same amount of protein.

(ii) As many as 100 assays may be performed in one day whereas more than 30 assays using the colourimetric technique proved cumbersome and laborious.

(iii) Once the trichloroacetic acid deproteinised supernatant of the reaction mixture was spotted and allowed to dry on the silica gel polyester thin-layer sheets, [^{14}C] ALA that was generated in the reaction did not appear to degrade with time.

6. Certain chemical agents, allylisopropylacetamide (AIA), and 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC) are known to increase the activity of δ-aminolaevulinic acid synthetase (ALA-S), the rate-limiting enzyme in porphyrin biosynthesis, in rat and chick embryo livers and cultured chick embryo liver cells. There is conflicting evidence on the mode of action of these drugs. It has been suggested that AIA acts at the transcriptional level. Initial investigations described in this thesis involved an attempt to isolate mRNA for ALA-S from porphyric rat livers and test its biological activity in cultured chick embryo liver cells. Although results from the colourimetric assay indicated some evidence for induction of ALA-S by RNA, the actual optical differences for RNA-treated cultured cells was extremely low (i.e. maximum = 0.05) and thus colourimetric assay was found to be inadequate.

7. Attempts were made to purify the mRNA for ALA-S from poly-ribosomal RNA and total hepatic cellular RNA, obtained from livers of AIA-treated rats, by such methods as sucrose density centrifugation, adsorption to nitrocellulose filters, or oligodeoxythymidylate-cellulose. However, the RNA fractions isolated by these methods did not increase

ALA-S activity, as measured by the radiochemical assay, in cultured chick embryo liver cells above control levels. Even the addition of either diethylaminoethyl-dextran or dextran sulphate to porphyric polyribosomal RNA prior to incubation with cells did not enhance ALA-S activity, as measured by the radiochemical assay method.

8. The failure by the radiochemical assay to detect any RNA mediated increase in ALA-S activity, contrary to previous reports by Hickman et al., (1967, 1968) and Skea et al., (1970) which indicated RNA induced ALA-S activity (measured by the colourimetric assay) in cultured liver cells, suggested two feasible explanations :-

(i) The cultured chick embryo liver cell system is not sensitive enough to detect mRNA for ALA.

(ii) The porphyrogenic drug, AIA, does not increase or stimulate synthesis of the mRNA for ALA-S.

9. The AIA- and DDC-induced ALA-S of rat and chick embryo livers and cultured chick embryo liver cells have been compared physiochemically in vitro.

(i) ALA-S in crude homogenates from AIA- and DDC-induced chick embryo liver appeared to require similar Na^{+} cation concentrations. In contrast, addition of Mg^{++} to the incubation mixtures decreased the enzyme activity for both drug induced enzymes but between 0.02 M and 0.1 M Mg^{++} there was a significant increase of the DDC-induced ALA-S but not for the AIA-S induced enzyme.

(ii) The AIA- and DDC-induced ALA-S in rat liver had identical pH optima (pH 6.7). In contrast, the DDC-induced ALA-S in chick embryo liver had a higher pH optimum (pH 7.7). Moreover, the AIA-induced ALA-S in chick embryo liver showed two distinct pH optima, one which coincided with the AIA-induced enzyme in rat liver (pH 6.7), and the other was identical to the DDC-induced ALA-S pH optimum (pH 7.7).

(iii) Heat stability curves for the induced enzyme were examined. The results suggested that the enzyme having ALA-S activity in rat and chick embryo liver may differ from the enzyme in cultured chick embryo liver cells.

MATERIALS AND METHODS

1. Biological Materials.

1. 1. Tissue Culture Material.

14-18 day old chick embryos were obtained from John R. Todd Ltd., Sidehead, Stewarton, Kilmarnock, Ayrshire KA3 5LN. Difco Laboratories, P.O. Box No. 1413 Central Avenue, East Mollesey, Surrey provided the trypsin.

Calf serum and penicillin/streptomycin were purchased from Flow Laboratories Inc., Irvine, Scotland.

1. 2. Composition of Culture Media and Standard Solutions.

1. 2. 1. Eagles Minimal Essential Medium (EC)

The Eagles minimal essential medium was obtained from Biocult Laboratories Ltd., 3 Washington Road, Abbotsinch Industrial Estate, Paisley, Scotland. The Glasgow modification of the medium described by Eagle (1959) containing 100 µg/ml Streptomycin and 100 µg/ml Penicillin and supplemented with 10% (V/V) calf serum was the growth medium EC10) for propagation of chick embryo liver cells.

1. 2. 2. Ca^{++} and Mg^{++} Free Balanced Saline Solution + Glucose (CMF)

NaCl	7.0 g
KCl	0.4 g
Na $H_2PO_4 . 2H_2O$	0.14 g
1% Phenol red	1.5 ml

This solution was made up to 1 litre and autoclaved in 45 ml aliquots. To each aliquot was added 5 ml Glucose (1% w/v)), 1 ml bicarbonate (5.6% (w/v)), and 0.5 ml Penicillin/Streptomycin (5000 units/ml) to complete the CMF necessary for the culture procedure.

1. 2. 3. Trypsin in CMF.

The pH of a 2.5% (w/v) Trypsin in 0.15M NaCl solution was adjusted to 7.8, followed by filtration through Whatman No. 1 filter paper, then sterilized by millipore filtration and stored at $-10^{o}C$. This solution was diluted one to ten with CMF just prior to use.

