BIOCHEMICAL STUDIES ON HAEM AND PORPHYRIN METABOLISM

Thesis submitted for the degree of Doctor of Philosophy

MICHAEL RITCHIE MOORE.

October, 1970

A series of studies has been carried out, examining the effect and mode of action of various compounds on the haem biosynthetic pathway.

a) (1) Allyl isopropyl acetamide (AIA) (300 mg/kg) elevated the activity of rat hepatic δaminolaevulic acid (ALA) synthetase activity tenfold, and also elevated ALA dehydrase activity and porphyrin and porphyrin precursor excretion.

(2) Animal sex had no significant effect on hepatic ALA synthetase activity.

(3) In the neonatal period of development hepatic ALA synthetase has a significantly higher activity than in the adult rat.

(4) Starvation of rats for 24 hours, with water 'ad lib' significantly elevates hepatic ALA synthetase activity.

(5) Vitamin E. significantly depresses porphyrin precursor and porphyrin excretion in rats intoxicated with AIA but has no significant effect on enzyme activities.

b) (1) Some barbiturates, when given to rabbits, cause a rise in urinary coproporphyrin excretion while others, suspected of provoking acute attacks of porphyria in man, do not. To investigate this anomaly, the levels of hepatic enzymes occurring early in the biosynthetic pathway have been examined in rats treated with barbiturates.

(2) Of the nine barbiturates tested all significantly raised the hepatic level of ALA synthetase activity in the rat. This elevation was abolished by the antibiotics, cycloheximide and actinomycin D.

(3) These barbiturates may be classified into four significantly different groups, which correspond to the groups of the earlier classification based on urinary coproporphyrin excretion.

(4) The chemically related compounds, cyanuric acid, glutethimide and pentazocine, also elevated hepatic ALA synthetase activity in the rat.

(5) It is suggested that these results show a direct reason for contraindication of any barbiturates, glutethimide and pentazocine in porphyria.

(6) The mechanism of this elevation of enzyme activity by these drugs is discussed.

c) (1) The activity of δaminolaevulic acid synthetase is significantly elevated in the liver of rats and the activity of δaminolaevulic acid (ALA) dehydrase is significantly depressed in the blood of man and rats intoxicated with ethanol.

(2) This depression of ALA dehydrase activity follows closely on the elevation of blood ethanol and returns to normal 'pari passu' with the ethanol level.

(3) In rats intoxicated with ethanol there is a significant depression of ALA dehydrase activity in the liver and kidney.

(4) A scheme is proposed whereby an increase in the intracellular redox potential by ethanol increases sulphydryl cofactor concentrations leading to an inhibition of ALA dehydrase activity.

d) (1) A series of twenty C_{18}, C_{19} and C_{21} steroids have been examined for their effects on rat hepatic ALA synthetase activity.

(2) The following: Dehydroepiandrosterone, 17 hydroxy-Pregnenolone, Androstenedione, Androstenediol and Etiocholanolone significantly elevated hepatic ALA synthetase activity in rats.

(3) Dehydroepiandrosterone and its sulphate were effective in elevating ALA synthetase activity yet the acetate and glucuronide forms were ineffective in raising activity.

(4) The antibiotics Actinomycin D and Cycloheximide abolished this DHA elevation of activity at all times during the induction, thus suggesting that this elevation of enzyme activity is due to a *de novo* synthesis of enzyme protein.

e) A study has been carried out on four patients with hereditary coproporphyria, two patients in attack and two in remission;

(1) All of the patients showed typical excessive excretion of urinary and faecal coproporphyrin with elevated excretion of porphyrin precursors in attack.

(2) Both patients in attack had elevated blood ALA dehydrase activity and one a highly elevated hepatic ALA synthetase activity.

(3) Both patients in attack had elevated urinary excretion of certain 17 oxosteroids.

These results and their relevance to the control and pathogenesis of the haem biosynthetic pathway are discussed.

INDEX

FIGURES

TABLES

TABLES

TABLES.

TABLES

TABLES

PREFACE

Over the three years that I have carried out this work in the Gardiner Institute of Medicine, I have become indebted to many people, to the Technicians who assisted me, to the Typists who struggled with my almost illegible handwriting and to many of the Medical staff.

I would particularly like to thank Mr. G.G. Thompson (George) for his technical assistance; Dr. A.D. Beattie (Gardiner Institute of Medicine) Professor J.N. Davidson and Dr.R.Y. Thompson (Department of Biochemistry) Professor C. Rimington (University College Hospital Medical School, London) and Professor S.D. Silvey (Department of Statistics) for their advice; and The department of Medical Illustrations, Western Infirmary, for the figures in this thesis; and Professor G.M. Wilson for his supervision and for the use of his laboratories.

Finally I should like to thank, most of all, Professor A. Goldberg for his advice, ideas, assistance and encouragement in the performance of this work.

ABBREVIATIONS

AIA	2 Allyl 2 isopropyl acetamide
A.I.P.	Acute Intermittent Porphyria
ALA.	δAminolaevulic Acid
ALA.S.	ALA. Synthetase (Activity)
ALA.D.	ALA. Dehydrase (Activity)
ATP	Adenosine Triphosphate
Copro.	Coproporphyrin
DDC.	Diethyl 1,4 dihydro 2,4,5, trimethyl Pyridine 3,5 dicarboxylate
DHA.	Dehydroepiandrosterone
DHA.S.	DHA. Sulphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamino Tetra-acetic Acid
EtOH	Ethanol
GSH	Reduced Glutathione
GSSG	Oxidised Glutathione
HS.CoA	Coenzyme A
PBG	Porphobilinogen
Proto	Protoporphyrin
RNA	Ribonucleic Acid
SD.	Standard Deviation
S.E.M.	Standard error on the Mean
UDP	Uridine Diphosphate
Uro	Uroporphyrin

SUMMARY

A series of studies has been carried out, examining the effect and mode of action of various compounds on the haem biosynthetic pathway.

a) (1) Allyl isopropyl acetamide (AIA) (300 mg/kg) elevated the activity of rat hepatic δaminolaevulic acid (ALA) synthetase activity tenfold, and also elevated ALA dehydrase activity and porphyrin and porphyrin precursor excretion.

(2) Animal sex had no significant effect on hepatic ALA synthetase activity.

(3) In the neonatal period of development hepatic ALA synthetase has a significantly higher activity than in the adult rat.

(4) Starvation of rats for 24 hours, with water 'ad lib' significantly elevates hepatic ALA synthetase activity.

(5) Vitamin E. significantly depresses porphyrin precursor and porphyrin excretion in rats intoxicated with AIA but has no significant effect on enzyme activities.

b) (1) Some barbiturates, when given to rabbits, cause a rise in urinary coproporphyrin excretion while others, suspected of provoking acute attacks of porphyria in man, do not. To investigate this anomaly, the levels of hepatic enzymes occurring early in the biosynthetic pathway have been examined in rats treated with barbiturates.

(2) Of the nine barbiturates tested all significantly raised the hepatic level of ALA synthetase activity in the rat. This elevation was abolished by the antibiotics, cycloheximide and actinomycin D.

(3) These barbiturates may be classified into four significantly different groups, which correspond to the groups of the earlier classification based on urinary coproporphyrin excretion.

(4) The chemically related compounds, cyanuric acid, glutethimide and pentazocine, also elevated hepatic ALA synthetase activity in the rat.

(5) It is suggested that these results show a direct reason for contraindication of any barbiturates, glutethimide and pentazocine in porphyria.

(6) The mechanism of this elevation of enzyme activity by these drugs is discussed.

c) (1) The activity of δaminolaevulic acid synthetase is significantly elevated in the liver of rats and the activity of δaminolaevulic acid (ALA) dehydrase is significantly depressed in the blood of man and rats intoxicated with ethanol.

(2) This depression of ALA dehydrase activity follows closely on the elevation of blood ethanol and returns to normal 'pari passu' with the ethanol level.

(3) In rats intoxicated with ethanol there is a significant depression of ALA dehydrase activity in the liver and kidney.

(4) A scheme is proposed whereby an increase in the intracellular redox potential by ethanol increases sulphydryl cofactor concentrations leading to an inhibition of ALA dehydrase activity.

d) (1) A series of twenty C_{18}, C_{19} and C_{21} steroids have been examined for their effects on rat hepatic ALA synthetase activity.

(2) The following: Dehydroepiandrosterone, 17 hydroxy-Pregnenolone, Androstenedione, Androstenediol and Etiocholanolone significantly elevated hepatic ALA synthetase activity in rats.

(3) Dehydroepiandrosterone and its sulphate were effective in elevating ALA synthetase activity yet the acetate and glucuronide forms were ineffective in raising activity.

(4) The antibiotics Actinomycin D and Cycloheximide abolished this DHA elevation of activity at all times during the induction, thus suggesting that this elevation of enzyme activity is due to a *de novo* synthesis of enzyme protein.

e) A study has been carried out on four patients with hereditary coproporphyria, two patients in attack and two in remission;

(1) All of the patients showed typical excessive excretion of urinary and faecal coproporphyrin with elevated excretion of porphyrin precursors in attack.

(2) Both patients in attack had elevated blood ALA dehydrase activity and one a highly elevated hepatic ALA synthetase activity.

(3) Both patients in attack had elevated urinary excretion of certain 17 oxosteroids.

These results and their relevance to the control and pathogenesis of the haem biosynthetic pathway are discussed.

INTRODUCTION

THE HAEM BIOSYNTHETIC PATHWAY

The initial elucidation of the early steps of this biosynthetic pathway was a result of a set of brilliant isotopic tracer studies. These are described by Shemin (1955) and laid the foundation for all further work in porphyrin biochemistry. Because of this and other studies the general pathway of Haem biosynthesis is now well understood.

Prior to 1946 the manner of biosynthesis of the porphyrins had been speculatory, but in that year Shemin and Rittenberg demonstrated a specific labelling of Haem by ^{15}N glycine. The initial stage of the pathway is the condensation of glycine and succinyl CoA (Shemin & Kumin, 1952) by ALA synthetase (ALA.S) (Fig.1.). The cofactors for this reaction are pyridoxal phosphate (Schulman & Richert, 1956) and ferrous iron (Brown, 1958). The pyridoxal phosphate activates the methylene group of glycine preliminary to Schiff Base formation. This glycine and 'activated' succinate, succinyl CoA, then condense to form αamino βketo adipic acid, which being a βketo acid readily decarboxylates to form δaminolaevulic acid (ALA).

To unify the reactions of glycine, Shemin & Russell, (1953) and Shemin (1955) postulated a series of reactions, the succinate-glycine cycle, now called the 'Shemin' cycle, whereby glycine in addition to its utilisation for ALA synthesis and porphyrin synthesis was also used to provide a single α carbon fragment in the synthesis of the ureido group of purines and the β carbon of serine, the remaining residue being reconverted to succinate. Although some experiments (Nemeth et al, 1957; Braunstein et al, 1964) support the existence of this cycle, its general metabolic significance remains to be evaluated. The enzyme which catalyses the conversion of ALA to 4.5 dioxovaleric acid, alanine dioxovalerate transaminase, has been demonstrated in Rhodopseudomonas spheroides (Neuberger & Turner, 1963) Bacterium diphtheriae (Bagdasarian, 1958) and in

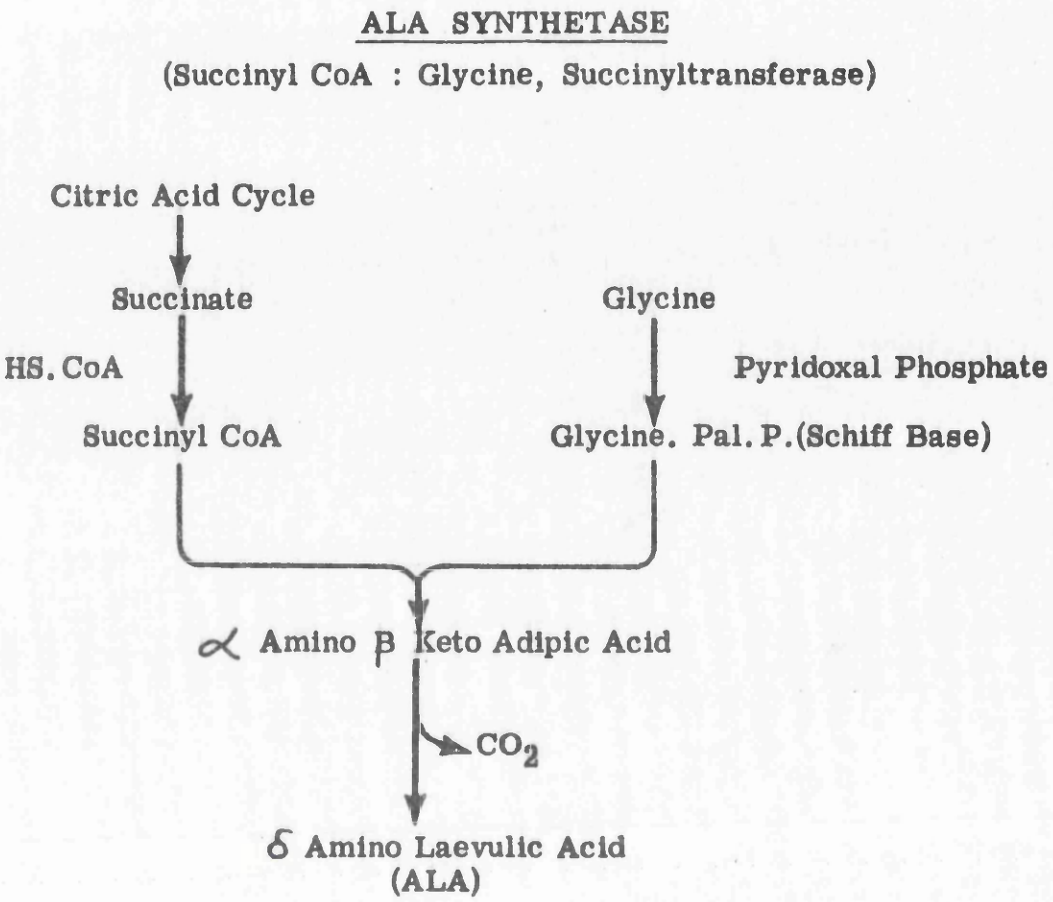

ALA SYNTHETASE
(Succinyl CoA : Glycine, Succinyltransferase)
Citric Acid Cycle
Succinate
HS. CoA
Succinyl CoA
Glycine
Pyridoxal Phosphate
Glycine. Pal. P.(Schiff Base)
α Amino β Keto Adipic Acid
CO_2
δ Amino Laevulic Acid
(ALA)

Fig. 1.

mammalian tissues (Kowalski et al, 1957). However, in the work on R. spheroides it was found that the reverse reaction was more easily studied because the equilibrium lay to the side of ALA, and in consequence this pathway must be of small importance in this biosynthetic sequence. (Gibson et al, 1961). , although other intermediates of this type have recently described by Shigesada et al (1970) from Rhodospirillum rubrum.

The monopyrrole precursor of the porphyrins, porphobilinogen (PBG), was first isolated from porphyria urine by Westall in 1952, and its structure determined by Cookson and Rimington (1953, 1954). The formation of PBG represents the first committed step in porphyrin biosynthesis. It is formed by the condensation of two molecules of ALA with elimination of water by the enzyme ALA Dehydrase (ALA.D) which was first described by Dresel & Falk (1953) and then by Gibson, Neuberger & Scott, (1955).

The subsequent steps of this pathway involve formation of the tetrapyrrole porphyrin ring from four units of PBG followed by the successive decarboxylation of the octacarboxylic porphyrin thus formed to the dicarboxylic porphyrin, protoporphyrin. The initial step of this sequence is catalysed by porphobilinogen deaminase which condenses the porphobilinogen monomer units into a polymer of indeterminate length, poly N. pyrryl methane. This polymer is then cyclised into uroporphyrinogen units, a tetrapyrrole (Bogorad, 1958a and b; Cornford, 1964). If the enzyme uroporphyrinogen isomerase or synthetase is present this reaction produces the biologically viable series 3 isomer; if not, it produces the series 1 isomer. In natural systems the series 1 isomer cannot be converted into protoporphyrin and will only produce uroporphyrin 1 and coproporphyrin 1 which are excreted in the urine.

Coproporphyrinogen the subsequent substance on the pathway is produced by the successive decarboxylation of uroporphyrinogen by uroporphyrinogen decarboxylase with the series 3 isomer being decarboxylated twice as rapidly as the series 1 isomer (Mauzerall & Granick, 1958).

By the action of the mitochondrial enzyme coproporphyrinogen oxidase and decarboxylase the tetracarboxylic coproporphyrinogen 3 is converted to protoporphyrin 9 through the intermediate tricarboxylic porphyrinogen and dicarboxylic porphyrinogen stages. It is these enzymes that cannot handle the series 1 isomer (Batlle et al 1965; Sano & Granick, 1961).

The final enzyme of the pathway is Haem Synthetase or Ferrochelatase (Goldberg, 1956; Labbe & Hubbard, 1960; Porra & Jones, 1963) which catalyses the insertion of ferrous iron into protoporphyrin to form Haem. This whole sequence, shown overleaf (fig.2) is reviewed in detail by Goldberg & Rimington (1962).

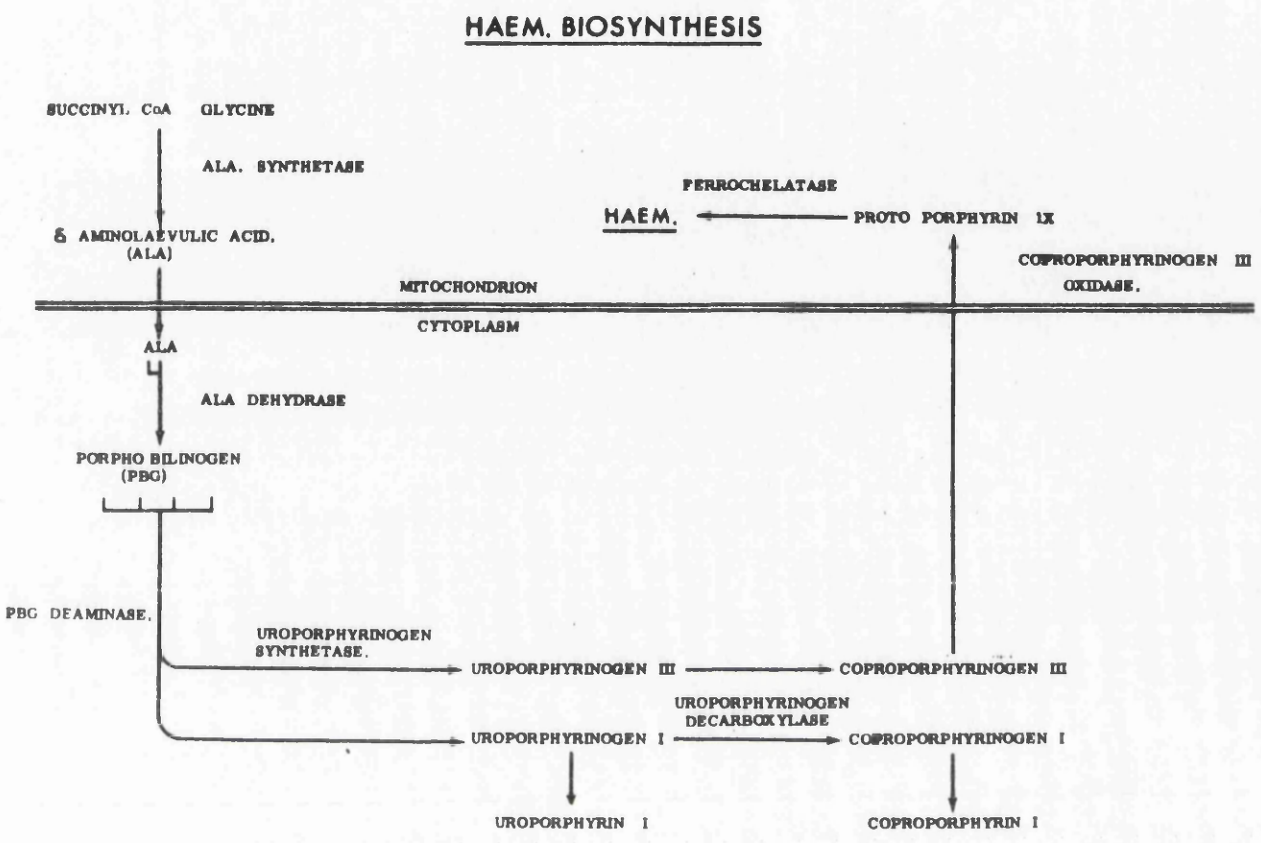


Fig. 2. The Haem Biosynthetic Pathway.

ALA SYNTHETASE

ALA synthetase is the initial and rate limiting enzyme of haem biosynthesis (Granick & Urata, 1963) and is the main subject of this study. By virtue of its properties, a short half life, 1 hour in mammalian systems (Marver et al, 1966; Stein et al, 1970) 4-6 hours in avian systems (Granick 1966) with an equally short half life of its messenger RNA; low endogenous activity, and its position at the beginning of a biosynthetic sequence, it might be expected that this enzyme would be one of the principal controlling influences on the haem biosynthetic pathway.

One endogenous controlling factor of this enzyme is Haem. End product repression of ALA.S by haem or haemin is well characterised. In bacteria, Lascelles (1960) and Burnham & Lascelles (1963) showed that haem participates in end product repression, whilst in eucaryotic cells a similar effect occurs (Granick, 1966; Marver et al, 1968). Welland et al (1966) showed that haemin inhibited or repressed ALA.S in liver at a concentration of 10^{-3}M and Vavra (1967) showed the same using chicken mitochondria. Other authors have been unable to detect this effect (Neuwirt et al, 1969 a and b) but this may in part be due to the relative crudity of their systems and to the presence of protein, since Scholnick et al (1969) have shown that haem is bound non-specifically by a number of proteins. ALA.S and Haem synthetase are mitochondrial enzymes (Granick & Levere, 1964) and although it had been suggested that compartmentalisation is a possible control point in the pathway there is little evidence that compartmentalisation is a controlling factor (Sano & Granick, 1961). Scholnick et al (1969) demonstrated cytoplasmic ALA.S in the liver cytosol and suggested that it was in transit to the mitochondrion. This is quite conceivable since it is possible that the enzyme is synthesised in the cytosol on the rough endoplasmic reticulum and transported from there

to the mitochondrion. This cytoplasmic enzyme is probably inactive since there is no extramitochondrial supply of Succinyl CoA in mammalian cells (Granick & Urata, 1963). The presence of the cytoplasmic enzyme could explain the discrepancy between the half lives of 1 hour for ALA.S. and about 7 days for the bulk of mitochondrial protein (Schimke et al, 1968; Druyan et al, 1969), McKay et al (1968, 1969) showed the mitochondrial enzyme to be positioned similarly to two other mitochondrial matrix enzymes, malate dehydrogenase and glutamate dehydrogenase and have concluded that it is also a mitochondrial matrix enzyme.

Within the mitochondrion various other aminoketones are synthesised from glycine other than ALA (Urata & Granick, 1963). Glycine combines with other compounds in their activated coenzyme A forms such as acetyl CoA to form these aminoketones. The general reaction for this series of reactions may be represented as :

$$R\ CO.S.\ CoA. + CH_2 . NH_2 . COOH \xrightarrow[PAL.P]{\text{synthetase}} R.\ CO.\ CH_2NH_2 + CO_2 + HS\ CoA$$

For ALA formation the enzyme is of course ALA.S, this being a different enzyme to aminoacetone synthetase although it has some aminoacetone synthetic activity (Dale, 1969) and the activated compound is succinyl CoA. This reaction was demonstrated in chicken erythrocytes by Gibson et al, (1958), and in R. spheroides by Kikuchi et al (1958). In addition to the formation of ALA, the other aminoketone produced in quantity is aminoacetone which uses as substrate Acetyl CoA (Nemeth et al, 1958). In this reaction sequence, pyridoxal phosphate (PAL.P) activates the glycine by forming a Schiff base with it. This is followed by condensation to a pyridoxalimine which decarboxylates whilst still on the pyridoxal with subsequent detachment of the δaminolaevulic acid. The Schiff base that is formed would be more stable when chelated by a metal ion, and indeed ferrous iron seems to fill this role. Brown, (1958) showed that ferrous iron at a concentration of 10^{-3}M depressed ALA.S activity which activity was restored on storage, presumably due to the

oxidation of the ferrous to ferric iron. These results were confirmed by Morrow et al (1969) who further showed that ferrous iron at a lower concentration, 10^{-6}M activated the enzyme and that ferric iron, at all concentrations used, caused a small depression of activity, although Stein et al (1970) have shown a synergistic effect of ferric citrate and AIA on ALA.S induction. In the scheme shown on the opposite page it is proposed that ALA.S is joined to the glycine-pyridoxal phosphate complex by an imine bond to the glycine nitrogen. The rest of the scheme is as described previously (fig.3.).

The Colourimetric determination of ALA depends on the formation of an'Ehrlich's positive' pyrrole. This is achieved by a condensation with acetylacetone followed by complexing of the α carbon of the pyrrole thus formed with Ehrlich's aldehyde reagent (p dimethyl amino benzaldehyde) (Mauzerall & Granick, 1956). This is of consequence since in biological systems other interfering aminoketones are present, principally aminoacetone, which also forms a pyrrole with acetyl acetone. This factor is taken into account in both ALA.S assays used in this work. In both assays conditions are such that they minimalise aminoacetone production whilst optimalising ALA production, and in one assay there is a further step which separates the pyrroles of these compounds by ion exchange chromatography.

At the beginning of the pathway, there is a possible relationship between the activities of ALA.S and succinyl CoA synthetase (E.C. 6.2.1.5.). This enzyme does not properly lie on the pathway but supplies succinyl CoA from the citric acid cycle which is essential to the working of the pathway. Most of the succinyl CoA required for this reaction is generated from succinate and coenzyme A in the presence of NADPH by the enzyme succinyl CoA synthetase (Shemin & Kumin, 1952; Brown, 1958; Granick & Urata, 1963). . Other

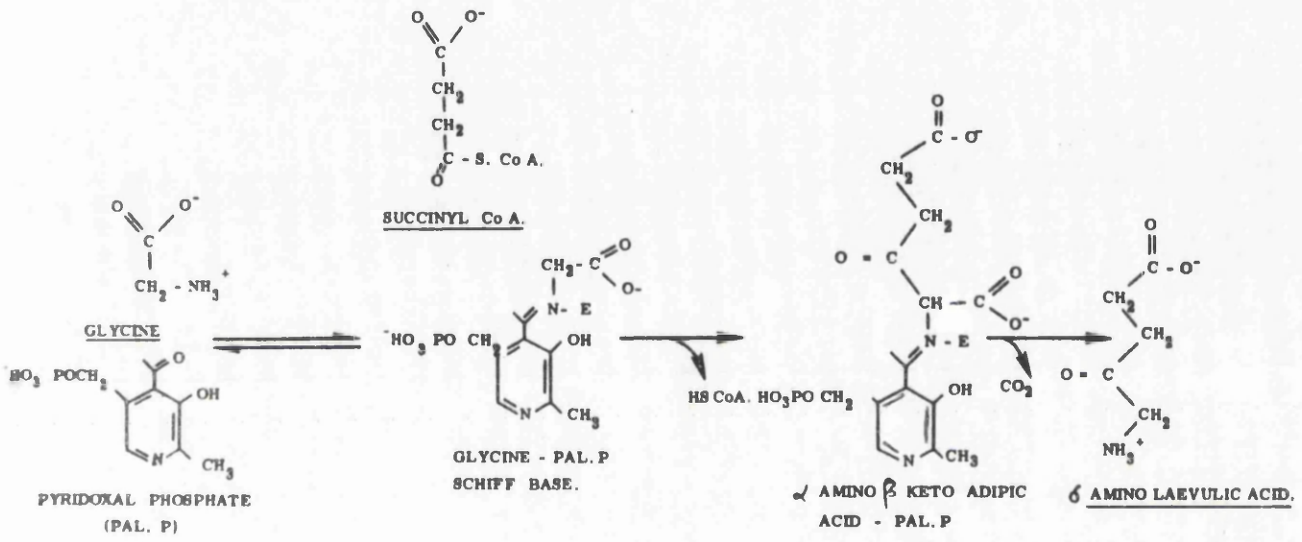


Fig. 3 Postulated Mechanism of Action of ALA synthetase.

E. in the figure represents the enzyme.

mechanisms for the formation of succinyl CoA from propionyl CoA through methylmalonyl CoA (Stadtman et al, 1960) and from acetoacetyl CoA via a CoA transferase to succinate (Stern et al, 1956) have been described in mammalian systems but their physiological relevance to ALA formation is uncertain. It was found that malonate used to inhibit succinate dehydrogenase, and thus leading to an accumulation of succinate, led to a 60% increase in ALA production (Miyakoshi & Kikuchi, 1963; Ludwig et al, 1965; Gajdos et al, 1966). This suggests that there had been some substrate induction of ALA.S by succinate. In experimental porphyria however, neither Granick (1966) nor Yoshida & Ishihara (1969) could find evidence for elevation of this enzyme although Labbe et al (1965) had shown a 70% increase in succinyl CoA synthetase.

All Porphyrias, hereditary, acquired and chemical are due to the overproduction rather that under-utilisation of porphyrins, and the de novo cause of the overproduction is the increased activity of the initial and rate limiting enzyme of haem biosynthesis, ALA.S. For this reason I choose to define porphyria in this thesis as an overproduction of ALA synthetase, rather than defining it in terms of the secondary products of the biosynthetic pathway. Thus it might be said that porphyria is due to the induction of ALA synthetase activity.

Many compounds have been used in the production of experimental porphyria in animals. The first observation was made by Stokvis (1955) who observed that excretion of 'haematoporphyrin' could be produced in rabbits by Sulphonal. Subsequently one compound described to be porphyrinogenic was sedormid (allyl isopropyl acetyl urea) (Schmid & Schwartz 1952). In the following years, Goldberg (1953) and Goldberg & Rimington (1954) described the compound allyl isopropyl acetamide (AIA) a non-hypnotic derivative of sedormid, with a very high porphyrinogenic activity. (fig.4.). This compound

2. ALLYL 2. ISOPROPYL ACETAMIDE.

(AIA)

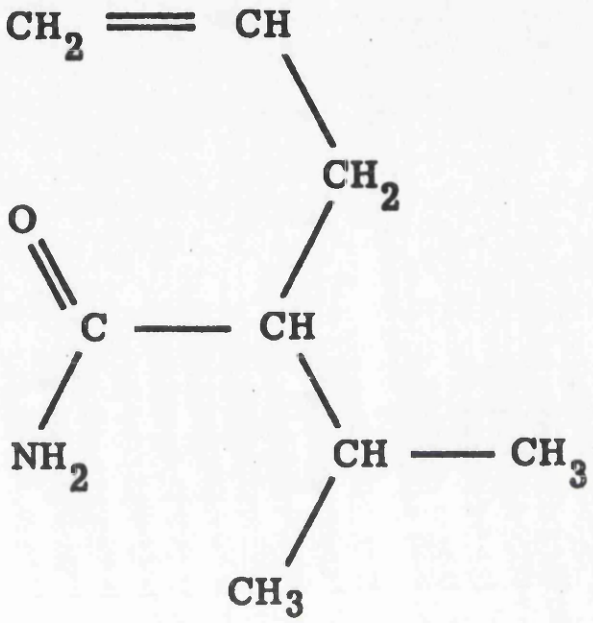


Fig 4. Structure of A.I.A.

is used in this thesis as a reference point in the measurement of porphyrinogenesis. Since then many other compounds have been described with porphyrinogenic activity (de Matteis, 1967). In 1966 Granick described a method of culture of chick embryo liver on coverslips, whereby he was able to screen a large number of compounds for porphyrinogenic activity, measuring this activity by the appearance of porphyrin fluorescence on the cover slips under ultraviolet light. Of the compounds tested in this manner, AIA, Meprobamate, Glutethimide, Methyprylon, Griseofulvin, DDC and some steroids were the strongest inducers of fluorescence. He further attempted to relate porphyrinogenic activity to their chemical structure, with some success.

Most papers subsequent to this quantitate the enzyme activity. Among the factors implicated in the exacerbation of porphyria is Diet. It was found that starvation increases the level of ALA synthetase and that carbohydrate feeding of rats made porphyric with AIA led to an alleviation of the porphyria and markedly inhibited the elevation of ALA.S (Rose et al, 1961; Welland et al, 1964; Tschudy et al, 1964). This was found to apply also to human porphyria (Welland et al, 1964). Simons (1970) describes a twofold induction of ALA.S by glucagon. In view of the above dietary work, this may be due to a depression of hepatic glycogen levels which is stimulated by glucagon mediated by cyclic AMP (Waxman et al, 1966).

In porphyria increased glycogenolysis and glucose consumption leads to lowered ATP levels in the cell. Thus Gajdos & Gajdos-Török (1969) showed that the ATP levels dropped before ALA rose and in consequence considered that the cellular redox state gave some control over ALA.S activity. They found in R. spheroides that ATP inhibited the production of porphyrins and further

that this depression was related to the ATP concentration. In rats treated with AIA and DDC similar findings were made, and in addition the level of NADH rose to a peak three hours after administration of these chemicals. These facts prompted Cowger and Labbe (1967) to suggest a block in the respiratory chain at the level of reoxidation of reduced NADH. There was no correlation however between these levels and the levels of porphyrins (Gajdos & Gajdos-Török, 1968; 1969). Thus it was shown that ATP depression precedes ALA synthetase induction in AIA induced porphyria. In comparison in AIA treated rats the induction of ALA.S in mitochondria could be abolished by atractyloside which was used to inhibit the translocation of adenine nucleotides across the inner mitochondrial membrane. (Bruni et al, 1964). Finally under low oxygen pressure, Gajdos et al (1969) found that DDC porphyrinogenesis was depressed. This effect was thought to be due to a stimulation of glycolysis with a subsequent elevation of ATP levels which lowered porphyrinogenesis and ALA.S induction.

Since purine derivates have been found to inhibit porphyrin biosynthesis (Gajdos & Gajdos-Török 1963) Adenosine 5 Monophosphate (AMP) has been used in the treatment of patients with porphyria, sometimes with apparently successful results but it is difficult to theorise on this disease because of natural remissions (Gajdos & Gajdos Török, 1961; Levrat et al, 1963; Oaks et al, 1969; Wetterberg, 1964). Recently however in a properly controlled study, Wetterberg (1970) concluded that it was not possible to show any difference in the overall state of health or biochemical tests of porphyria in patients receiving AMP or a placebo. In consequence it is doubtful whether these nucleotides play an important role in porphyria.

Bottomley & Smithee (1968) described a method for the measurement of ALA synthetase in bone marrow cell mitochondria, in the following year they published a paper showing that erythropoietin stimulated ALA synthetase but was ineffective in elevating haem synthetase in rabbit bone marrow culture. The work of Paul & Hunter (1969) would appear to corroborate this since they demonstrated an induction of haem synthesis by erythropoietin in mouse embryo liver culture. Thus the stimulation of erythropoiesis by erythropoietin would appear to require the initial production of haem, this process being initiated at the level of ALA synthetase by erythropoietin. The progress in this work to date is reviewed by Gordon (1970).

Woods & Dixon (1969; 1970) have shown that in the perinatal period the normal activity of rabbit ALA.S was 4-8 times greater than that of the adult animal. Song et al, (1968) found that newborn rats are refractory to induction of ALA synthetase by DDC and AIA. This was confirmed by Woods & Dixon in rabbits. These facts are of consequence in the problems of haem metabolism in the neonate where physiological jaundice and the inability to metabolise various drugs may be associated with an inability to regulate the activity of ALA synthetase.

Of metals other than iron, only the heavy metals would appear to affect porphyrinogenesis. Lead is well known to inhibit ALA.S (Morrow et al, 1969) and arsenic in the same grouping will do the same (Schwartz & Zagaria, 1951). Rubino (1961) found that the inhibiting concentration of lead in vivo corresponds to an 'in vitro' concentration of 10^{-5} - 10^{-6} M leading to an inhibition of 12 - 22%.

Much of the work carried out on control of haem biosynthesis has been carried out on micro-organisms. Indeed the general concept of induction and repression (Jacob & Monod, 1961) has only been demonstrated in bacteria.

Lascelles (1959) showed that under conditions of low aeration Bacteriochlorophyll synthesis in R. spheroides begins after a short lag and this is preceeded by a rapid rise in ALA synthetase activity. It was then shown that when these cultures were aerated this bacteriochlorophyll synthesis and ALA synthetase activity fell rapidly (Lascelles, 1960; Higuchi et al, 1965; Higuchi et al, 1968). Lascelles (1968) considers that this is in fact a repression by high aeration and further that haem also acts as a regulatory factor. In addition ALA acts as an inhibitor although this may be due to its conversion into haem. Marriot (1968) and Marriot et al (1968) suggested that in R. spheroides this decrease in ALA.S is caused by a decrease in a low molecular weight activator and increase in a low molecular weight inhibitor, to account for the gross changes in ALA.S.

This inhibition is reversible and Tuboi et al (1969) showed that this inhibitor was labile and *not* identical with haem . One problem at this stage is that it would appear that in R. spheroides at least, there are two different ALA synthetases of similar molecular weight but varying in physical properties which means that in this bacterium there are two control systems available for the generation of this enzyme (Tuboi et al, 1970 a). Finally in S. Aureus, Tien & White (1968 showed that a population of mutants for the production of protoheme fell into five classes, and these five classes represented five enzymatic lesions in the haem biosynthetic pathway. The genes for these lesions were found to be tightly linked and co-transducible and arranged in the same sequence as the reactions of the biosynthetic pathway. These facts fall in well with the concept of induction starting at ALA synthetase and proceeding through the genes to haem synthetase.

In the liver the process of control is postulated to be of a similar form, with the genes for this pathway linked in a similar manner.

Chemical porphyrinogens are thought to derepress the operator gene and lead to uncontrolled porphyrin biosynthesis. The only physiological substances yet shown to have porphyrinogenic activity are steroids; some of these compounds are active in the free or sulphate form but inactive in the glucuronide form (Granick & Kappas 1967 a and b). Further, it has been shown that the induction was prevented by metalloporphyrins such as haemin; UDP glucuronic acid and inhibitors of protein and nucleic acid biosynthesis (Kappas & Granick, 1968). Finally, Goldberg et al (1969) have demonstrated that some of these steroids are elevated in the urine of porphyric patients in attack and that these steroids are porphyrinogenic in rats. On the basis of this work a proposed model of control in porphyrin biosynthesis is shown overleaf (Fig. 5). ALA synthetase production is controlled by an aporepressor and a co-repressor, haem. Haem combines with the aporepressor to form firstly the active repressor which acts on the operator gene, repressing the biosynthetic pathway which in the final analysis stops producing haem. If drugs or chemicals or endogenous compounds such as the steroids, displace this haem from the repressor or by other means stop production of aporepressor or block the operator gene, the whole pathway is derepressed and there is an uncontrolled production of porphyrins. The endogenous steroids may be inactivated by glucuronide formation; the other compounds are either oxidised by systems such as cyt P450 or detoxified by glucuronide formation and rendered inactive. Thus it is likely that in the liver porphyrin synthesis is controlled by induction and repression. In the absence of an inducer the activity of ALA synthetase falls rapidly due to its very short half life since it has never been shown that ALA synthetase can be activated or that changed conditions can alter its half life (Stein et al, 1970).