2. Chemical Materials.

2. 1. General.

All chemicals, unless otherwise stated, were of Analar grade, and were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

2. 2. Reagents for Purification of RNA.

Baycovin was a gift from the Bayer Chemicals Ltd., Leverkusen, Germany. Phenol was redistilled before use, while m-cresol was twice redistilled under reduced temperature and pressure. Tri-isopropylnapthalenesulphonic acid was purchased from Kodak Ltd., Kirkby, Liverpool, U.K.

Millipore filters, 2.5 cm diameter and 0.22 μ pore size were obtained from Millipore Filter Corp., Bedford, Mass., U.S. Collaborative Research Inc., 1365 Main Street, Waltham, Massachusetts, 02154, U.S., supplied the oligodeoxythymidylate (oligo d(T))-cellulose. This cellulose was Whatman CF-11, washed with acid, base and ethanol and the fines removed. Oligo d(T) had chains up to 10 nucleotides long, covalently attached via the terminal 5'-phosphates. 1 g of oligo d(T)-cellulose bound at least 33.5 OD_{256} poly rA.

2. 3. Acrylamide gel Materials.

Acrylamide and NNN'N'-tetramethylethylenediamine (TEMED) were obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. The acrylamide was purified as described by Loening (1967). Acrylamide was dissolved at a concentration of 70 g/l in chloroform at 50°C. The crystals were collected by filtration at 0°C in a chilled filter funnel.

NN'-Methylene bisacrylamide was purified according to the method of Loening (1967). The solid was dissolved at 10 g/l in acetone

at 50°C, filtered hot and recrystallized by slowly cooling to -20°C. The crystals were recovered by filtration and washed with cold acetone.

2. 4. Drugs and Enzymes.

A generous gift of 50 g of allylisopropylacetamide (AIA) from Roche Products Ltd., Welwyn Garden City, Herts., is gratefully acknowledged. Diethyl 1,4-dihydro-2,4,6-trimethylpyridine-3,5-dicarboxylate (DDC) was obtained from Eastman Kodak Co., Rochester, New York 14650 U.S. δ-aminolaevulinic acid and electrophoretically purified RNase were purchased from Sigma London Chemicals Co. Ltd., Norbiton Station Yard, Kingston-upon-Thames, Surrey KTZ 7BH.

2. 5. Materials used in Radiochemical Assay.

Silica gel thin layer sheets (Mallinckrodt "Chromar 1000" glass fibre mat, impregnated with silica gel; total thickness 1 mm) were purchased from Camlab., Cambridge, U.K. Polyester sheets, 400 mm x 200 mm, coated with silica gel F1500, manufactured by Camag, 4132 Matternz, Switzerland, were supplied by the U.K. distributors. Griffin & George, Braeview Place, Nerston, East Kilbride, Scotland. The Camag high-voltage electrophoresis (HVE) apparatus consisted of one HVE cell (Camag 6100) which contained a single water cooled plate, one safety case (Camag 62000) and a HV-power supply (Camag 63000) (output 0-5000v d.c. at maximum current of 250mA). All accessories for this Camag HVE apparatus were obtained from Griffin & George, Braeview Place, Nerston, East Kilbride, Scotland. Ninhydrin was purchased from Koch-Light

Laboratories Ltd., Colnbrook, Bucks., U.K. Microfuge tubes (polyethylene), 46 mm x 4.6 mm, were supplied by Beckman, Glenrothes, Fife, U.K.

2. 6. Reagents for the Colourimetric Assay.

4-Dimethyl-aminobenzaldehyde was supplied by Anderman & Co. Ltd., Battlebridge House, 87-95 Tooley St., London S.E.1, the U.K. distributors of E. Merck Laboratory Chemicals.

2. 7. Radioisotopes and Materials for Liquid Scintillation Counting.

[2-^{14}C] glycine (52-56 mCi/mmol) and [3,5-^{3}H] δ-amino-laevulinic acid (2 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh, supplied 1M hyamine hydroxide in methanol and Koch-Light Laboratories, Colnbrook, Bucks., supplied 2,5-diphenyl oxazole. Toluene based scintillation fluid was prepared by dissolving 5 g 2,5-diphenyl oxazole in 1 litre of Analar toluene at room temperature.

2. 8. Materials used during Incubations of Cultured Liver Cells with RNA.

DEAE-dextran (M.W. 2 x 10^6) and dextran sulphate (M.W. 500,000) were obtained from Pharmacia, Uppsala, Sweden.

2. 9. Miscellaneous

Beckman Spinco Ltd., Palo Alto, California, U.S., supplied the cellulose nitrate tubes 1.6 cm x 7.6 cm for the Titanium 50 Rotor and 2.54 cm x 8.89 cm for the SW 27 rotor. 15 ml and 30 ml Corex high speed centrifuge tubes made by Corning Glassware, Nutley, New Jersey, U.S., were supplied by their U.K. distributors, A. Gallenkamp & Co. Ltd., Braeview Place, Nerston, Glasgow G74 3XJ.

METHODS

1. Biological methods

1. 1. Cultured Chick Embryo Liver Cell System.