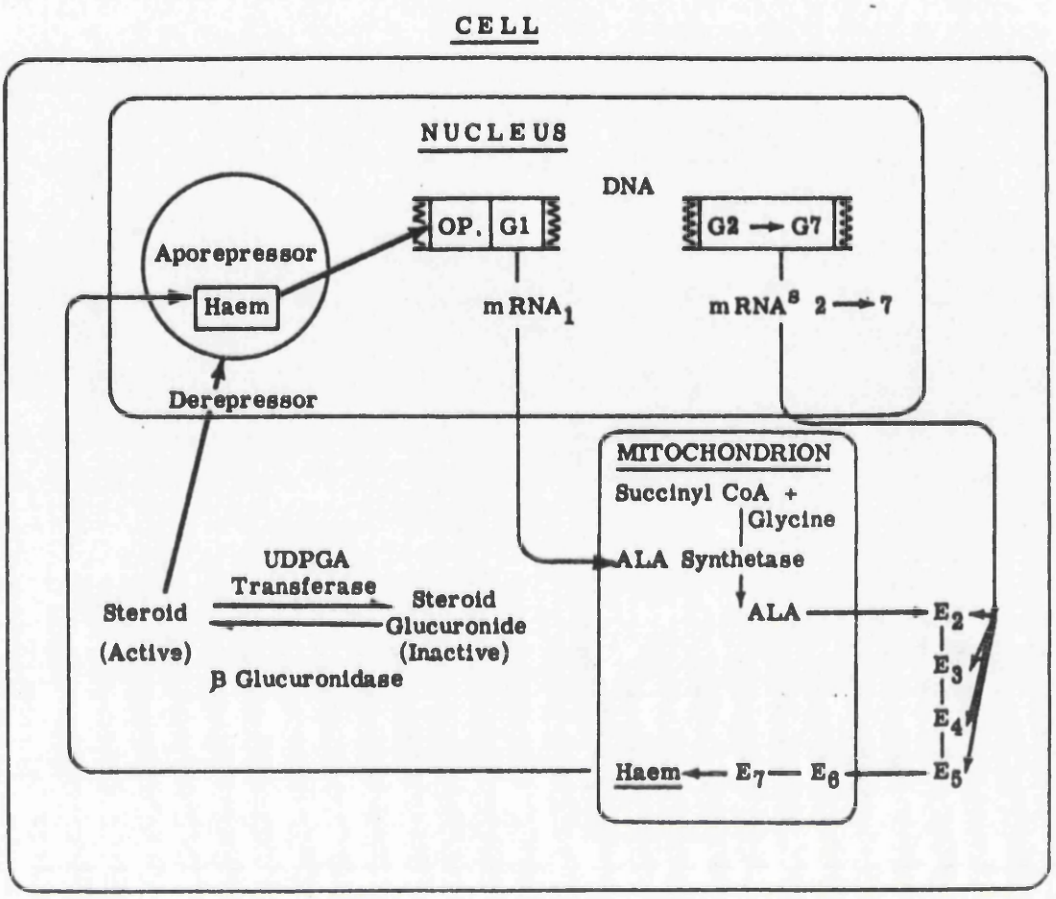


Fig. 5. Possible scheme of control of the Haem biosynthetic pathway. OP - Operator gene: G1 → G7 :- Structural genes: $E_2 \rightarrow E_7$:- enzymes of the pathway: UDPGA - Uridine diphosphoglucuronic acid.

ALA DEHYDRASE

The second enzyme of the haem biosynthetic pathway catalyses the condensation of two molecules of ALA to form the monopyrrole Porphobilinogen (PBG). Although two bonds are formed, this requires only one enzyme, ALA dehydrase (5 aminolaevulinate hydrolyase, EC 4,2,1,24) (Granick & Mauzerall 1958). Bogorad (1960) has summarised the properties of several purified preparations of this enzyme. Unlike ALA.S this enzyme is firstly detectable in quantity in many animal, plant and bacterial cells, at a very much higher specific activity than ALA.S. It is secondly a cytoplasmic enzyme which means that in eucaryotic cells, ALA produced within the mitochondrion by ALA.S must pass out of the mitochondrion before it can be used by ALA.D for PBG production. Finally Doyle and Schimke (1969) showed that the half life of the mouse enzyme is 5-6 days as opposed to the 1 hour of ALA.S. They further showed that the perinatal development of the enzyme, proceeds from a high specific activity just before birth (about twice adult level) decreases rapidly after birth and then rises slowly to adult levels after about 3 weeks.

The enzyme has been purified from many sources; from ox liver (Gibson et al, 1959); cow liver (Batlle et al, 1967); human erythrocytes (Callissano et al, 1966); yeast (De Barriero, 1968); Soy-bean callus tissue (Tigier et al, 1968); wheat leaves (Nandi & Waygood, 1967); R. spheroides (Burnham & Lascelles 1963; Nandi et al, 1968) and tobacco leaves (Shetty & Miller, 1969).

In some cases the preparation thus produced is electrophoretically homogeneous. ALA dehydrase is a sulphydryl enzyme in which activity can only be demonstrated in the presence of thiol compounds and requires highly reducing conditions for activity.

Although these factors are common in general for ALA.D, the effect of EDTA and various cations varies with the enzyme source. Coleman (1966) noted activation by EDTA in mouse preparations, yet in most other preparations EDTA inhibits enzyme activity (Gibson et al, 1955). Nandi & Waygood (1967) reported that the inhibition was reversed by Mn^{2+}, and Fe^{2+} was reported to activate the mouse enzyme liver (Coleman, 1966), yet to inhibit in oxliver (Gibson et al, 1955) and in tobacco leaves (Shetty & Miller, 1969). In wheat leaves the enzyme was unaffected by Fe^{2+} and activated by Mn^{2+} and Mg^{2+} over a wide range of concentrations (Nandi & Waygood, 1967), yet in Rhodopseudomonas spheroides Mn^{2+} activated only at low concentrations and inhibited at higher concentrations (Nandi et al, 1968). In addition the enzyme from R. spheroides requires K^{+} for activation (Burnham & Lascelles, 1963; Nandi et al, 1968).

An activator of ALA.D is reduced glut athione (Nandi et al, 1968) and most assays of ALA.D use some reduced sulphydryl compound (De Barriero, 1969). In these experiments reduced glut-athione (GSH) is used. In Rhodopseudomonas spheroides Nandi & Shemin (1968) showed the enzyme to have the properties of an allosteric enzyme (Monod et al, 1965) with an equilibrium mixture of three species, a monomer, dimer and trimer in the presence of K^{+} Rb^{+} or NH_4^{+} ions, In the absence of these only monomer is present. The partially purified ox liver enzyme has been found to be highly specific with respect to substrate, neither higher nor lower homologues of ALA acting as substrate (Gibson et al, 1955).

The formation of porphobilinogen from two molecules of ALA is essentially a Knorr readion which involves firstly an aldol condensation, an elimination of water from the carbon atom β to the carbonyl group and the adjacent carbon atom, and the formation of a Schiff base between the carbonyl group of one molecule and the amino group of the other with the elimination of another molecule of water. Since this reaction is catalysed by one enzyme the mechanism of the synthesis must take

account of the sequence of these reactions. Shemin (1968) and Nandi & Shemin (1968) suggested the sequence shown overleaf (fig.6) using the formation of heterologous pyrroles to prove the sequence. The sequence then is that one molecule of ALA forms a Schiff base with the enzyme. This is followed by a nucleophilic attack by the enzyme - ALA anion on the carbonyl of a second ALA molecule. This aldol eliminates water and the free amino group of the 2nd molecule displaces the amino group of the enzyme by transamination or transaldimination to form porphobilinogen.

In animals several factors are known to affect ALA.D activity. Of these lead is the best known inhibitor of activity (Dresel & Falk, 1956 a and b; Lichtman & Feldman, 1963; Gibson & Goldberg, 1970; Morrow et al, 1970). The finding that the enzyme activity is depressed in erythrocytes by lead provides a sensitive index of lead poisoning in humans (Hernberg & Nikkanen, 1970; De Bruin, 1968; Bonsignore, 1966; Nakao et al, 1968). Although it has been reported that GSH will reverse the depression of ALA.D by lead (Lichtman & Feldman 1963; De Barriero, 1969), Gibson & Goldberg (1970) found that it was only partially reversed and suggested that it had another inhibitory role.

Other factors implicated in affecting of ALA dehydrase are monoamine oxidase inhibitors. Meo et al (1965) showed an increase in PBG in rat tissues and in man subsequently to an increase in the blood and urine content of amino ketones and PBG (Bonzanio & Meo, 1965). Iproniazid leads to an increased in vitro synthesis of PBG in rat liver (Rubino, 1965) and similarly to an increase of blood and urine levels of aminoketones and PBG and the same results were found with Nialamide [(1, (2 benzcarbonyl) ethyl) 2 iso nicotinyl (hydrazine)] (Pisani et al 1968. They explain the mechanism of action by suggesting that inhibition of monoamine oxidase and therefore of ALA oxidase (Pisani et al, 1968) which as a step of the Shemin cycle oxidatively deaminates ALA to 2 keto glutaraldehyde, leads to a rerouting of this ALA through ALA.D to PBG. A more likely explanation

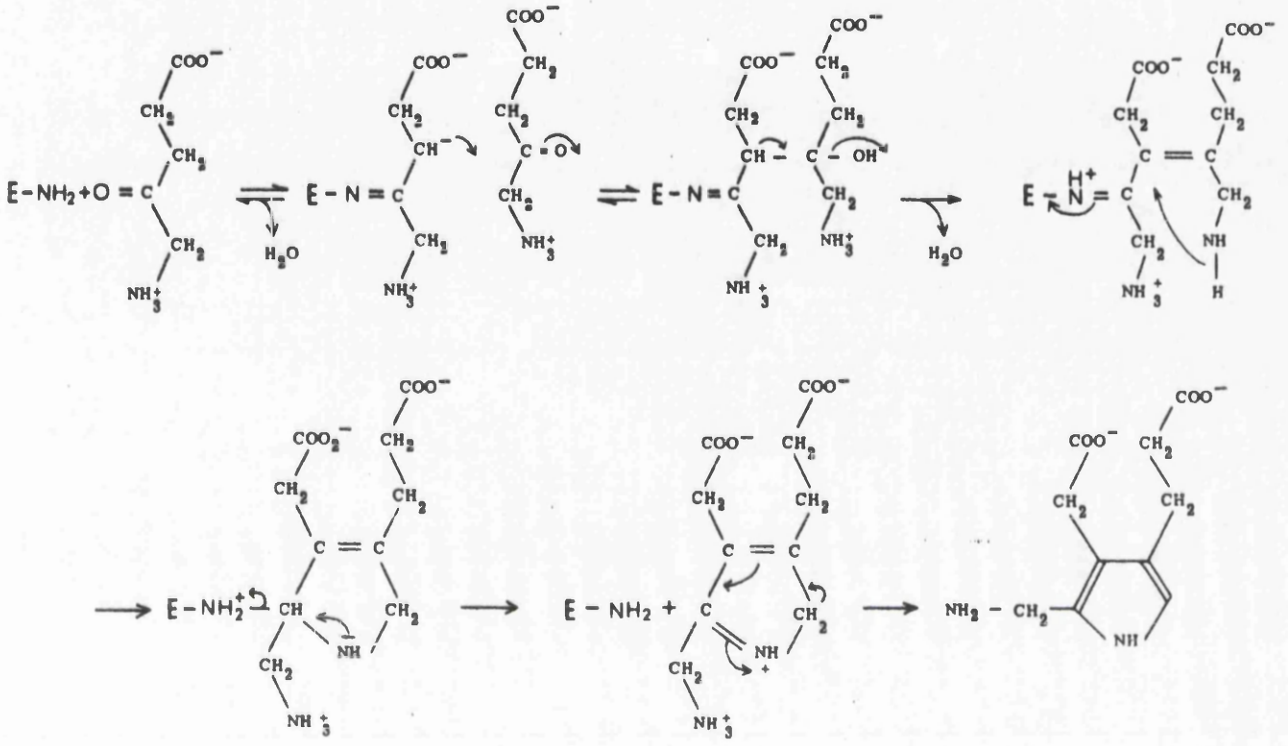


Fig. 6. Mechanism of action of ALA dehydrase, E represents the enzyme.

is however that these compounds are acting in this manner similarly to EDTA, by virtue of their chelating ability and thus reducing enzyme activity.

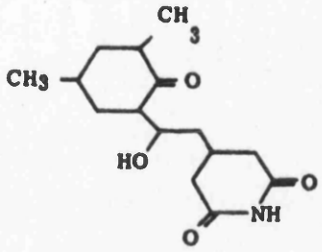
In humans certain forms of anaemia appear to affect ALA dehydrase in the blood. Battistini et al (1970) described an elevated level of ALA dehydrase in macrocytic anaemias both in vitamin B12 and folic acid deficiency, although there was no significant difference from normal in iron deficiency anaemia or in the anaemias of rheumatoid arthritis and carcinomatosis. In another form of cancer, Leukemia, Takaku et al (1968) found depressed levels of PBG production in the leucocytes in acute and chronic myelogenous leukemia this being associated with elevated ALA.S and depressed haem synthetase.

ANTIBIOTICS

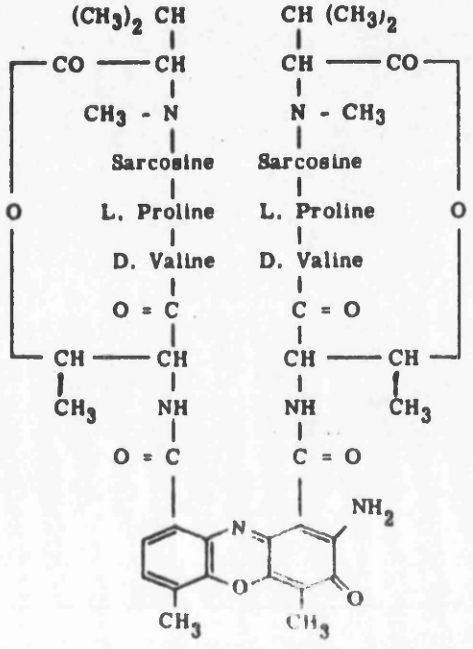
In much of the work carried out on animals in these studies it was important to determine whether or not the elevation of enzyme activity by some substance was an induction or was due to activation or decreased breakdown of the enzyme. Induction, by definition, is the 'de novo' synthesis of enzyme protein. To determine whether or not there was synthesis of protein, it was decided to use the antibiotics Actinomycin D and cycloheximide, inhibitors of nucleic acid and protein synthesis respectively (fig.7.)

The ability of Actinomycin D to bind to the DNA molecule has long been recognised, and it is on the basis of this that it has its inhibitory effect on the DNA dependent synthesis of RNA (Reich & Goldberg, 1964), blocking the use of DNA as a template for RNA polymerase. Further, at higher concentrations it inhibits DNA polymerase. It has a toxic effect on the cell, which may be determined by the degree of binding to DNA. In this context there may be some similarity between the effects of ionising radiation and Actinomycin D (Elkind, 1967; Elkind, 1968). The studies of Bachetti and Whitmore (1969) suggest that Actinomycin D is a potent cytostatic and cytotoxic reagent which is a potent inhibitor of RNA synthesis but which inhibits at concentrations which have an effect on DNA synthesis and cell viability. At lower concentrations Actinomycin D acts as an activator (Schwochau & Hadwiger 1968; De Matteis 1968; Rosen et al 1964). The action in this way may cause derepression of the gene by combination of the Act.D. with the regulator gene leading to a derepression of the structural gene (Pollock, 1963). On the basis of their data, Schwochau & Hadwiger (1969) proposed that the mode of activation was by preferential reaction with the guanine moiety of double stranded DNA changing the base configuration over at least 6 base pairs and resulting in an improper attachment of the respective repressor to this site leading to a derepression of the gene.

ANTIBIOTICS



CYCLOHEXIMIDE



ACTINOMYCIN D.

Fig. 7. The Structures of Cycloheximide and Actinomycin D.

Cycloheximide (Actidone; 2 hydroxy 2 - [2 oxo 3. 5 dimethyl cyclohexyl] ethyl glutarimide) is an antibiotic, extracted from Streptomyces griseus (Sisler-Siegel 1967) which inhibits protein synthesis in yeasts (Kerridge, 1958; Siegel & Sisler 1964, 1965; Widuczynski & Stoppani, 1965) and higher organisms, rabbits (Young et al, 1963) mice (Trakatellis et al, 1965) rats (Gorski & Axman, 1964; Tornheim et al 1969; Yeh & Shils, 1969) and in mammalian cell culture (Bennet et at, 1964) yet it does not affect bacterial protein synthesis (Fukumara, 1965). The mechanism of action of this compound involves the processes of protein synthesis at more than one point. It does not affect the charging of transfer RNA with amino acids (Ennis & Lubin, 1964) but in fact acts on two of the sites of a postulated 3 sites on the ribosome. (Culp et al, 1969). The sites acted on are the donor and acceptor sites. Cycloheximide inhibits the movement of peptidyl t. RNA from the aminoacyl (donor) to the peptidyl (acceptor) sites on the ribosome. (Baliga et al, 1969; McKeehan & Hardesty, 1969).

Baliga et al (1969) in their studies on rat liver have also shown an inhibition of polysome aggregation at the initiation of protein biosynthesis and a reduction of inactivation by GSH suggesting the inactivation of the enzyme involved at this stage, aminoacyl transferase II. Tornheim et al (1969) also found that in addition to causing a 69% inhibition of protein synthesis in rat liver with a slight inhibition of RNA synthesis, there was a two fold increase in DNA, Phosphorus. However this occurred as a gentle rise as opposed to the abrupt cessation of protein synthesis and in consequence suggested a separate effect of cycloheximide. Since cycloheximide is often used to determine the rate of protein turnover it has been suggested that its effect might be due to change in the rate of protein degradation. The work of Feldman & Yagil (1969) showed that in the presence of cycloheximide, there is no change in the rate of protein turnover in cell culture. Further this inhibitory effect is reversible after 12 hours at a dosage of 1 µg/g. body weight which causes an initial 95% inhibition of protein

synthesis. Similarly in a study of quantitative aspects Yeh & Shils (1969) have shown that at a dosage of 0.5 mg/kg the level of protein synthesis is minimal for about 3 hours, rising to normal levels after about 16 hours. In view of the results from this paper cycloheximide has been used at a dosage of 1mg/kg to inhibit protein synthesis in the following studies. Since larger dosage of this drug leads to secondary changes in organ function such as renal change. Fig.7 shows the structure of the two antibiotics used in these experiments. Their mode of action as explained previously is shown in the diagrammatic representation of protein biosynthesis in fig.8.