Instruments were sterilized by immersing in 70% ethanol for 10 min. The eggs were swabbed with 70% ethanol. The egg was cracked near the air space and the pieces of shell removed. Using sterilized forceps, a portion of egg membrane and chorioallantoic membrane were punctured. The embryo was removed through this hole into a sterile petri dish (10 cm diameter). Using forceps and scissors, the liver was removed asceptically and immersed in CMF in another weighed petri dish. This procedure was repeated with the other eggs until enough tissue had been obtained. These livers were then cut into small pieces using fine scissors and forceps. The CMF medium was discarded and replaced with 20 ml of 0.25% trypsin in CMF at 37°C. Cells were trypsinized for 15 min at 37°C in a humidified incubator. The trypsin solution was

discarded and the tissue was washed twice with fresh CMF at room temperature in a universal bottle. The tissue was immediately dissociated in a small volume of CMF by repeated (10x) aspiration and then the concentrated cell suspension was added to 10 ml EC and the aspiration procedure (10x) repeated. The undissociated tissue fragments were allowed to sediment and then removed. Using a Coulter counter 0.1 ml was counted. The volume of the suspension was adjusted (with EC at 37°C) to give a cell density of approximately 3×10^6 cells per ml. Nine ml aliquots of cell suspension were transferred to 10 cm petri dishes, followed immediately by 1 ml of calf serum. The dishes were swirled quite vigorously to ensure thorough mixing and then incubated for 18 to 22 hours in a humidified incubator at 37°C maintained at an atmosphere of 5% (v/v) CO_2 in air. 20 to 30 x 10^6 cells per plate gave good dense monolayers. After the appropriate time of incubation, the dishes were removed and the viability of the monolayers was checked using an inverted microscope.

1. 2. Incubation of Cultured Chick Embryo Liver Cells with RNA, AIA or DDC.

When the colourimetric assay was used to measure the ALA-S activity, it was necessary to use 2 x 10 cm petri dishes of cultured cells and 10 ml of EC, containing approximately 2 mg RNA per dish. Therefore, the RNA to be tested was dissolved in 18.5 ml of EC (which contained no glucose, vitamins and amino acids). It was important that the culture medium (EC) used to dissolve the RNA should be free from glucose, which gives a dark colour in the conditions of the orcinol reaction. 0.5 ml of this solution was removed for accurate determination

of the RNA concentration by the orcinol method (Hutchison & Munro, 1961). 2 ml of Eagles glucose, vitamins and amino acids solution (10x) were then added to complete the EC medium. Only 2 dishes, each time, were removed from the incubator. The medium was suctioned off by a pasteur pipette and the monolayers in both dishes washed 3 times with EC (37°C). 10 ml of the EC/RNA solution were added to each dish and both dishes were returned to the same humidified incubator (37°C) for 3.5 hours. Since the radiochemical assay, when used to measure the ALA-S activity, required only 1 x 2.5 cm dish of cultured cells, then 2 ml of the EC/RNA was added to each dish.

AIA (10 mg/ml water) or DDC (20 mg/ml ethanol) was administered either at the same time as the addition of cell suspension to the petri dishes, or after the monolayers had formed, with the final concentration being 200 µg AIA/ml EC or 100 µg DDC/ml EC for optimal increase in ALA-S activity.

Experiments involving addition of DEAE-dextran and dextran sulphate to RNA prior to incubation with the cultured chick embryo liver cells were performed as follows. DEAE-dextran and dextran sulphate dissolved in 0.15 M phosphate-buffered saline and the pH adjusted to 7.2 with NaOH, were added to each RNA preparation in quantities varying from 0.2 mg/ml EC up to 2.0 mg/ml EC (1/1 to 10/1 ratio of RNase inhibitor/RNA) as suggested by Maes et al., (1967) and Deckers & Pilch (1971).

1. 3. Harvesting of the Cultured Chick Embryo Liver Cells for Determination of ALA-S Activity.

The medium was removed via pasteur pipette, rinsed twice with

150 mM Tris-HCl and 20 mM EDTA (pH 7.2) and then 1 ml of the same Tris-EDTA buffer was added and the cells removed using a rubber scraper. The cell suspension was transferred to centrifuge tubes and recovered by centrifugation (1500 rpm for 5 min at 4°C, using a MSE Mistral 6L centrifuge. 450 µl or 10 µl of 150 mM Tris-HCl (pH 7.2) containing 20 mM EDTA were added to the pellet of cells for use in the colourimetric and radiochemical assay, respectively. The cell suspension was transferred to a home-made glass homogenisation tube (70 mm x 10 mm) and hand homogenized with a close fit pestle. The homogenate was then assayed for ALA-S activity by either the colourimetric or radiochemical assay.

1. 4. Experimental Animals.

Male rats, which were derived from the Wistar strain, and bred at Glasgow University, were fed ad libitum on diet 41 B. They were used only at weights of 250 g or greater.

1. 5. Administration of AIA and DDC to Experimental Animals.

Rats received intraperitoneal injections of 0.25 ml (400 mg AIA per kg body weight) in propylene glycol, or 0.25 ml of DDC (200 mg per kg body weight) in 95% ethanol and were starved during the last 36 - 48 hours of the experiments. Control animals received propylene glycol or 95% ethanol, depending on which drug had been administered.

Induction of ALA-S in livers of whole chick embryos by AIA or DDC was as follows. A fertilized egg, incubated for 14 - 18 days, was inoculated under sterile conditions through the air sac with 0.5 ml of AIA (10 mg) in water or 0.2 ml of DDC (4 mg) in propylene glycol, via a pinhole made in the shell. The hole was covered with scotch tape, and the egg was incubated in an upright position in a humidified incubator at 37°C.

2. Chemical methods.

2.1. Colourimetric Assay Method for Determination of ALA-S Activity in Crude Liver Homogenates.

The colourimetric assay method of Dowdle *et al.*, (1967), slightly modified, was used to determine the ALA-S activity in crude homogenates of rat and chick embryo liver and cultured chick embryo liver cells. The tissue was homogenized in 150 mM Tris-HCl, pH 7.2, containing 20 mM EDTA. 4 x 100 µl of this homogenate were transferred to 4 microfuge tubes (46 mm x 4.5 mm), each tube containing 200 mM glycine in 100 mM sodium citrate, pH 7.4. 2 of these microfuge tubes had an additional 100 µl of 15% (w/v) trichloroacetic acid. The latter pair of assay tubes enabled the determination of the original amount of ALA present (termed 'trichloroacetic acid treated'), while the former pair of tubes measured the incubation generated ALA (termed 'test'). After incubation of the 'test' and 'trichloroacetic acid treated' homogenates in a shaking water bath at 37°C for 1 hour, the tubes were placed in ice and 100 µl of 15% trichloroacetic acid added to the 'test' samples. All mixing of the

microfuge tubes was performed using the Beckman 154 Micromixer. After 10 min at 0°C, the tubes were centrifuged for 3 min using the Beckman 152 Microfuge, at room temperature. 200 µl of the trichloroacetic acid supernatants were transferred to glass tubes (8 cm x 1 cm) which already contained 100 µl of 0.6 M sodium acetate, 200 µl water, and 20 µl acetyl-acetone (redistilled twice before use). The tubes were sealed off with No. 7 rubber stoppers, mixed thoroughly on a Vortex and heated for 10 min at 100°C. After a rapid cooling in ice water, the contents were mixed again, and the stoppers removed. 1 ml of special Ehrlichs reagent (described below) was added to each tube and the contents mixed immediately. The glass tubes were centrifuged at room temperature for 5 min at 2000 rpm using the MSE Mistral 6L in order to sediment any contaminating particles and dust. The pink colour resulting from the complex of the ALA-pyrrole with Ehrlich reagent was allowed 10 min to develop and the optical density at 552 nm was read against a reagent blank using 4 cm cuvettes. The nmolar extinction coefficient for ALA was assumed to be 13.7 at 552 nm. Amounts of ALA formed were determined by subtracting the control values found at zero time incubation ('trichloroacetic acid treated') from the values obtained at 60 min of incubation ('test'). Protein concentration in the homogenate was determined by the Lowry et al., (1951) method.

2. 2. Ehrlichs Reagent for Use in the Colourimetric Assay.

This reagent was prepared fresh just prior to its use. 2.5 g of 4-dimethylaminobenzaldehyde was dissolved in 40 ml of glacial acetic acid. 4 ml of the $HgCl_2$ solution (0.25 g $HgCl_2$ dissolved in 5 ml of glacial acetic acid by gentle heating) was added to the 4-dimethyl-

aminobenzaldehyde/acetic acid mixture followed by addition of 24.5 ml of perchloric acid (sp. gr. = 1.7) with the final volume made up to 100 ml with glacial acetic acid. This reagent was further diluted by an equal volume of distilled water and stored at 0°C until used.

2. 3. Radiochemical Assay Method for Measurement of ALA-S Activity in Crude Liver Homogenates.

The radiochemical assay procedure used for measurement of ALA-S activity in crude liver homogenates was developed independently of the existing isotopic methods of Irving & Elliott (1969), Ebert et al., (1970), and Freshney & Paul (1970). The tissue was homogenised in 150 mM Tris-HCl, pH 7.2, containing 20 mM EDTA. 10 µl of homogenate were transferred to microfuge tubes (polyethylene, 46 mm x 4.5 mm) which contained 10 µl of 20 mM glycine in 100 mM sodium citrate, pH 7.4, 1 µCi/assay tube of [2-^{14}C] glycine (52-56 mCi/mmol). The tubes were then sealed, mixed using the Beckman 154 Micromixer, and incubated for 40 min in a shaking water bath at 37°C. The reaction was stopped by addition of 5 µl of 30% (w/v) trichloroacetic acid, followed by an addition of 5 µl of 15 mM ALA, which acted as a marker in the electrophoresis procedure. After 15 min at 0°C, the trichloroacetic acid precipitate was pelleted by centrifugation for 3 min using the Beckman 152 Microfuge. The protein of the sediment was determined (Lowry et al., 1951). 5 µl of the deproteinized supernatant was dissolved in 10 ml of a toluene based scintillant to determine the total dpm of counts in the 5 µl aliquots that were applied to the thin-layer sheet. Another 5 µl sample was applied to a 400 mm x 200 mm silica gel thin-layer sheet

(Camag, Switzerland) and electrophoresed, using the Camag HVE apparatus, for 30 min at 130-195 mA and 3 KV, at 3-5°C in 0.05 M phthalate buffer (pH 4.0) containing 0.01 M EDTA. The thin-layer sheet was dried with hot air, sprayed with 0.1% (w/v) ninhydrin in 95% ethanol, and heat dried again until the ALA (yellow) and glycine (purple) spots had fully developed. Each ALA spot was cut out, placed in a scintillation vial containing 1 ml of 1M hyamine hydroxide in methanol and left at 60°C for 30 min. 10 ml of toluene based scintillator was then added to each vial and the radioactivity assayed by liquid scintillation spectrometry using a Phillips Liquid Scintillation Analyser PW4510. ^{14}C was counted with efficiencies of 63-72%.