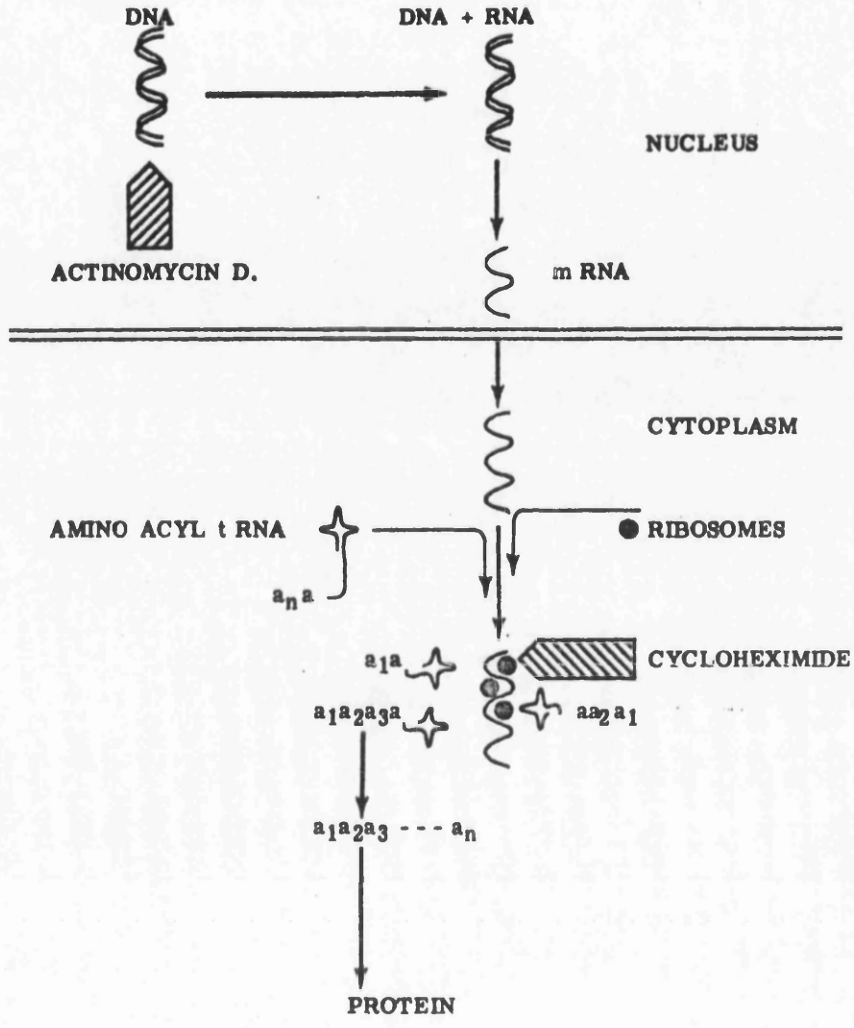


Fig. 8. Sites of action of Actinomycin D and Cycloheximide on the protein biosynthetic chain.

MATERIALS and METHODS

1) MATERIALS

Allylisopropyl acetamide was kindly donated by Roche, Welwyn Garden City, and CIBA laboratories Limited, Horsham, Sussex, donated Metopirone as its ditartrate salt. Actinomycin D, as 'Lyovac Cosmogen' and Dexamethasone as 'Decadron' its sodium phosphate salt were supplied by Merck, Sharp & Dohme, Hoddesdon, Hertfordshire. Coenzyme A, Acetyl Coenzyme A, Cycloheximide, Pyridoxal Phosphate and UDP Glucuronic Acid were supplied by the Sigma Chemical Company, St. Louis, Missouri. Vitamin E was used as DLαtocopherol acetate as supplied by British Drug Houses, Poole, England. Ethanol was used as Burroughs 95% ethanol diluted with distilled water. All steroids except DHA sulphate were supplied by Koch Light Limited, Colnbrook, Buckinghamshire. DHA sulphate was kindly donated by Dr. J.K. Grant of the department of Steroid Biochemistry, Royal Infirmary, Glasgow. The barbiturates used were supplied by the pharmacy, Western Infirmary, Glasgow, as were glutethimide, as 'Doriden' (CIBA); and Pentazocine as 'Fortral'. All other reagents were supplied by BDH.

Before commencing these experiments various means of assaying ALA synthetase were examined. The difficulty that previous workers experienced in the measurement of enzyme activity in non porphyric tissue may be explained by their difficulty in producing an enzyme preparation sufficiently quickly, since, due to its very short half life, the enzyme activity would have decayed to such an extent in any delay, as to make the activity unmeasurable. This failing is specific mainly to such assays that use mitochondrial preparations as the enzyme source; these assays also have the failing that some provision must be made for the production of one of the substrates, succinyl CoA. For these reasons it was decided to use a whole homogenate assay for ALA synthetase as described by Marver et al (1966).

ALA synthetase was assayed in rat and human liver by measurement of ALA production from glycine and endogenously produced succinyl CoA. Iron free glassware was used in both assays and was produced by soaking all glassware overnight in 'Cristaclean' (Hawksley) and washing in distilled deionised water. For rat tissues, the animals were killed by decapitation or cervical dislocation and the tissues rapidly excised and chilled in ice cold saline.

In the micromethod (Assay 1) the livers were then weighed and a weighed aliquot removed for enzyme assay. A 1 in 4 homogenate of liver was then made in 0.15M tris buffer pH 7.2 containing 0.02 M EDTA; , using a piston homogeniser (Tri R). An aliquot, 100 µl of this homogenate was then incubated anaerobically in the dark with 20 µmoles glycine and 10 µmoles sodium citrate at pH 7.4 in a final volume of 200 µl for 1 hour at 37°C. One tube had 200 µl 0.3M TCA added immediately to stop the reaction and thus to measure endogenous ALA; in the other tubes the reaction was stopped similarly after the 1 hour incubation.

The tubes were then allowed to stand in ice after thorough mixing for fifteen minutes; then spun at 2,000 g. for 5 minutes at 0°C and an aliquot of 200 µl of supernatant removed from each tube for assay and put into a boiling tube containing 200 µl H_2O 50 µl Sodium acetate (0.6M) and 20 µl acetyl acetone. The tubes were stoppered and boiled for exactly 10 minutes, cooled and 50 µl water added, and 500 µl of special Ehrlich's reagent added (Ehrlich/Mercuric chloride reagent). The tubes were allowed to stand for fifteen minutes to allow colour development and they were then read at 552 mµ in micro cells or semi-microcells. The ALA content was measured from a standard graph.

In the macro-method (Assay 2), a lobe of liver was taken, weighed and homogenised in 3 volumes of ice cold saline (0.9% NaCl) containing 0.5 mM EDTA and 10 mM Tris at pH 7.2. 1 ml of this was taken and incubated with 3 ml. incubation mixture (100 mM Glycine; 50 mM Tris 6mM EDTA) in 25 ml Erlenmeyer flasks in a shaking water bath. The reaction was stopped with 2 ml 2 M HCl and the flasks stood in ice for 10 minutes. The contents were then transferred to centrifuge tubes, spun at 2000 g for 10 minutes, and the supernatant taken, pH adjusted to 4-5 and 0.05 ml acetyl acetone added to the mixture. The tubes were boiled for 10 minutes and then cooled. This solution was then passed through a Dowex 1 x 8 column (2 cm x 1 cm) which had previously been equilibrated with 0.1M HCl/4M NaCl. The column absorbed the pyrroles and the eluate was discarded and the column washed through again with 0.1M HCl/4M NaCl and 10 ml H_2O. Aminoacetone pyrrole (2.4 dimethyl 3 acetyl pyrrole) was eluted from the column with 0.01 M NH_4OH in dry n. Butanol. ALA pyrrole (2 methyl 3 acetyl 3 propionic acid pyrrole) was then eluted with 10M acetic acid, 1 ml special Ehrlich's reagent was then added, the volume made up to 3 ml and read at 553 mµ after 15 minutes.

The details of both of these assays are given in the Appendix. Table 1 shows that there is no significant difference between the results of these

TABLE 1.

ALA Synthetase activities in normal Rats -

Comparison of Assays 1 & 2

Assay No.	No. of Rats.	ALA.S. Activity (µg/g/hr.)	Significance
1	12	6.87 ± 1.85	No Significant Difference.
2	12	6.42 ± 1.94	

two assays. In consequence the results are thought to be interchangeable. The important factor in both of these assays is that the conditions are determined such that one has firstly, maximal production of ALA with minimal production of aceto-acetate and little or no conversion of ALA to PBG. Figure 9 shows that at a concentration of 10mM EDTA there is little production of porphobilinogen. This inhibition of ALA dehydrase by EDTA is non-competitive and in addition in this system there are no added thiol activators of ALA dehydrase. There is a maximal production of ALA at a glycine concentration of 100 mM (Fig.10) and the concentration of succinyl CoA and its production in this whole homogenate system is non-limiting. For the 1 in 4 homogenate of liver ALA production is linear. (fig.11).

ALA dehydrase activity was assayed in erythrocytes and tissues by measurement of the porphobilinogen production from δaminolaevulic acid. Lead-free glassware was used throughout the estimation, since as other authors have shown the lead markedly inhibits ALA dehydrase activity 'in vivo!' The effect of lead on ALA dehydrase activity 'in vitro' is shown in fig.12. The physiological level of lead is in the region of 10^{-7} molar. For the assay 10 ml. of blood was withdrawn by venepuncture, transferred to a heparinised tube in ice and centrifuged at 1000 g. at $4^{o}C$ for twenty minutes. The plasma and white cell layer were discarded and the red cells washed in 0.15 M potassium chloride and centrifuged at 1000 g. for ten minutes. 8 ml. of 0.15 M potassium chloride was added to 2 ml. of the packed washed cells and 1 ml. of the resulting suspension removed for a red cell count. The sample was centrifuged again and the potassium chloride carefully removed. 5.4 ml. distilled water was added to the packed cells and the sample left on ice for twenty minutes to haemolyse. 1.8 ml. 0.6 M potassium chloride was then added to restore isotonicity. For tissues the enzyme preparation was obtained from an appropriate tissue homogenate in 0.15 M KCl at $4^{o}C$.

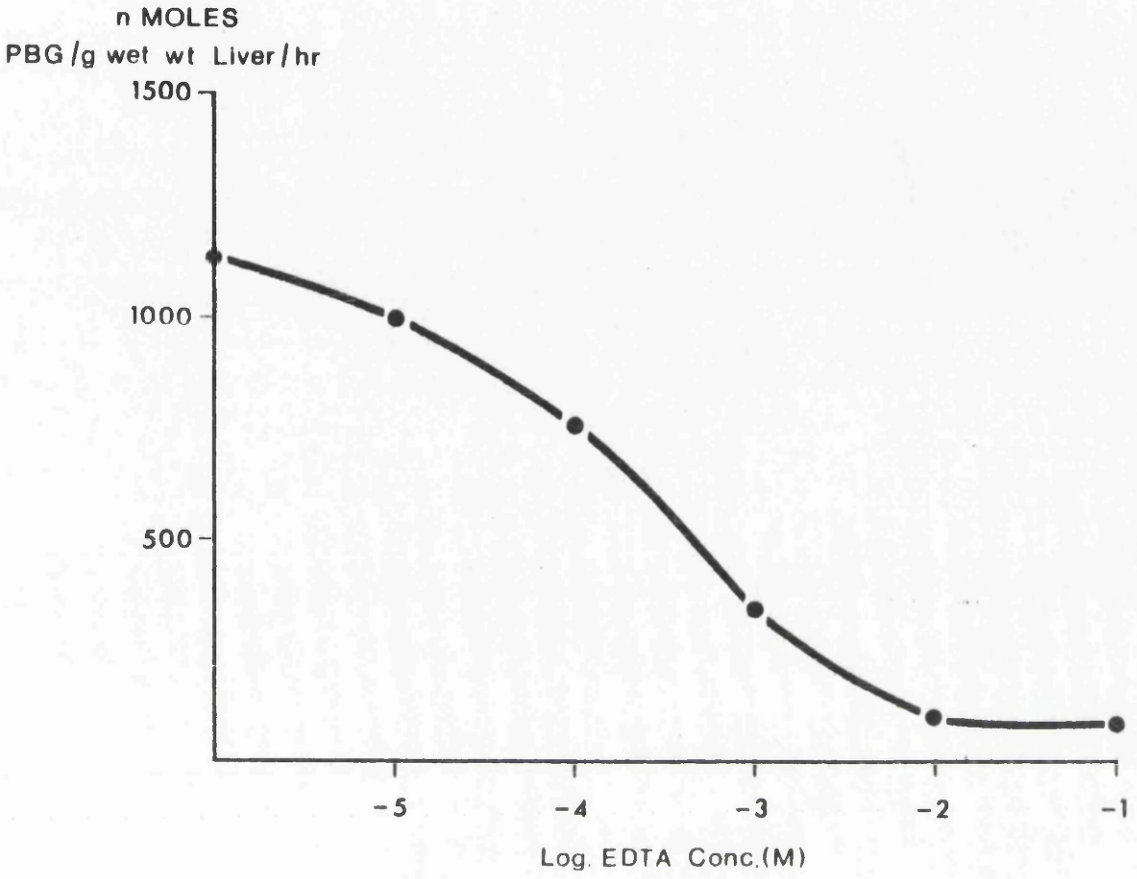


Fig. 9. Inhibition of Porphobilinogen production by EDTA. Porphobilinogen was assayed as for ALA dehydrase with varying concentrations of EDTA added.

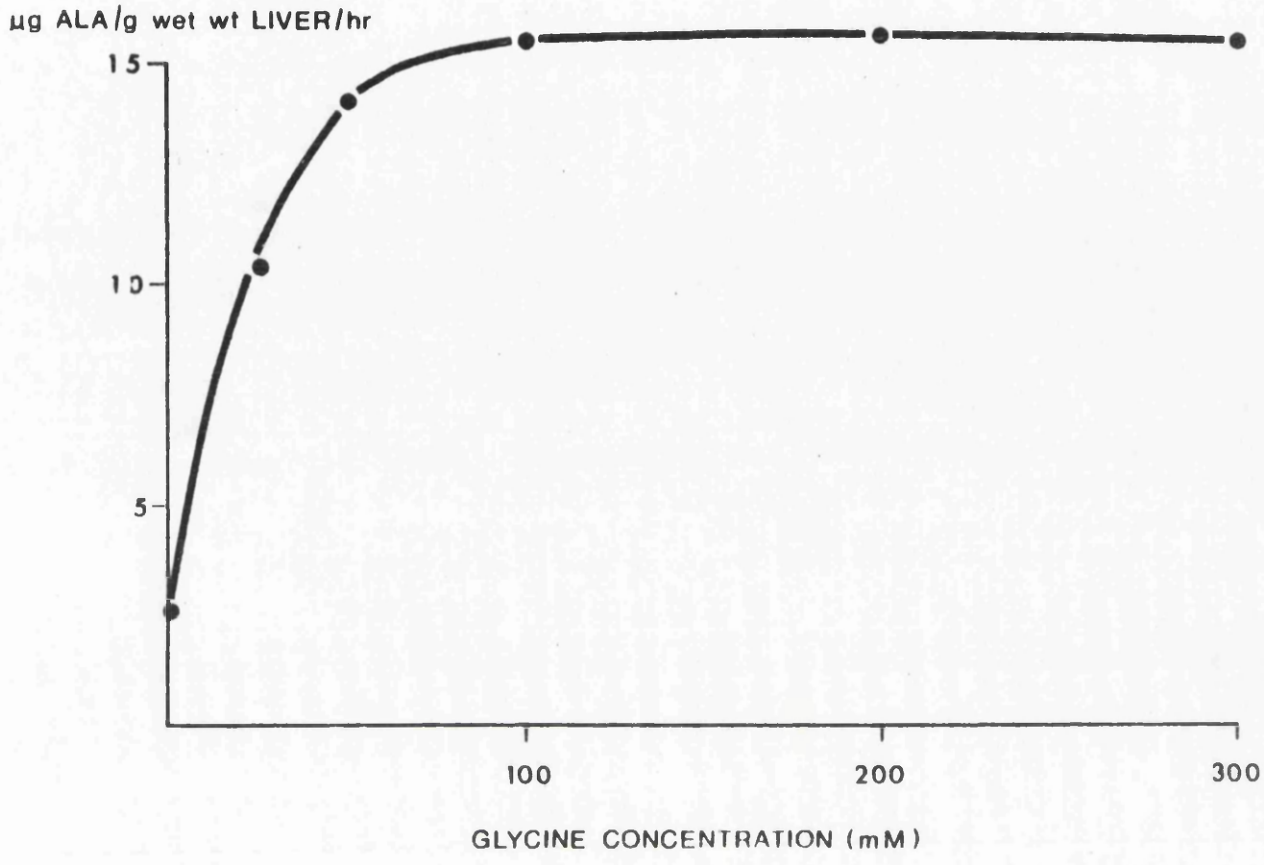


Fig.10. The effect of Glycine Concentration on ALA Synthetase activity. ALA.S. was assayed by the standard method 2 using various concentrations of Glycine in the system.

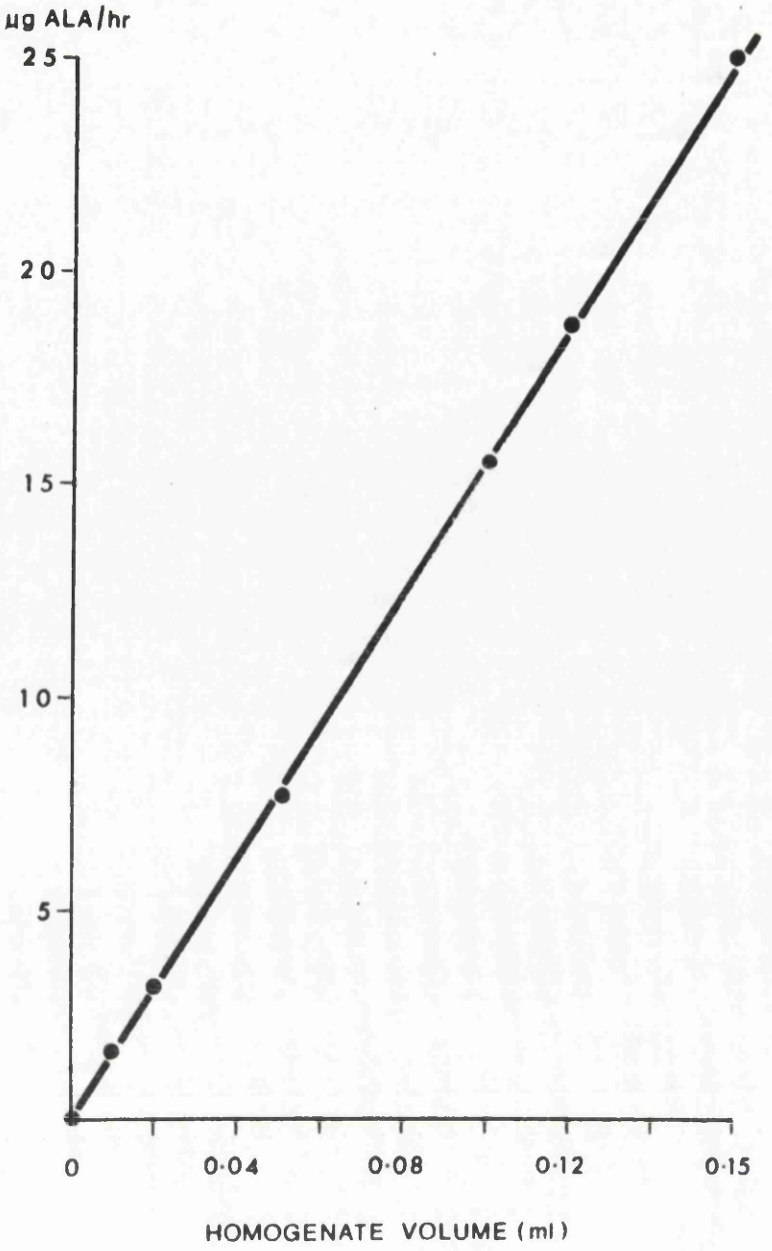


Fig. 11. The effect of enzyme concentration on the system of ALA synthetase assay.