3. Preparation of RNA.

3. 1. Glassware and Solutions.

All glassware used in the preparation was either rinsed in methylated spirit and flamed before use or autoclaved at 15 p.s.i. for 30 min, and all solutions, when possible, were sterilized by autoclaving to remove contaminating nucleases.

3. 2. Preparation of Ribosomal RNA.

With minor modifications, ribosomal RNA (rRNA) was isolated from rat liver and purified by the method of Parish & Kirby (1966). Treated and control rats, fasted for 36 to 48 hours to deplete liver of glycogen, were anaesthetised with ether and killed by exsanguination.

The liver was immediately buried in cubed ice and freed from fibrous material and any adhesions. About .01 g of each liver was removed for assay of endogenous ALA-S activity. The remainder was weighed as quickly as possible (a total of 12 - 14 g was convenient) and homogenized at 0°C in 10 volumes of ice cold 6% 4-aminosalicylate, 1% NaCl, 1% sodium tri-isopropylnapthalene sulphonate and 6% 2-butanol by 6 passes in a close fit teflon/glass homogeniser. The homogenate was transferred to a 500 ml conical flask and an equal volume (i.e. 11 x wt. of liver) of phenol/m-cresol/water/8-hydroxyquinoline (500:70:55:0.5, by volume) at room temperature was added. The flask was shaken at room temperature for 1 min. The mixture was centrifuged for 15 minutes at 12,000 g (10,000 rpm in a MSE high speed centrifuge) at 0 - 4°C. Better separation of the phases was obtained if, after the first spin, the top layer plus some of the material from the fluffy layer is transferred and centrifuged again. The upper layer was removed by pasteur pipette, the volume measured and sufficient solid NaCl added (deproteinisation agent) to give a concentration of 3%. After a second extraction with phenol/m-cresol/water/8-hydroxyquinoline at room temperature (this time for 10 min with 0.5 volumes of the phenol mixture), the upper layer was separated by centrifugation in 30 ml Corex tubes, and the volume measured. Two volumes of ice-cold ethanol/m-cresol (9:1, v/v) was added, the flask shaken and allowed to stand in ice for 30 - 60 min. The white precipitate was then spun down (2500 rpm for 10 min at 0°C in a MSE Mistral 6L), washed twice with 2% sodium acetate in 75% ethanol and dissolved in 0.1 M sodium acetate (0°C). Solid NaCl was added to give a final concentration of 3 M and the solution was stored at -5°C overnight. The very fine

precipitate of RNA was spun down (15 min at 12,000 g), washed twice with 3 M sodium acetate (pH 6) to remove NaCl, residual DNA and glycogen, twice with 2% sodium acetate in 75% ethanol and twice with ethanol ($0^{\circ}C$). The rRNA was resuspended in approximately 5 ml of ethanol, and stored at $-10^{\circ}C$. When required, the appropriate volume of freshly agitated suspension was spun at 2500 rpm for 10 min at $0^{\circ}C$ and the precipitate allowed to drain. The RNA content of the RNA/ethanol suspension was often estimated by measurement of UV absorption at 260 nm so that the amount of RNA added to cultured cells, when dissolved in EC, would be approximately 0.2 mg/ml EC (1 OD = 20 μg RNA/ml).

3. 3. Preparation of Cytoplasmic, Nuclear, Polyribosomal, and Transfer RNA.

The procedure used was essentially the method described by Henshaw (1968). AIA-treated and control rats were fasted for 36 - 48 hours to deplete liver of glycogen, and killed by exsanguination. The livers were buried in cubed ice and homogenized as quickly as possible in 2 volumes of TEAMN homogenisation medium (0.25 M sucrose, 0.02 M triethanolamine, 0.001 M $MgCl_2$, 0.01 M NaCl, 0.1% Baycovin, pH 7.6) in a close fit teflon/glass homogeniser at $0^{\circ}C$. About 30 - 50 g of liver was sufficient. The homogenate was spun for 10 min at 15,000 g (12,000 rpm in an 8 x 50 rotor in a MSE 18 high speed centrifuge) to remove nuclei, mitochondria and lysosomes, and the upper 3/4 of supernatant was retained as the cytoplasmic fraction. Nuclear and cytoplasmic RNA, when required, was extracted from the pellet and the upper 3/4 supernatant, respectively, with phenol/m-cresol mixture as in the Parish & Kirby (1966)