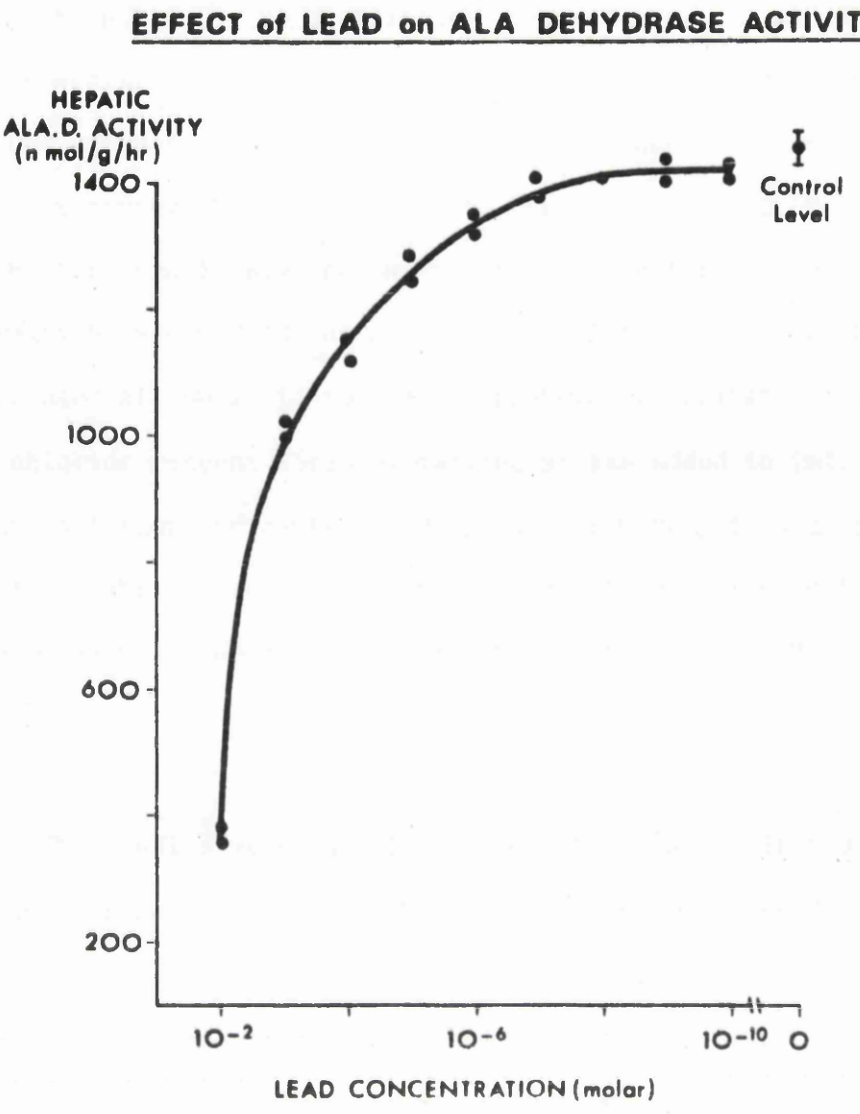


Fig. 12. Effect of Lead on Rat hepatic ALA Dehydrase activity. The enzyme was assayed under standard conditions with varying amounts of lead acetate added.

Thunberg tubes containing 10^{-5} moles reduced glutathione and 10^{-5} moles ALA in 2 ml. 0.1 M phosphate buffer (pH 6.8) were preincubated at 37°C for fifteen minutes to equilibrate temperatures. 1 ml. of temperature equilibrated enzyme preparation was then added to the substrate and the tube evacuated. Endogenous porphobilinogen was measured by immediately stopping the reaction in one tube with 2 ml. 0.3M trichloracetic acid. The test sample was incubated with shaking for one hour at 37°C and the reaction stopped in the same way. After ten minutes the solution was centrifuged at 1000g. to remove the protein precipitate. 2ml. Ehrlich's mercuric chloride reagent (Special Ehrlich's) was added to 2ml. of the supernatant solution and centrifuged again to remove potassium perchlorate precipitate. The optical density of the resulting supernatant solution was then read between 15 and 45 minutes at the Soret band of 553 mμ. against a reagent blank.

The results were calculated from the molar extinction coefficient of 6.8×10^4 for porphobilinogen and expressed as nanomoles PBG produced per 10^{10} RBC per hour, or as nanomoles PBG produced per g. wet weight tissue per hour. The conditions for ALA dehydrase activity determinations, in the system used, have been shown to be an incubation of the sample at physiological temperature of 37°C (fig. 13) at a GSH concentration of 0.33×10^{-3}M (fig. 14). The optimal pH for the system is 6.8 and it has been shown that activity increases linearly as the vacuum increases to 760 mm Hg. (fig. 15). Porphobilinogen production proceeds linearly up to one and a half hours. (fig. 16).

Protein, RNA and DNA were determined on a combined extraction. Protein was measured using the technique of Lowry et al (1951) on a 1 in 800 dilution of liver homogenate. The standard graph was calibrated using Bovine serum albumin. The diluted homogenate was then digested in alkali

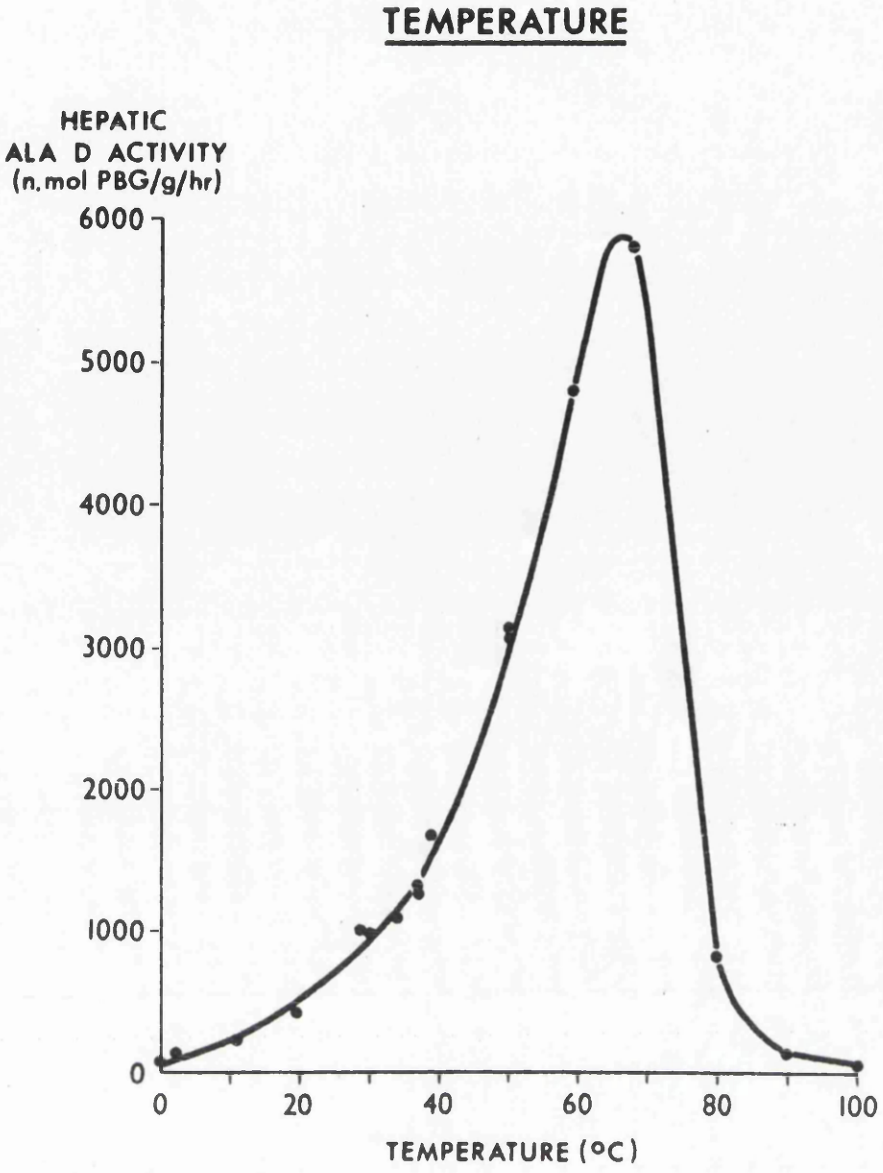


Fig. 13. The effect of temperature on rat hepatic ALA dehydrase activity. The enzyme was assayed under standard conditions.

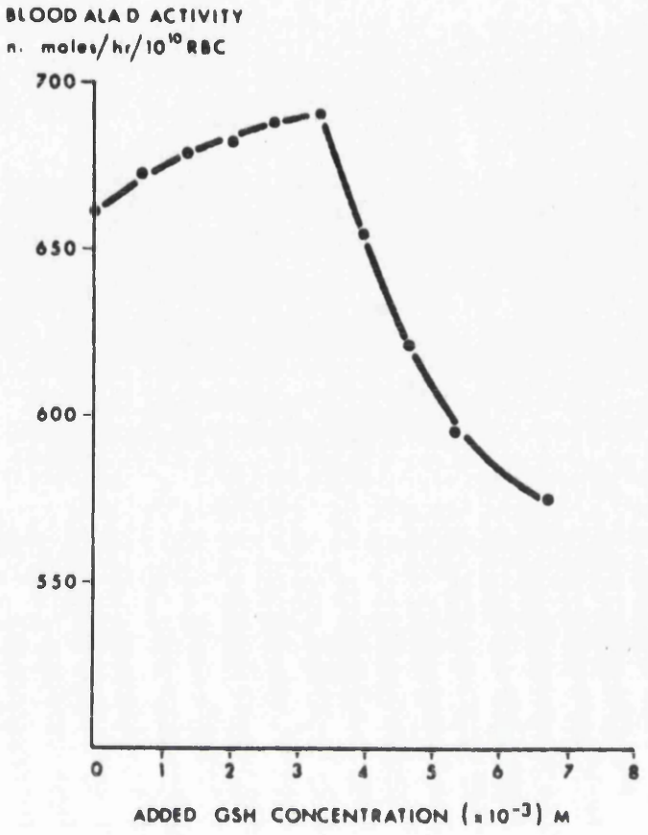


Fig. 14. The effect of added glutathione on rat blood ALA dehydrase activity. Each point represents one assay.

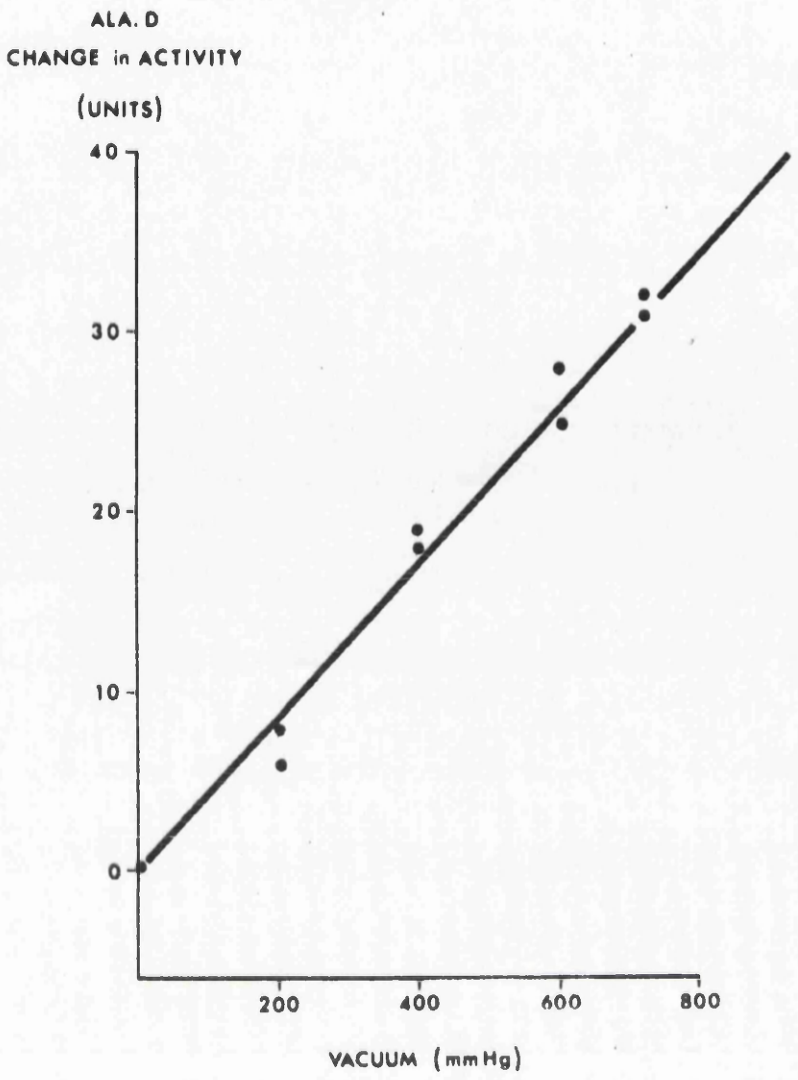


Fig. 15. The effect of vacuum on Rat hepatic ALA dehydrase activity. Each point represents one assay. Vacuum was measured on a Bourdon Gauge.

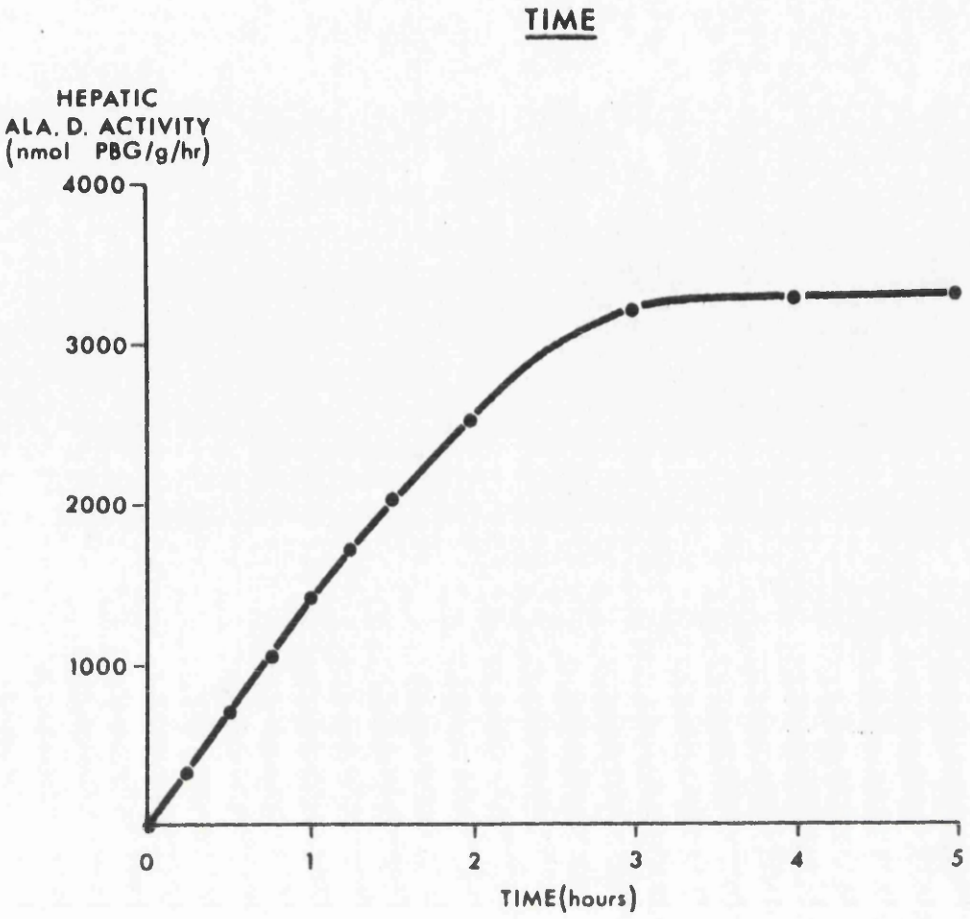


Fig. 16 The effect of time of incubation on ALA dehydrase activity. Each point represents one assay.

for one hour to hydrolyse RNA which was then measured by its extinction at 260m μ. DNA was measured by the method of Ceriotti (1953, 1955) calibrated with calf thymus DNA.

Reduced glutathione was measured in blood by the method of Beutler et al (1965) and ethanol was measured by gas liquid chromatography. Porphyrins and porphyrin precursors were measured by the method of Rimington (1962).

Results were calculated and expressed as means $\pm$ standard deviation (S.D.), the standard deviation being calculated from the expression

$$S.D. = \sqrt{\frac{\sum(x - \bar{x})^2}{n - 1}}$$

Where x is any of the values, $\bar{x}$ is the mean value and n the number of observations.

The significance of results was calculated by Students 't' test using the formula

$$t = \frac{\bar{x}_1 - \bar{x}_2}{s\sqrt{\frac{1}{n_1} - \frac{1}{n_2}}}$$

Where s is an estimate of the combined standard deviation of both groups calculated from

$$s^2 = \frac{\sum(x - \bar{x}_1)^2 + \sum(x - \bar{x}_2)^2}{n_1 + n_2 - 2}$$

$\bar{x}_1$ and $\bar{x}_2$ are the means of both groups and n_1 and n_2 the number of observations in each group

SECTION 1

NORMAL RATS

The effects of sex age diet and allylisopropylacetamide.

INTRODUCTION

Experimental porphyria in animals and human porphyria have historically followed closely on each other chronologically (Goldberg, 1968; 1970). The drug used principally in this study as a porphyrinogenesis reference point is Allylisopropyl acetamide (AIA) (fig.1.1.) (Goldberg, 1953; 1954). AIA is porphyrinogenic by virtue of its ability to induce ALA synthetase and produces an experimental porphyria very like acute intermittent porphyria (A.I.P.). The mechanism of this induction is not yet fully understood, but certain facts are known about it. It is known that inhibitors of nucleic acid and protein biosynthesis abolish its induction of ALA synthetase which would suggest that it is in fact an induction and not an activation (Granick, 1965; Narisawa & Kikuchi, 1966; Stein et al 1970). During the AIA induced increase of ALA.S it was found that haemin (Granick, 1965) and bilirubin lowered the increase and that this depression might have been due to an inhibition of messenger RNA synthesis. Further, the level of ALA.S activity in normal animals was also lowered, whilst the level of succinyl CoA synthetase was unaffected. (Hayashi et al, 1968). This accords well with the concept of feedback repression rather than alternative pathways as the source of increased porphyrin synthesis. Indeed AIA does not appear to affect PBG utilisation or ALA utilisation by the Shemin cycle or for single carbon fragment (C_1) metabolism, the tricarboxylic acid cycle or glycolysis in the liver of animals (Tschudy et al, 1962). The actual mechanism whereby AIA acts on the system is not clear. Various attempts have been made to relate structure to function (Goldberg & Rimington, 1955; Granick,1966; Scheck et al, 1968) but this does not completely explain the mechanism in view of the diverse structures of the various porphyrinogenic drugs.

In this section, an attempt has been made to show the normal levels

2. ALLYL 2. ISOPROPYL ACETAMIDE.

(AIA)

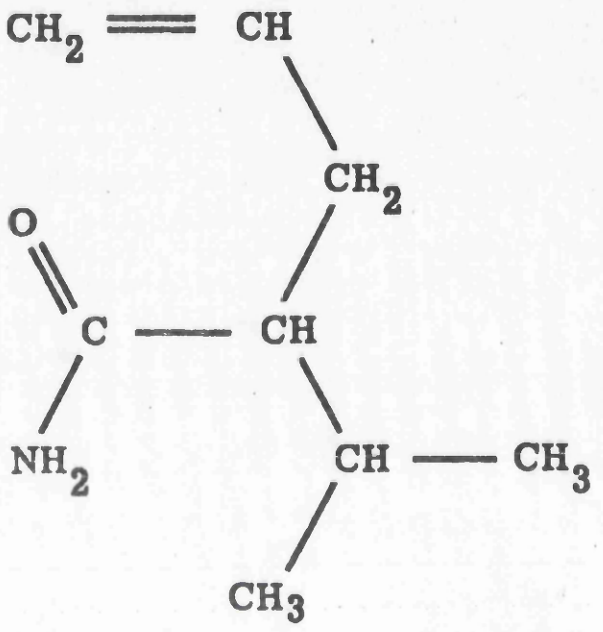


Fig 1.1. Structure of A.I.A.

of the various parameters measured in the subsequent sections in rats, and to show the sorts of elevations of activity shown by AIA, and finally to report another compound which can diminish the elevation of porphyrins and precursors in experimental porphyria, produced by A.I.A.

MATERIALS AND METHODS.

AIA was supplied by Roche Limited, Welwyn Garden City, and used as a propylene glycol syrup of concentration 300 mg/ml. It was administered to rats intraperitoneally at a dosage of 300 mg/kg unless otherwise stated. Rats used were male Sprague Dawley weighing about 200-250g. Methods are given previously and in the appendix section. Urine and faeces were collected in a metabolism cage (fig. 1.2.) and results are expressed as the mean $\pm$ standard deviation.

RESULTS.

The normal rat hepatic levels of ALA synthetase activity and ALA dehydrase activity are shown in Table 1.1., and Table 1.2. shows the normal levels of urinary excretion of ALA and PBG in rats. Rat sex does not appear to have any significant effect on ALA synthetase activity, although females have a slightly higher level of activity together with a higher variance (Table 1.3.). Opposed to this age does have a factor to play in this work, since the activity of hepatic ALA.S in neonates is significantly higher than that of adult rats ($P < 0.001$), yet the enzyme is very much less inducible in the neonates than in the adult rats. AIA raised the adult activity ten-fold ($P < 0.001$) yet did not significantly alter the activity of the enzyme in the neonate (Table 1.4.).

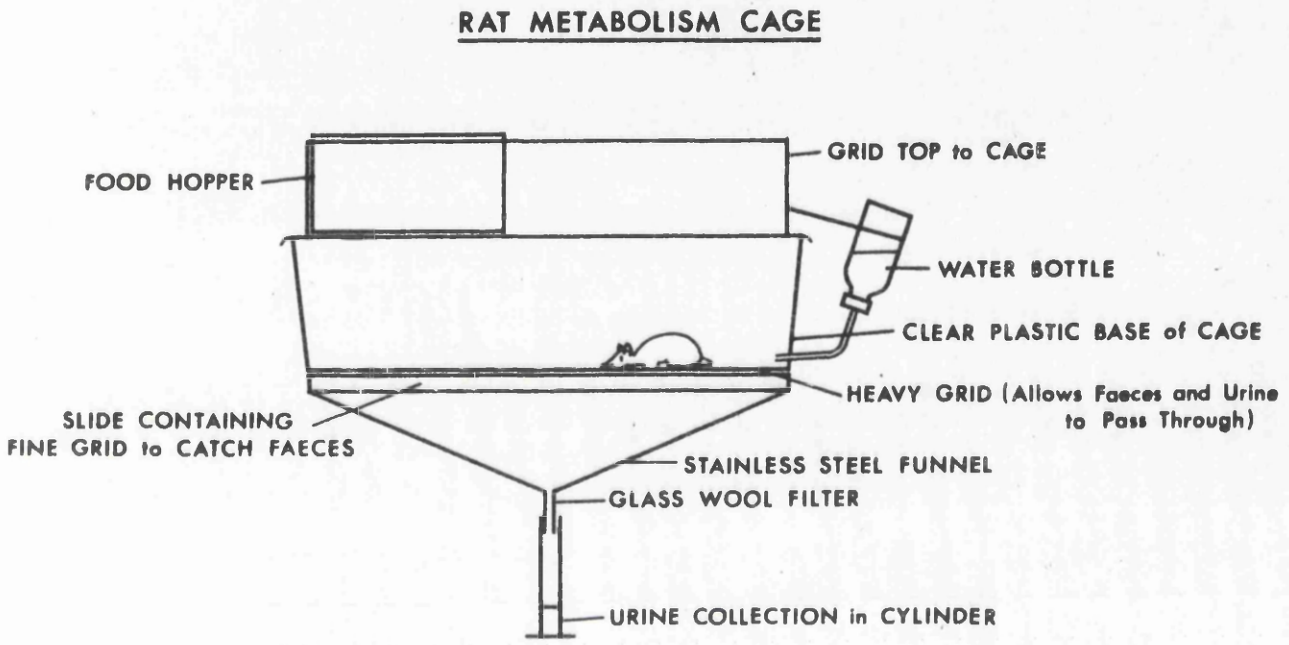


Fig. 1.2. RAT METABOLISM CAGE

This cage is designed to collect Urine and faeces with minimal contamination from food and water.

TABLE 1.1.

NORMAL LEVELS OF HEPATIC ALA SYNTHETASE & ALA DEHYDRASE IN RATS

ENZYME	UNITS	No. of RATS	MEAN ACTIVITY ± STANDARD DEVIATION
δAminolaevulic acid Synthetase (ALA.S.)	µg ALA produced / g wet wt, liver / hr.	100	6.79 ± 2.58
δAminolaevulic acid Dehydrase (ALA.D.)	n.mol. PBG. produced / g.wet wt. liver / hr.	80	1038 ± 266

TABLE 1.2.

LEVELS OF URINARY ALA. & PBG. EXCRETION IN RATS

Treatment	No. of Rats	ALA. (µg/24hr)	PBG. (µg/24hr)
Control	40	9.5 ± 4.7	4.8 ± 3.6
AIA. (300mg/kg. for 4 days)	10	102.0 ± 10.9	106 ± 15.4

Results are given as means ± standard deviation

TABLE 1.3.

THE EFFECT OF SEX ON HEPATIC ALA SYNTHETASE ACTIVITY IN ADULT RATS (250g)

SEX	No. of RATS	ALA HEPATIC SYNTHETASE ACTIVITY (µg/g/hr)
Male	6	6.84 ± 1.45
Female	6	7.96 ± 2.72

TABLE 1.4.

THE EFFECT OF AGE ON HEPATIC ALA.S. ACTIVITY IN RATS AND ON ENZYME INDUCTION BY AIA.

TREATMENT	No. of RATS	HEPATIC ALA SYNTHETASE ACTIVITY		SIGNIFICANCE
		NEWBORN (2 days)	ADULT (250g)	
Normal	12	43.60 ± 3.46	7.75 ± 2.10	$P<0.001$
AIA. (300mg/kg for 2 days)	12	48.40 ± 5.52	38.26 ± 19.70	N.S.
Significance		N.S.	$P < 0.001$	

The dietary state of the rats is also important since the level of ALA synthetase activity in starved rats is significantly greater ($P < 0.05$) than that of rats in a normal nutritional state (Table 1.5.) The effects of AIA on this system are summarised in Table 1.6. AIA significantly elevates both hepatic ALA synthetase activity and ALA dehydrase activity ($P < 0.001$) and also ALA ($P < 0.01$) and PBG ($P < 0.05$) Uroporphyrin coproporphyrin and protoporphyrin ($P < 0.001$).

Vitamin E(∝tocopherol) (fig.1.3.) was administered both to normal animals and AIA treated animals and was found to significantly depress ALA and PBG excretion in the AIA treated animals ($P < 0.001$) bringing these levels back to the base line control level. On its own Vitamin E had no significant effect on the normal levels of excretion or on the levels of the hepatic enzymes in both normal and AIA treated rats (Table 1.7).

The abolition of the elevation of ALA.S activity by AIA when these rats were treated with antibiotics (Table 1.8) indicates that this elevation was due to 'de novo' protein synthesis.

DISCUSSION.

It can be seen from these results that several endogenous factors are involved in the control of ALA synthetase activity; the effects of diet on porphyrinogenesis are already well documented both in experimental animals (Dent & Rimington, 1947; Tschudy et al, 1964) and in humans (Delena & Brown, 1969; Loriaux et al, 1969; Perlroth et al, 1968; Welland et al, 1964). These results confirm that ALA synthetase is elevated in animals starved for 24 hours. It is difficult to relate this dietary effect to porphyria and porphyrin metabolism. It has been suggested however, that it is related

TABLE 1.5.

THE EFFECT OF DIETARY STATE ON HEPATIC ALA SYNTHETASE ACTIVITY IN ADULT RATS

DIETARY STATE	No. of RATS	ALA. HEPATIC SYNTHETASE ACTIVITY (µg/g/hr	SIGNIFICANCE (with respect to normal)
Normal	6	5.95 ± 2.16	
Starved (24 hrs.) with water ad.lib.	6	8.89 ± 1.22	$P < 0.05$

TABLE 1.6.

THE EFFECT OF ALLYLISOPROPYL ACETAMIDE (AIA.) ON HEPATIC ALA.S. ALA.D., ALA, PBG, & PORPHYRINS IN THE RAT

TREATMENT	No. of RATS	ENZYMES		PORPHYRIN PRECURSORS†		PORPHYRINS†		
		ALA.S.*	ALA.D.†	ALA	PBG.	URO	COPRO	PROTO
AIA. (300 mg/kg. for 4 days)	12	65.72 ± 21.20	1347 ±190	15.5 ±7.0	10.6 ±8.2	0.29 ±0.18	1.35 ±0.91	1.77 ±0.56
Control	12	7.48 ±1.52	945 ±56	5.9 ±2.9	4.7 ±3.7	0.03 ±0.02	0.12 ±0.12	0.10 ±0.09
Significance (P <)		0.001	0.002	0.05	0.05	0.001	0.001	0.001

* ALA. Synthetase, µg/g/hr + ALA. Dehydrase n.mol /g/hr

† Porphyrin precursors & Porphyrins in µg/g wet wt.

TABLE 1.7.

THE EFFECTS OF VITAMIN E AND AIA ON BODY AND LIVER WEIGHT HEPATIC ENZYMES & URINARY PORPHYRIN PRECURSORS

TREATMENT	No. of RATS	INCREASE IN BODY WT.	WT. LIVER/100G BODY WT. (g)	ALA. S. ACTIVITY	ALA. D. ACTIVITY	ALA. (µg/24hrs)	PBG. (µg/24hrs)
Control	6	58 ± 12	4.31 ± 0.50	6.62 ±1.51	996 ±66	14 ±7	4 ±1
Vitamin E (40mg/kg)	6	54 ± 15	4.20 ± 0.40	5.96 ±0.79	973 ±127	11 ±5	3 ±1
AIA (300mg/kg)	6	52 ± 10	4.00 ± 0.29	27.81 ±3.25	1325 ±92	102 ±23	106 ±31
AIA (300mg/kg) + Vitamin E (40mg/kg)	6	54 ± 9	4.38 ± 0.34	23.61 ±5.40	1253 ±174	18 ±8	13 ±5

Dosage for 3 days Results are Mean ± S.D.

TABLE 1.8.

THE EFFECT OF CYCLOHEXIMIDE & ACTINOMYCIN D ON AIA INDUCED ELEVATION OF ALA.S. IN RATS.

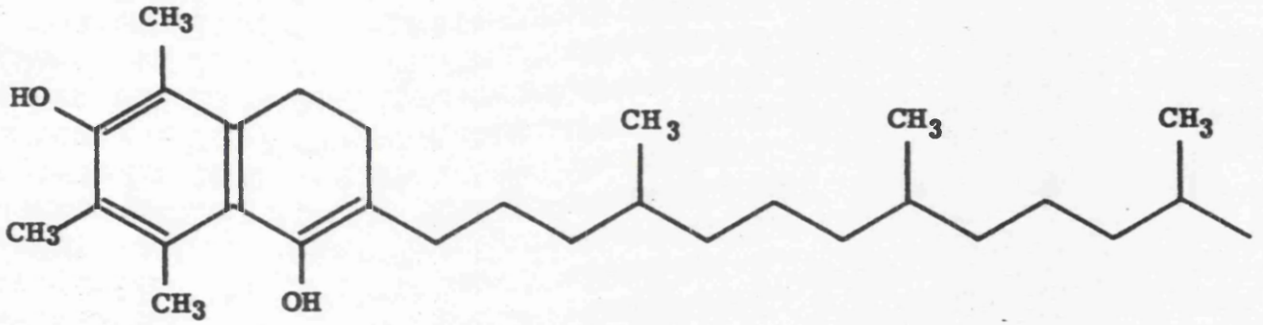
TREATMENT	No. of RATS	HEPATIC ALA SYNTHETASE ACTIVITY (µg/g/hr)
Control	5	7.48 ± 1.53
AIA. (300mg/kg)	5	55.84 ± 14.58
Control + Act.D. (1mg/kg)	5	2.39 ± 1.95
AIA. + Act.D.	5	2.86 ± 2.10
Control + Cyclohex. (1mg/kg)	5	3.25 ± 2.08
AIA + Cyclohex	5	5.79 ± 4.31

Actinomycin D & Cycloheximide were administered together with the last dose of AIA. 6 and 3 hours respectively before death. AIA was administered daily for 4 days.

to catabolite repression (Magasanik, 1961) by glucose metabolites, but in view of the fact that it is impossible to produce compounds within the biosynthetic pathway without passing through the agency of ALA synthetase, the initial and rate limiting enzyme, this is unlikely.

Although sex differences play some part in human porphyria (Goldberg & Rimington, 1962) it does not significantly alter the level of ALA synthetase activity in rats. There is however, an age difference. Woods & Dixon (1969; 1970) have already demonstrated that there is a perinatal variation in ALA synthetase activity; these results confirm this in the neonatal rat. This high activity in the newborn rat, coupled with the lack of ability of the enzyme to be induced, suggests that at birth this system is in a highly 'derepressed' state with a consequent high activity and inability to be derepressed further.

In AIA induced porphyria the primary defect is an increase in the activity of ALA synthetase by 'de novo' synthesis of enzyme protein (Granick, 1965). Other defects are induced by this compound such as increases in the synthesis of L ascorbic acid (De Matteis, 1964) and a loss of cytochrome P.450 and haem. In this work it has been shown that there is an approximately tenfold increase in ALA synthetase activity by AIA. This increase in activity is unaffected by vitamin E (fig.1.3.) although it lowers the levels of excreted porphyrin precursors and porphyrins induced by AIA (Murty & Nair, 1969). In this it is possible that its action is due to its role as an antioxidant, possibly acting in a manner similar to β carotene in the alleviation of porphyrin photosensitivity (Mathews-Roth et al, 1970). Although AIA induces a ninefold increase in ALA synthetase activity (as can be seen from the molar figures (Table 1.9.; as derived from Table 1.6) there is more than sufficient activity of ALA dehydrase to metabolise all of the ALA produced by ALA synthetase.



VITAMIN E. (α TOCOPHEROL)

Fig. 1.3. Structure of Vitamin E.

TABLE 1.9.

MEAN MOLAR ACTIVITIES OF RAT HEPATIC ENZYMES (n.mol/g.wet wt./hr)

TREATMENT	ALA.S.	ALA.D.	$\frac{\text{ALA.D.}}{\text{ALA.S.}}$
Normal	48.7	945	19.5
AIA. (300mg/kg)	428.1	1347	3.1
Ratio $\frac{\text{AIA}}{\text{Normal}}$	8.8	1.4	

In consequence it is not surprising that the activity of the ALA dehydrase is only marginally raised whilst that of ALA synthetase is elevated to such high levels.

In total, as can be seen from fig. 1.4., the balance of production of ALA in rats against its excretion shows that in the normal state, this pathway is operating very efficiently with little excretion of the metabolites on the pathway to haem, and thus showing that the tightly linked pathway is efficiently controlled at the level of ALA synthetase. When this balance is upset there is a gross alteration in this state of affairs with subsequent over excretion of all these substances.

BALANCE SHEET FOR THE HAEM. BIOSYNTHETIC PATHWAY IN RATS.

(Rat, weight, 200g., MALE)

PRODUCTION		EXCRETION	n mol. Excreted/24hr.	ALA Equivalents Excreted/24hr. (n. mol.)
ORGAN	ALA SYNTHESIS/ORGAN/24hr. (n. mol.)	URINE		
LIVER	11,716	A.L.A.	64	64
HEART	251	P.B.G.	12	24
KIDNEY	206	UROPORPHYRIN	0	0
SPLEEN	2079	COPROPORPHYRIN	27	217
BONE MARROW	2611			
BRAIN	164	FAECES		
BODY	5703	UROPORPHYRIN	0	0
TOTAL SYNTHESIS	22,736	COPROPORPHYRIN	55	438
		PROTOPORPHYRIN	87	697
			TOTAL EXCRETION	1440

THIS REPRESENTS A 6.33% LOSS OF METABOLITES WITHIN THE PATHWAY.

Fig. 1.4.

SECTION 2

BARBITURATES GLUTETHIMIDE

CYANURIC ACID AND OTHER DRUGS.

Soon after the introduction of barbiturates at the beginning of this century, they were associated with the onset of acute attacks of porphyria. Waldenstrom (1937) put forward the theory that they could precipitate latent porphyria into the active form. Since then it has been shown conclusively that barbiturates adversely affect acute intermittent porphyria either by provoking attacks or, if taken after the beginning of an attack, by profoundly aggravating the severity of the neurological symptoms. (Goldberg 1959). When barbiturates were administered to rabbits, it was found that out of nine barbiturates, three, each possessing one or two allyl groups caused a marked increase in urinary coproporphyrin excretion whilst the remaining six caused a slight or no increase in porphyrin excretion although all of these drugs have been associated with exacerbation of the human disease. (Goldberg 1955).

As has already been shown, a constant finding in experimental porphyria is an elevation of ALA.S.: for this reason, it seemed profitable to examine the effects of these same nine barbiturates on ALA.S. activity particularly noting the effect of these barbiturates that had failed to cause any increase in porphyrin excretion. With a view to correlating structure and function, it was also decided to examine the effects of the related compounds cyanuric acid and glutethimide together with pentazocine, an analgesic containing an allyl group. The subsequent enzyme ALA.D. is also thought to be rate limiting in some systems of porphyrin biosynthesis (Nandi & Shemin 1967) and also exhibits some characteristics of an allosteric enzyme suggestive of a regulatory role (Nandi et al. 1968). For these reasons ALA D was also measured in these experiments.

MATERIALS AND METHODS.

The experimental animals were male Sprague Dawley rats, weighing about 200g. at the commencement of the experiments. These were used in groups of 12, 6 test and 6 control animals, and fed on standard rat diet 41. Drugs were administered intraperitoneally as an aqueous solution or orally as a propylene glycol syrup. Livers were excised under ether anaesthetic, immediately chilled in ice cold saline, weighed, and aliquots removed for enzyme and porphyrin assays. The animals were killed by exsanguination before consciousness. Liver weights are expressed as g wet weight.

Table 2.1 lists the drugs used, the method and level of dosage. Hereafter they will be named by their approved name. These drugs were administered in the morning to allow minimal disturbance of the animals nocturnal feeding habits.

RESULTS

The time course of barbiturate stimulation of A.L.A. synthetase activity was studied in detail with phenobarbitone which was administered (at anaesthetic dosage) every 24 hours for 12 days. The activity of hepatic A.L.A. synthetase rose rapidly to a peak at about 70 hours plateauing about 200 hours (fig. 2.1.) From these results it was decided to administer the drugs for six days. The levels of A.L.A. synthetase activity following phenobarbitone administration by the two different methods of drug administration were not significantly different (Table 2.2). The parameters measured on each liver were A.L.A. synthetase, A.L.A. dehydrase, coproporphyrin and protoporphyrin (Table 2.3.) Also measured were body weight and liver weight. There was no significant difference in body weight change or liver weight per 100 g. body weight in any of the groups.

The following were significantly elevated; hepatic A.L.A. synthetase activity in all samples, with levels of significance, for Thiopentone ($P < 0.02$), for Amylobarbitone ($P < 0.01$) and for the rest ($P < 0.001$); hepatic A.L.A. dehydrase activity in Dial with a level of significance ($P < 0.05$); hepatic coproporphyrin in Dial ($P < 0.001$) and in Allobarbitone and Quinalbarbitone ($P < 0.01$).