preparation. However, if polyribosomal and transfer RNA were desired, the pellet was discarded. The cytoplasmic fraction was divided 3:1 for preparation of cell sap and polyribosomes respectively: the larger volume was spun for 2 hours at 150,000 g (49,000 rpm) at 2°C in a Ti 50 rotor using the Beckman Spinco L-2 65B and the supernatant retained as the cell sap. Meanwhile, the remaining 1/4 of cytoplasmic fraction was distributed in 2 ml portions in 13.5 ml (7.6 cm x 1.6 cm) cellulose nitrate spinco tubes. To each tube was added 4.2 ml of 2.3 M sucrose in TEAMN. The tubes were well mixed and kept on ice until the cell sap was prepared. Half of the cell sap was retained as the "cell sap fraction" and the remainder was used for preparation of polyribosomes as follows. Sucrose was dissolved in the cell sap at 0°C to give a concentration of 2.0 M (prolonged stirring was required), and 5 ml portions of this solution were carefully introduced under each cytoplasmic preparation in the tubes. These tubes were spun for 4 hours at 150,000 at 0-2°C and the upper layers were removed. In order to prevent contamination by membranous elements adhering to the wall, the top of the tube was cut off (about 1/2 inch from the base). The tubes were quickly inverted and drained and the pellets taken up in TEAMN solution by stirring with a sterile glass rod followed by gentle homogenisation by hand in a close fit teflon/glass homogeniser. The "cell sap" and "polyribosome" preparations obtained as above were extracted twice with phenol/m-cresol as in the Parish & Kirby (1966) rRNA preparation. RNA was precipitated from the aqueous phases by the addition of 2 volumes of ethanol/m-cresol at 0°C, and the flask allowed to stand in ice for 30 - 60 min. The precipitate was spun down, washed twice with 2% sodium acetate in 75% ethanol, and twice with ethanol. The RNA was stored under ethanol at -5°C.

The alternative technique used for isolation of RNA from polyribosomes was the method described by Brawerman et al., (1972). All operations were carried out at 0-4°C. For extraction of the total polyribosomal RNA, the polyribosomes were diluted in sterile water (concentration was lower than 50 A_{260} unit/ml), 0.1 volume of 5% SDS and of 1 M Tris (pH 9.0) followed by 1 volume of 88% (v/v) phenol equilibrated with water. The mixture was shaken for 10 min and the aqueous phase (interphase plus phenol phase) was re-extracted with 0.1 M Tris (pH 9.0) and the two aqueous phases were combined. The aqueous phase was re-extracted at least 3 times with 88% phenol. This served to remove residual protein as well as the remaining SDS. The RNA was precipitated by addition of 2.5 volumes of ethanol and 0.1 volumes of 1 M NaCl and stored overnight at -10°C. The precipitate was collected by centrifugation and washed twice with 2% sodium acetate in 75% ethanol, twice with ethanol, and stored in ethanol at -5°C until used.

3. 4. Hot Phenol - Sodium Dodecyl Sulphate Technique for Polyribosomal and Total Hepatic Cellular RNA.

This was carried out essentially as described by Burdon & Clason (1969). Polyribosomes were isolated from rat livers by the Henshaw (1968) method. Polyribosomal RNA or total cellular RNA from rat livers were isolated by the following procedure. The polyribosomes or rat liver was homogenised in 0.05 M ammonium acetate (pH 5.1) containing 0.1% SDS (w/v) buffer and shaken at 60°C for 5 min with an equal volume of 80% (v/v) phenol equilibrated with the same buffer. The resultant emulsion was separated by centrifugation at 10,000 g for 10 min

in a MSE 18 high speed centrifuge at 4°C. The aqueous phase plus interphase was then re-extracted with an equal volume of phenol/0.05 M ammonium sulphate/0.1% SDS (pH 5.1). The aqueous phase from the extraction was precipitated with 2 volumes of 95% alcohol containing 0.2 M NaCl and stored at -20°C for 18 hours. The precipitate was collected by centrifugation at 2500 rpm for 10 min at 4°C, washed twice with 2% sodium acetate in 75% ethanol, twice with absolute alcohol and stored in ethanol at -5°C.

3. 5. Purification of mRNA by Adsorption on Millipore Filters.

The RNA, either polyribosomal isolated by the Henshaw (1968) Burdon & Clason (1969) or Brawerman et al., (1972) methods, was dissolved in 500 mM KCl, 10 mM Tris (pH 7.6) and 1 mM $MgCl_2$, at concentration of 0.1 to 0.3 mg/ml, was passed slowly through a nitrocellulose millipore filter (25 mm diameter, 0.22μ pore size) presoaked in the same solution, at a rate of approximately 1 drop per second. The quantity of RNA adsorbed on a single filter was not determined. The filter was cut into small pieces and the adsorbed material was eluted with an ice-cold solution of 0.5% SDS (v/v) in 0.1 M Tris (pH 9.0). The filter was kept in 1 ml of this solution for about 30 min with occasional shaking. The fluid was removed and the filter washed with 1 ml of 0.1 M Tris (pH 9.0). This was combined with the fluid. The RNA solution was chilled to 4°C and repeatedly centrifuged to remove all traces of SDS. The supernatant was made 200 mM with NaCl and the RNA precipitated with 2 volumes of 95% ethanol. After 24 hours at -10°C, the RNA was pelleted, washed twice with 95% ethanol, twice with absolute alcohol, and stored in ethanol at -5°C.

3. 6. Oligodeoxythymidylate-Cellulose Chromatography of Total Cellular Hepatic RNA.

The procedure used here is essentially that described by Aviv & Leder (1972). All chromatographic operations were performed at room temperature with all glassware and reagents (except for oligo d(T)-cellulose) having been autoclaved. Approximately 240 OD_{260} unit of total cellular RNA isolated from rat livers by the method of Burdon & Clason (1969), was dissolved in the application buffer containing 0.01 M Tris-HCl (pH 7.5), 0.5 M KCl was applied to a 2 ml (about 1 g dry weight) oligo d(T)-cellulose column previously washed with the application buffer. The non-adsorbed material was eluted by continued washing with the application buffer. The material retained by the column was eluted by 0.01 M Tris-HCl (pH 7.5), immediately precipitated in 2 volumes of ethanol and stored at -10°C for 24 hours. The RNA was pelleted, washed twice with 2% sodium acetate in 75% ethanol, twice with ethanol and stored in ethanol at -5°C.

3. 7. Fractionation of RNA on Polyacrylamide Gels.

Gels were prepared as described by Loening (1967). High molecular weight RNA was examined by electrophoresis on gels containing 2.5% (w/v) acrylamide and low molecular weight material was examined in gels containing 7.5% (w/v) acrylamide in a vertical tube system by the following procedure. The following stock solutions were prepared (acrylamide and methylene bisacrylamide were purified as described in MATERIALS 2. 3): - (A) 30% (w/v) acrylamide, 1.5% (w/v) methylene

bisacrylamide in distilled water, (B) 30% (w/v) acrylamide, 1% (w/v) methylene bisacrylamide in distilled water, (C) 1% (v/v) NNN'N'-tetramethylethylenediamine in distilled water, (D) 2.8% (w/v) ammonium persulphate in distilled water, (E) electrophoresis buffer consisting of 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, and 0.2% SDS (pH 7.7 - 7.8). The 2.5% gels were prepared by mixing these solutions as follows: - 0.83 part (A), 1 part (C), 0.4 part (D), 1 part (E), and 6.8 parts distilled water. 7.5% gels were prepared as follows: - 2.5 parts (B), 1 part (C), 0.4 part (D), 1 part (E), and 5 parts water. The prepared solutions were carefully mixed to avoid aeration and 3 ml aliquots were rapidly pipetted into vertical 150 mm x 10 mm perspex tubes. Water was then carefully layered over the solution using a syringe. The 2.5% gels set in approximately 15 min and the 7.5% gels in about 2 min. All gels were pre-electrophoresed at 5 mA/gel for 30 min before RNA (40 - 100 µg), dissolved in 0.05 ml of electrophoresis buffer containing 2 drops of glycerol and 1 drop of 1% bromophenol blue marker dye, was layered on the top. The gels were electrophoresed at 5 mA/gel until the marker was approximately 1 cm from the end of the gel (usually 1 hour). The gels were then removed, stained in 0.1% (w/v) toluidine blue in distilled water for 30 min and after destaining overnight, were scanned at 260 nm in the linear transport attachment for the Gilford 240 recording spectrophotometer.

3. 8. Fractionation of RNA by Sucrose Density Centrifugation.

The method used was a modification of that described by Girard *et al.*, (1965). Up to 5 mg of RNA in approximately 1 ml of LETS buffer (0.005 M Tris-HCl, pH 7.2, 0.01 M EDTA, 0.1 M LiCl, and 0.55% SDS)

was layered onto a 37.5 ml, 15-40% linear sucrose density gradient in LETS buffer in a 2.54 cm diameter x 8.89 cm cellulose nitrate tube. Centrifugation was for 32 hours at 131,000 g (27,000 rpm) and at 2°C in the SW 27 rotor of a Beckman model L2 65B ultracentrifuge. Gradients were eluted, by the use of a peristaltic pump, through the flow cell of a Gilford 240 recording spectrophotometer and the extinction at 260 nm was continuously monitored. 28S, 18S, and low molecular weight RNA fractions were collected and the RNA was precipitated by 2 volumes of 95% ethanol and stored at -10°C for 24 hours. The RNA was collected by centrifugation, washed twice with 2% sodium acetate in 75% ethanol, twice with ethanol, and stored in ethanol at -5°C.