No other parameters were significantly elevated. On the basis of the results these barbiturates may be separated into four significantly different groups (Fig. 2.2) In the first group (Dial) the hepatic ALA synthetase activity was significantly greater than the activity in the second group (Allobarbitone and Quinalbarbitone ($P < 0.001$). The activity in the second

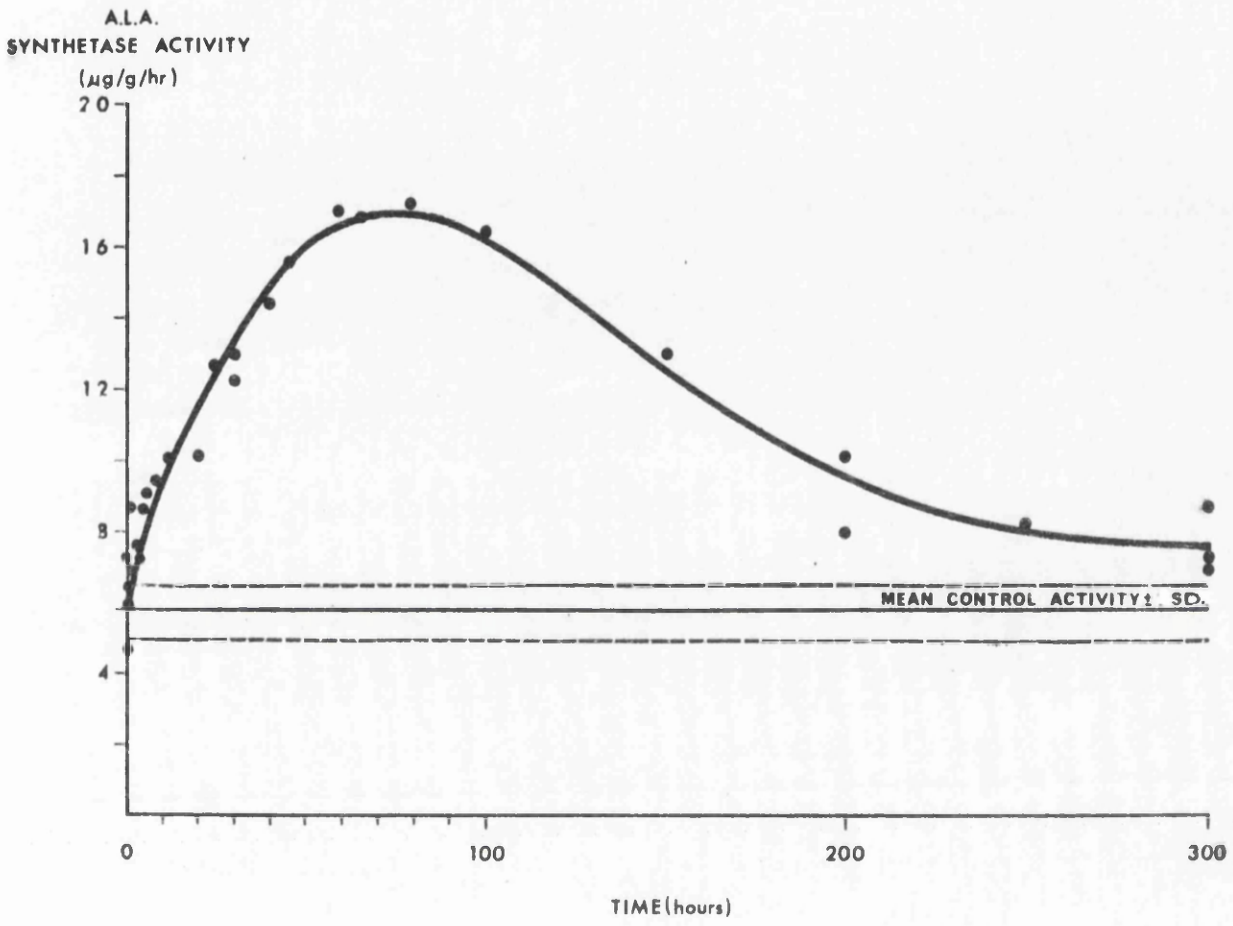


Fig. 2.1.: Time course; Effect of phenobarbitone on ALA synthetase activity. Each point represents one rat. Phenobarbitone was administered at anaesthetic dosage every 24 hr during the period of the experiment.

TABLE 2.1

BARBITURATES AND OTHER DRUGS - AND ANIMAL EXPERIMENTAL PLAN

Approved Name	Chemical Name	No. of Animals	Method of dosage	Dosage mg/kg
Dial	Diallyl barbituric acid	5	Oral	100
Allobarbitone	5 Allyl 5 isopropyl barbituric acid	5	Oral	100
Quinalbarbitone	5 Allyl 5.1 methyl butyl barbituric acid	5	Oral	100
Barbitone	5.5 diethyl barbituric acid	5	i.p.	190
Pentobarbitone	5 ethyl 5.1 methyl butyl barbituric acid	5	i.p.	50
Phenobarbitone	5 ethyl 5 phenyl barbituric acid	6	i.p.	100
Butobarbitone	5 butyl 5 ethyl barbituric acid	6	Oral	100
Thiopentone	5 ethyl 5.1 methyl butyl thio barbituric acid	5	i.p.	40
Amylobarbitone	5 ethyl 5 isopentyl barbituric acid	6	i.p.	100
Cyanuric Acid		6	i.p.	50
Glutethimide	5 ethyl 5 Phenyl glutarimide	6	oral	200
Pentazocine	2' hydroxy 5.9 dimethyl 2 (3.3' dimethyl allyl) 6.7 benzomorphan	8	i.p.	15

TABLE 2.2

EFFECT OF DIFFERENT MEANS OF DOSAGE

Barbiturate	No. of Rats used	ALA synthetase activity (µg ALA/g wet wt. Liver/hr)
Phenobarbitone (intraperitoneally)	6	12.42 ± 2.27
Phenobarbitone (orally)	5	12.01 ± 2.28

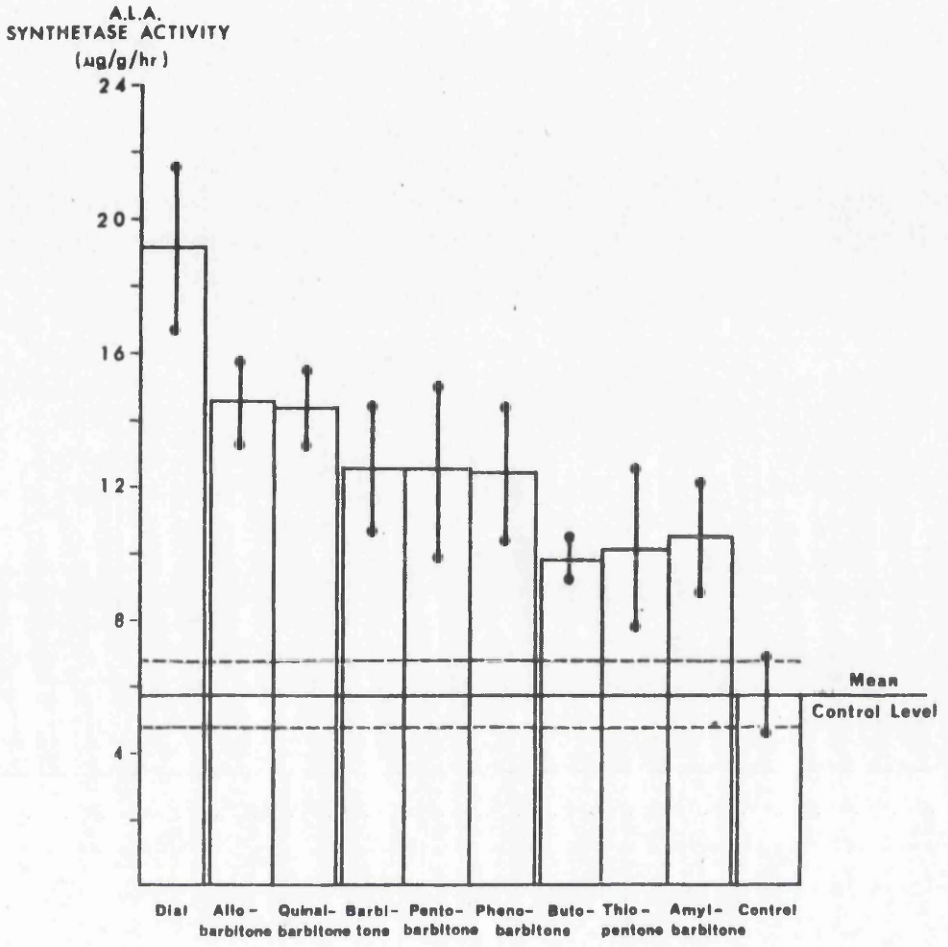


Fig. 2.2. The effect of certain barbiturates on hepatic ALA synthetase activity in the rat. Each block of the histogram represents the mean activity. Vertical bars show the standard deviation.

group was greater than the activity in the third group (Phenobarbitone, Barbitone, and Pentobarbitone) ($P < 0.05$) and the activity in this third group was greater than in the fourth group (Thiopentone, Amylobarbitone and Butobarbitone) ($P < 0.001$) which was greater than the activity in the control group ($P < 0.001$).

Following dosage with glutethimide, cyanuric acid and pentazocine in a similar manner, it was found that all of these significantly elevated ALA synthetase activity ($P < 0.001$). Whilst neither ALA dehydrase activity or body or liver weights were significantly altered.

As a measure of the means of elevation of ALA Synthetase, the effects of the antibiotics Actinomycin D and cycloheximide were examined in this system. It was found that both of these antibiotics abolished the elevation of ALA.S. by phenobarbitone, figs. 2.3 and Fig. 2.4. It was similarly found that these antibiotics abolished the stimulating effect of glutethimide. The urinary excretion of ALA was elevated significantly by phenobarbitone ($P < 0.001$) Cyanuric acid ($P < 0.01$) and Glutethimide ($P < 0.001$) whilst PBG excretion was similarly elevated ($P < 0.001$) ($P < 0.05$) ($P < 0.001$). (Table 2.5.)

TABLE 2.3

THE EFFECT OF CERTAIN BARBITURATES ON BODY AND LIVER WEIGHTS, PORPHYRINOGENIC ENZYMES AND PORPHYRINS IN THE LIVER.

Drug	No. of rats	Increase in body wt. (g)	Wt. liver/ 100 g body wt. (g)*	ALA synthetase activity (µg/g/hr)*	ALA dehydrase activity nMoles/g/hr*	Copropor-phyrin (µg/g)*	Protopor-phyrin (µg/g)*
Dial	5	55.0 ± 7.1	4.12 ± 0.50	19.16 ± 2.50	1348 ± 119	7.50 ± 0.770	8.00 ± 0.100
Allobarbitone	5	55.0 ± 12.1	4.76 ± 0.40	14.52 ± 1.24	1226 ± 61	0.91 ± 0.090	0.89 ± 0.410
Quinalbarbitone	5	58.0 ± 20.8	3.99 ± 0.58	14.40 ± 1.12	1189 ± 156	1.03 ± 0.610	0.83 ± 0.350
Barbitone	5	54.0 ± 8.2	4.52 ± 0.41	12.53 ± 1.96	1197 ± 124	0.152 ± 0.112	0.137 ± 0.101
Pentobarbitone	5	64.1 ± 12.0	4.10 ± 0.22	12.52 ± 2.72	1204 ± 233	0.171 ± 0.156	0.149 ± 0.150
Phenobarbitone	6	47.0 ± 15.0	4.75 ± 0.39	12.42 ± 2.27	1293 ± 89	0.074 ± 0.010	0.056 ± 0.014
Butobarbitone	6	58.0 ± 10.1	3.67 ± 0.42	9.85 ± 0.84	1117 ± 84	0.110 ± 0.030	0.090 ± 0.050
Thiopentone	5	65.0 ± 12.7	4.26 ± 0.52	10.14 ± 2.53	1150 ± 67	0.040 ± 0.020	0.076 ± 0.010
Amylobarbitone	6	60.0 ± 54.0	4.21 ± 0.43	10.56 ± 1.86	1065 ± 153	0,038 ± 0.024	0.043 ± 0.030
Control	25	64.5 ± 10.1	4.26 ± 0.93	5.76 ± 2.08	1134 ± 169	0.111 ± 0.050	0.088 ± 0.075

* In all cases g/wet weight liver.

TABLE 2.4

THE EFFECT OF CYANURIC ACID GLUTETHIMIDE & PENTAZOCINE ON BODY AND LIVER WEIGHTS & PORPHYRINOGENIC ENZYMES

TREATMENT	No. OF RATS	INCREASE IN BODY WT. (g)	Weight LIVER / 100g BODY WT. (g)	ALA. SYNTHETASE ACTIVITY (µg/g/hr)	ALA. DEHYDRASE ACTIVITY (n.mol/g/hr)
Control	6	50 ± 10	4.00 ± 0.52	7.75 ± 1.53	1132 ± 111
Cyanuric acid	6	52 ± 11	4.23 ± 0.31	13.87 ± 2.01	1153 ± 116
Glutethimide	6	47 ± 8	4.12 ± 0.44	14.69 ± 2.81	1189 ± 95
Pentazocine	6	52 ± 13	4.41 ± 0.26	11.00 ± 1.95	1106 ± 155

g: in all cases g. wet weight

TABLE 2.5

URINARY EXCRETION OF ALA AND PBG IN RATS TREATED WITH PHENOBARBITONE CYANURIC ACID & GLUTETHIMIDE

Drug	No. of Rats	URINARY µg/24 hrs.	
		ALA	PBG
Control	6	9.3 ± 2.7	1.84 ± 0.91
Phenobarbitone	6	37.2 ± 8.4	5.80 ± 1.48
Cyanuric acid	6	15.5 ± 3.9	2.85 ± 0.77
Glutethimide	6	44.4 ± 6.2	8.75 ± 2.90

Drug dosage is as in table 2.1

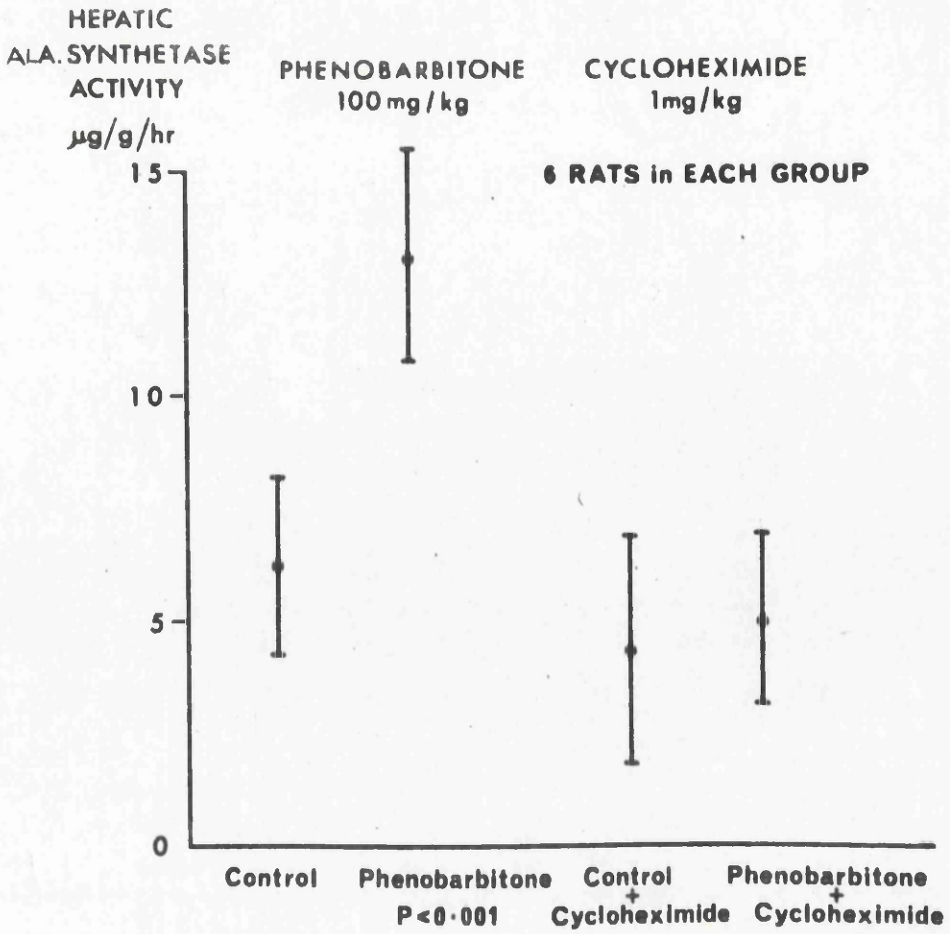


Fig. 2.3. The effect of Cycloheximide on hepatic ALA synthetase in phenobarbitone treated rats. Each bar represents the mean ± the standard deviation.

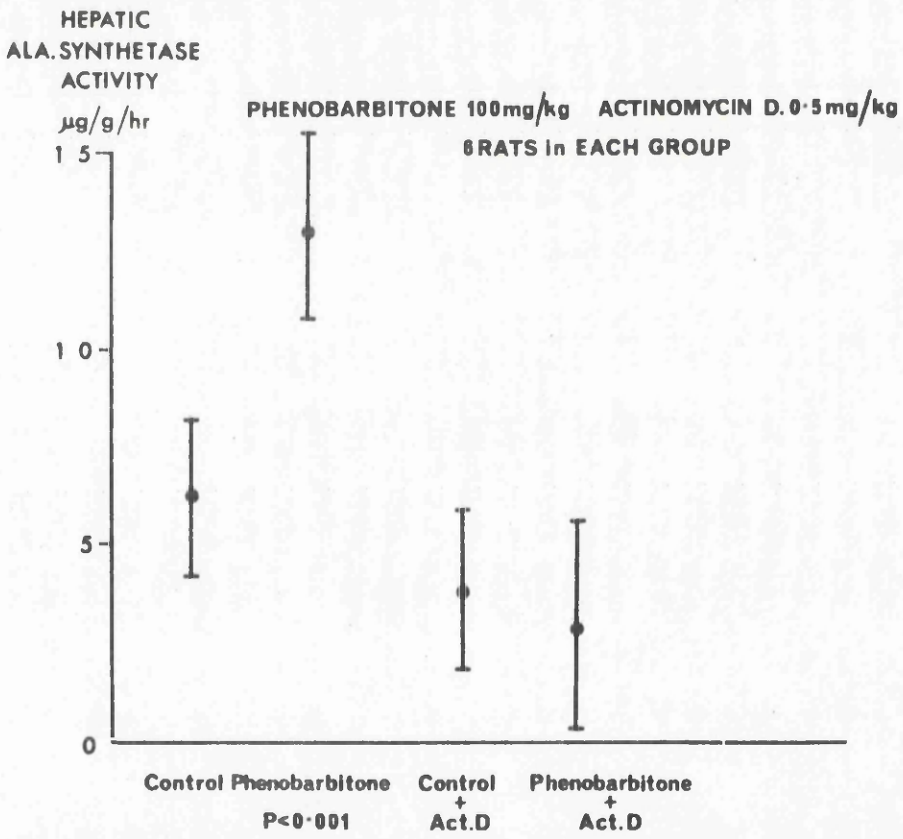


Fig. 2.4. The effect of Actinomycin D on hepatic ALA synthetase activity phenobarbitone rats. Each bar represents the mean ± the standard deviation.

DISCUSSION.

This work has shown that of these nine barbiturates tested, all significantly elevated the activity of hepatic ALA synthetase in the rat. There is also an increase in hepatic porphyrins with three of these drugs, Dial, Allobarbitone and Quinalbarbitone, that is the three which previously caused the greatest excretion of urinary porphyrins in rabbits. There was also an elevation of ALA dehydrase in the case of Dial.

On the basis of the elevations of ALA synthetase activity, these drugs may be classified into four groups.