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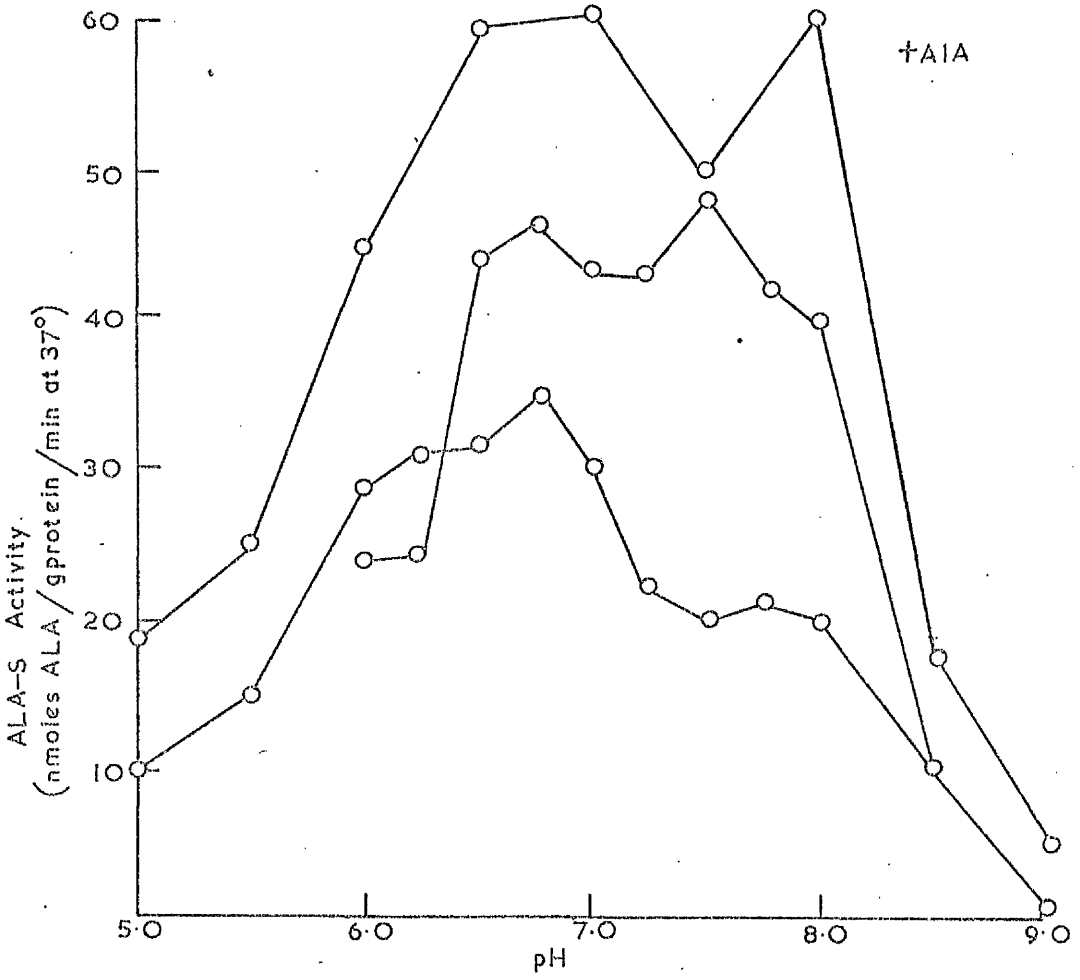
Fig 13a

CHICK EMBRYO LIVER



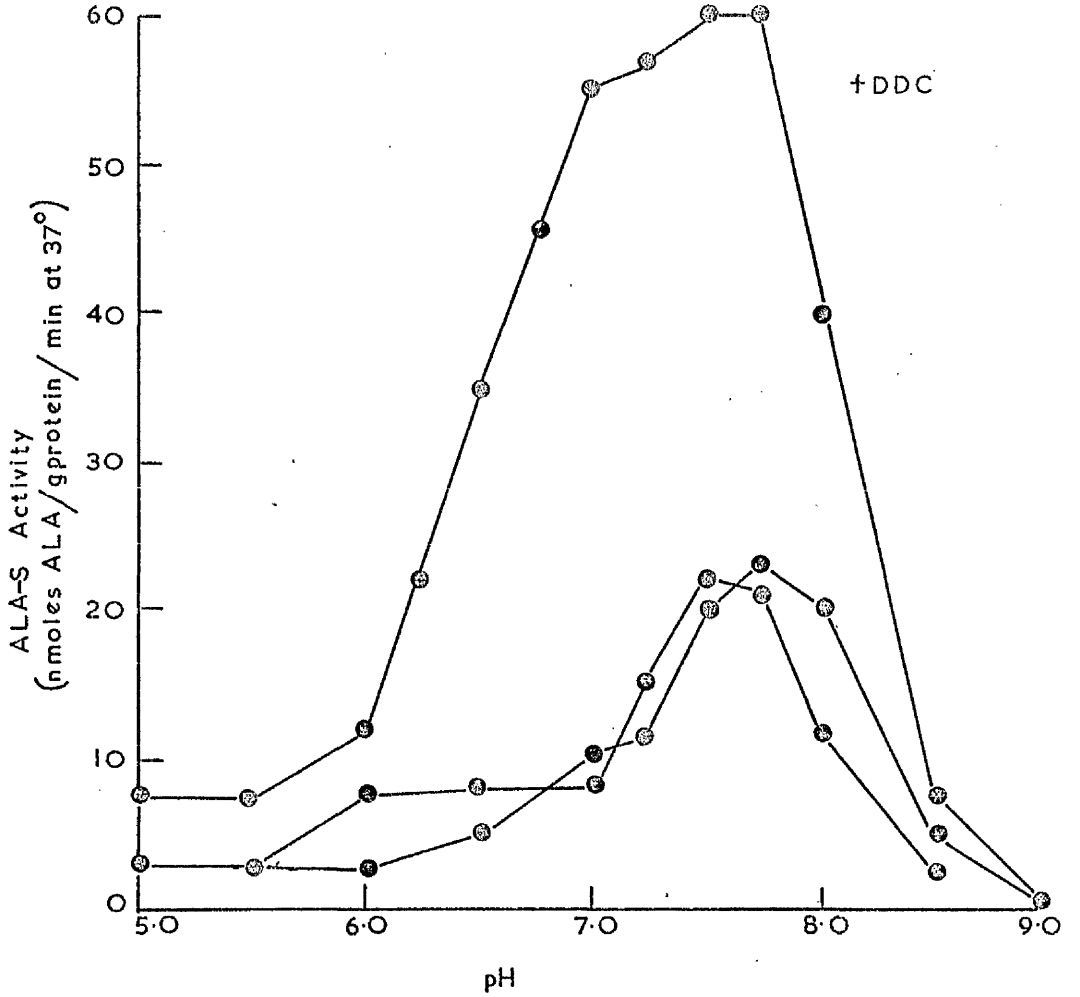


Fig 13b

RAT LIVER