(1) Dial, causing a large rise of hepatic ALA synthetase activity and a rise in hepatic ALA dehydrase and of hepatic copro- and protoporphyrin.

(2) Allobarbitone and Quinalbarbitone, which caused a lesser rise of hepatic ALA synthetase activity and a small rise of hepatic copro- and protoporphyrins.

(3) Barbitone, Pentobarbitone and Phenobarbitone which caused a rise of hepatic ALA synthetase activity.

(4) Thiopentone, Amylobarbitone and Butobarbitone which caused a smaller but significant rise of hepatic ALA synthetase activity.

It is noteworthy that this classification is the same as that previously based on urinary coproporphyrin excretion (Goldberg, 1954).. fig. 2.5. The main difference is that in group 4 there was no elevation of urinary coproporphyrin excretion in the previous study, whilst in the present study, the level of ALA synthetase is significantly elevated in all groups including group 4. This underlines the rule that all barbiturates are contraindicated in patients with hepatic porphyrias since in them there is already an elevation

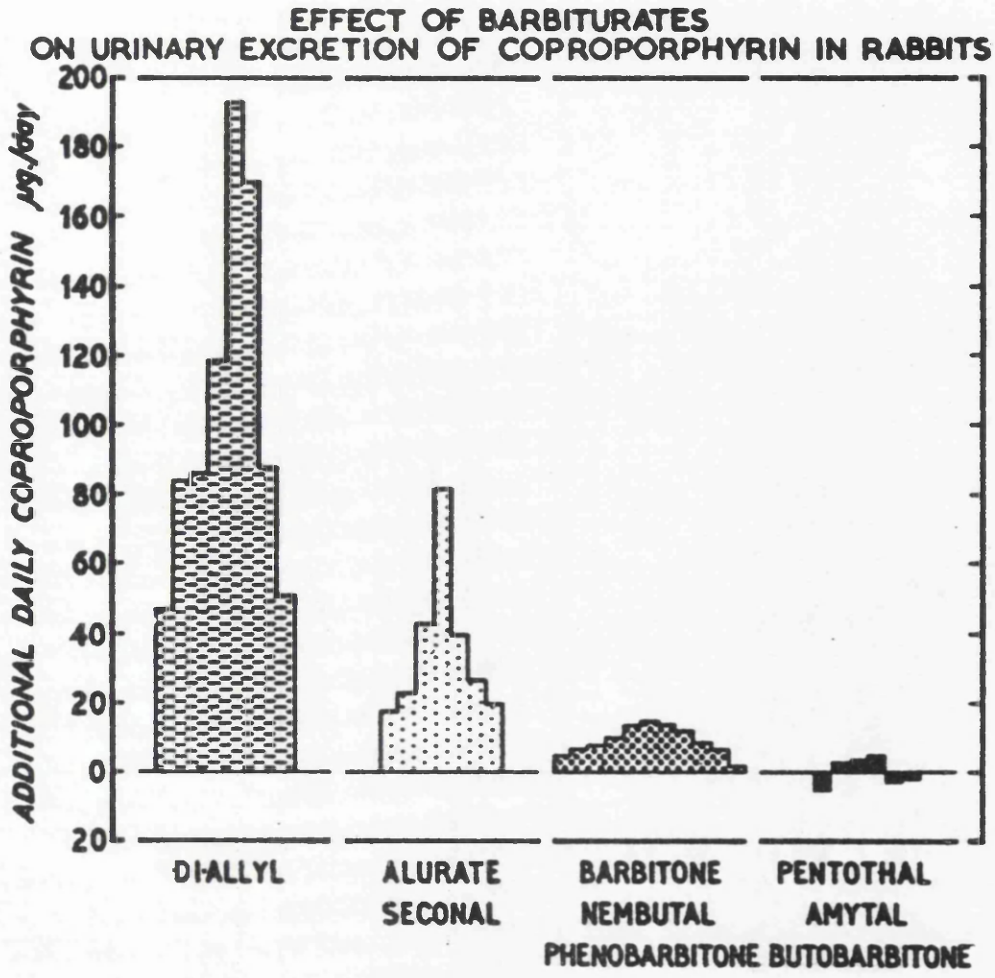

EFFECT OF BARBITURATES
ON URINARY EXCRETION OF COPROPORPHYRIN IN RABBITS
ADDITIONAL DAILY COPROPORPHYRIN µg./day
200
180
160
140
120
100
80
60
40
20
0
20
DI-ALLYL
ALURATE
SECONAL
BARBITONE
NEMBUTAL
PHENOBARBITONE
PENTOTHAL
AMYTAL
BUTOBARBITONE

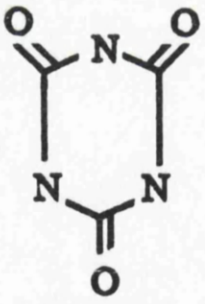
Fig. 2.5

of hepatic ALA synthetase.

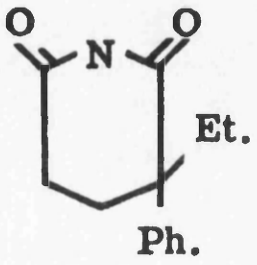
Hepatic ALA dehydrase was also raised in the case of Dial. This enzyme is normally found in abundance in all tissues of the body, and is located in the cell sap. On the other hand ALA synthetase is mitochondrial in location, has a short half life of about 1 hour (Marver, 1966) and is found in very much lesser quantities, mainly in the liver and haemopoietic system. It is partially by virtue of these characteristics that ALA synthetase is the rate limiting enzyme of this pathway. The relative quantities of these enzymes are such, that a large rise in ALA synthetase activity does not necessitate a parallel rise in the activity of ALA dehydrase, to cope with the increased production of ALA. It is consequently not surprising that the levels of ALA dehydrase have not been raised.

The hepatic levels of copro- and protoporphyrin were only raised by the drugs containing an allyl group, which was also found by Goldberg (1954). When the level of porphyrin formation is higher there is clearly some accumulation of porphyrins leading to these higher hepatic levels.

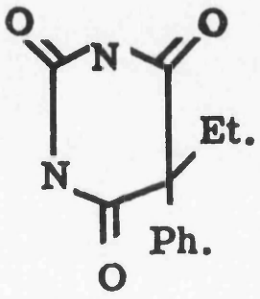
The significant elevation of ALA synthetase activity by cyanuric acid, glut ethimide and pentazocine is not surprising. Fig. 2. 6 shows the structure of the first two of these, and, as can be seen there is a close structural relationship between these two compounds, especially gluthethimide and phenobarbitone. In this case the inducing ability of these compounds would appear to reside in the cyclic lactam grouping associated with all three of these compounds, although there is probably some contribution from the rest of the structure since the inducing ability of these groups does not appear to be related to the number of groups in the molecule. Pentazocine (Fig. 2.7) is not structurally a surprise either, since in its structure is an allyl group which has



CYANURIC ACID

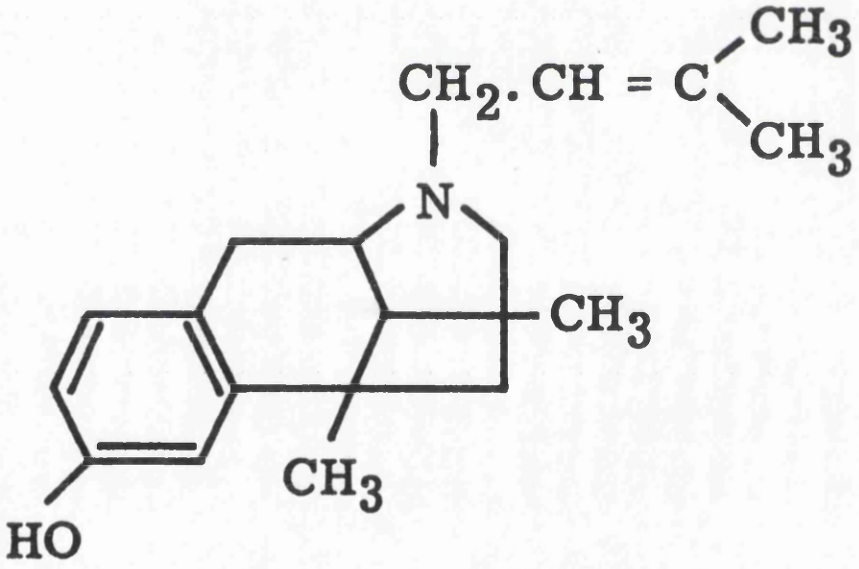


GLUTETHIMIDE



PHENOBARBITONE

Fig. 2.6 Structures of Cyanuric acid, Glutethimide and Phenobarbitone.



PENTAZOCINE

Fig. 2.7. Structure of Pentazocine.

already been shown to be associated with porphyria in the allyl barbiturates and AIA.. Pentazocine is an analgesic with properties similar to morphine and other opium dervatives (Laroche and Remy 1970; Jasinski et.al. 1970) and in view of these results must be included in the list of drugs contraindicated in porphyria (Appendix).

The biochemical relationship between acute porphyria and its sensitivity to barbiturates has in the past been ascribed to blocks in the pathway of purine biosynthesis (Labbe, Talman & Aldrich 1954) or to impairment of acetycholine synthesis by restriction of acetyl CoA through a deficiency of ATP limiting its synthesis (De Matteis and Rimington, 1962). The latter hypothesis was strengthened by the work of Kyogoku, Lord and Rich (1968) who showed that barbiturates form highly specific hydrogen bonded adenine derivatives, and suggested that this strong complex is an explanation of the physiological activity of barbiturates.

It is now known that in hepatic porphyrias there is an overproduction of the initial and rate limiting enzyme of porphyrin biosynthesis, A.L.A. synthetase. It has further been shown that the biochemical disturbance of human porphyria is associated with the overproduction of hepatic A.L.A. synthetase (Tschudy et.al, 1965; Nakao et. al, 1966; Dowdle et. al, 1967). Thus the possible mechanisms of drug action in haem biosynthesis are three in number:

1) *By control of production of Succinyl CoA*

The importance of this mechanism is not clear; it must however have some part in control of haem biosynthesis since the experiments of Ludwig et.al. (1965), have shown that malonate, an inhibitor of succinate dehydrogenase, increases porphyrin production 10 fold in the rat.

2) *End product inhibition*

Is a much more clearly established method of control in this system. Marriot (1968) showed that in Rhodopseudomonas spheroides a decrease in A.L.A. synthetase activity is caused by increase of a low molecular weight inhibitor, and a decrease of a low molecular weight activator and suggested that a reversal of this mechanism might account for activation of the enzyme. This method works by change of enzyme activity directly, possibly by alteration of the enzyme's spatial conformation.

3) *End product repression*

Which involves repression of the de novo synthesis of A.L.A. synthetase. This is more indirect in its action involving the apparatus for protein synthesis. Barbiturates could involve any of these 3 mechanisms in order to increase the activity of hepatic A.L.A. synthetase. Phenobarbitone induces an increase in activity very rapidly within an hour of starting drug administration; the increase becomes statistically significant at about 2 hours after initiation. This means that the method of action must be a rapid one.

The method of control of this pathway both normally and in drug treated systems is thought to be by de novo induction of enzyme synthesis, as suggested by the work using nucleic acid and protein synthesis inhibitors. In addition, phenobarbitone has been shown to increase the microsomal mixed function oxidase, cythochrome P.450 (Schmidt, Marver and Hammaker, 1966) a haemoprotein central in hepatic oxidative transformations. As a haemoprotein its increased synthesis probably relies on the increased function of the haem biosynthetic pathway. Whether this occurs initially, due to an increase in ALA synthetase activity by methods such as citric acid cycle control or due to a decrease in the repressor haem which is being drawn off to produce cytochrome P.450 required to oxidise these drugs, remains to be elucidated. Thus, although rigorous proof of de novo induction is not yet available and evidence does suggest that it is

the main means of regulation, rather than citric acid cycle control, it is possible that drug stimulation of this biosynthetic pathway may be through either of these control mechanisms.

SECTION 3.

ETHANOL.

INTRODUCTION

Ethanol is known to affect haem and porphyrin metabolism. Some of the porphyrias are provoked into attack or episodes of photosensitivity by alcoholic excess (Goldberg and Rimington 1962) and the administration of ethanol causes a significant elevation of δ-aminolaevulic acid (ALA) synthetase (Shanley Zail and Joubert 1968; 1969), the initial and rate limiting enzyme of haem biosynthesis. There have been no studies up till now on the effect of ethanol on ALA dehydrase (δ-aminolaevulic hydrolyase, E.C. 4. 2. 1. 24.) the second enzyme of the haem biosynthetic pathway which catalyses the condensation of two molecules of δ-aminolaevulic acid to form porphobilinogen (Gibson, Neuberger and Scott, 1955; Schmid and Shemin, 1955; Granick and Mauzerall, 1958). This enzyme can now be readily and accurately measured in peripheral blood, where it is contained in the erythrocytes, and in other tissues. The present study was undertaken to examine the effect of ethanol, in man and rats, on blood ALA dehydrase activity and to determine whether any changes observed in the blood reflect similar or dissimilar changes of the same enzyme in other tissues.

MATERIALS & METHODS.

Human Studies:

Estimations of ALA dehydrase and blood alcohol levels were made in 28 male patients who attended the casualty departments of the Western Infirmary and the Royal Infirmary, Glasgow. Each patient was thought, on clinical grounds, to be intoxicated with alcohol and this was confirmed by blood alcohol measurement. Their ages ranged from 18 to 68 years. The clinical presentation in 14 cases was a head injury but only one patient was unconscious from this cause. One patient was admitted to hospital with burns to his arms. The remainder had injuries of a trivial nature requiring little attention.

Control male subjects, matched for age, were tested at the same time, each control having a blood alcohol of nil.

A further investigation was devised to study the effect of alcohol during a 24 hour period in normal male volunteers. 2 normal males aged 55 and 77 were admitted to the metabolic ward of the Western Infirmary. A full medical history was taken and a physical examination was performed in each case. Haemoglobin, white cell count, serum folate, blood urea, serum proteins, liver function tests and serum transaminases were shown to be normal at the commencement of the experiment. The 55 year old subject was re-admitted after an interval of four weeks and the test was repeated. Initial blood samples were taken for alcohol and ALA dehydrase levels. The subjects were then asked to drink 300 ml. whisky (96 g. absolute alcohol) during the course of the first hour of the experiment. Estimations of blood alcohol and ALA dehydrase were made during the course of the next twenty-four hours.

Animal Studies:

Groups of male Sprague Dawley rats weighing between 200 and 250 g. were injected intraperitoneally with 1.5 ml. 50% ethanol/water solution eighteen hours and two hours before the termination of the experiments. For each group, an equivalent number of control animals were given 1.5 ml. dextrose solution of the same caloric value as the ethanol solution, by the same route. Blood samples were obtained by direct cardiac puncture under ether anaesthesia. Tissue samples were obtained immediately after killing the animals by cervical dislocation.

RESULTS

In studies carried out on intoxicated patients a negative correlation ($r = -0{\cdot}86$) was found between the blood ethanol concentration and the blood ALA dehydrase activity (fig.3,1.) This line has equation $y = 374.4 - 0.62x$. Statistically there was a significant depression ($p < 0.001$) of blood ALA dehydrase activity at a mean alcohol level of 225mg/100ml. Similarly in rats administered 1.5 ml. 50% ethanol orally there was a significant depression of blood ALA dehydrase activity ($p < 0.001$) (Table 3,1).

Human volunteers ingested 300 ml. whisky in one hour. When ALA dehydrase activity and blood ethanol levels were followed during the subsequent 24 hours, as the blood ethanol level was raised so pari-passu was the blood ALA dehydrase lowered. Fig.3.2 shows typical results from one of these subjects.

Tissue ALA dehydrase activities, measured in rats administered 1.5 ml. 50% ethanol intraperitoneally 2 hours and 18 hours before death (Table 3.2) were significantly depressed only in the liver and kidney; the spleen and heart enzyme activities were unaffected. At the same time a highly significant elevation of ALA S activity was noted, in rat liver(fig. 3.3)

In 'in vitro' studies using human blood haemolysate prepared as for the blood enzyme assay, it was found that exogenous GSH reached an optimal activating concentration of 3.3×10^{-3} molar followed by a rapid decline in activity with increasing GSH concentration. When ethanol at a concentration of 200mg/100ml was added to the system the optimal activating GSH concentration was halved to 1.65×10^{-3} molar and additionally the maximal level of activation was lowered. This effect was shown additionally by an enzyme preparation of rat liver homogenate. (fig.3.4.) In this system ALA dehydrase activity fell linearly with rising ethanol concentration with

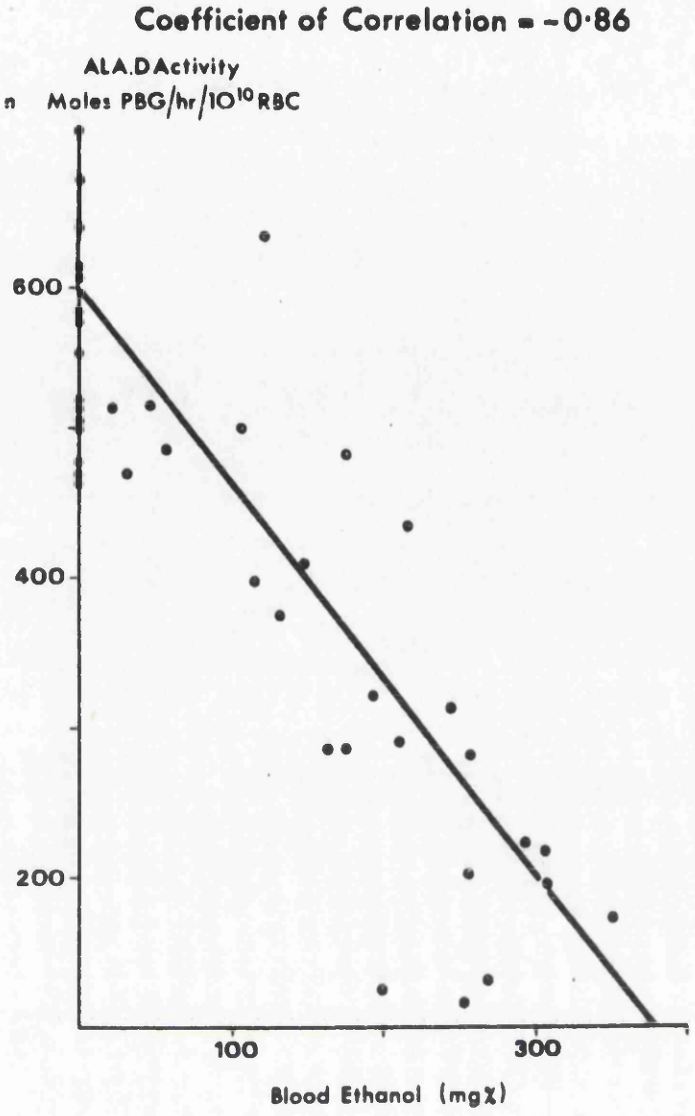


Figure 3.1: Correlation between blood ethanol and blood ALA dehydrase activity in humans.
Each point represents the enzyme activity and blood ethanol level in one patient.

TABLE 3.1.

DEPRESSION OF BLOOD ALA DEHYDRASE ACTIVITY IN HUMANS AND RATS BY ETHANOL

Subjects	Blood Ethanol Level (mg%)*	ALA Dehydrase Activity (n.moles PBG/hr/10^{10} RBC)	Significance
27 Humans (drunk)	225 $\pm$ 72	374 $\pm$ 161	P < 0.001
20 Humans (normal)	0	545 $\pm$ 90	
10 Rats (Ethanol oral)	-	60 $\pm$ 16	P < 0.001
10 Rats (control)	-	134 $\pm$ 22	

* mg/100 ml. whole blood.

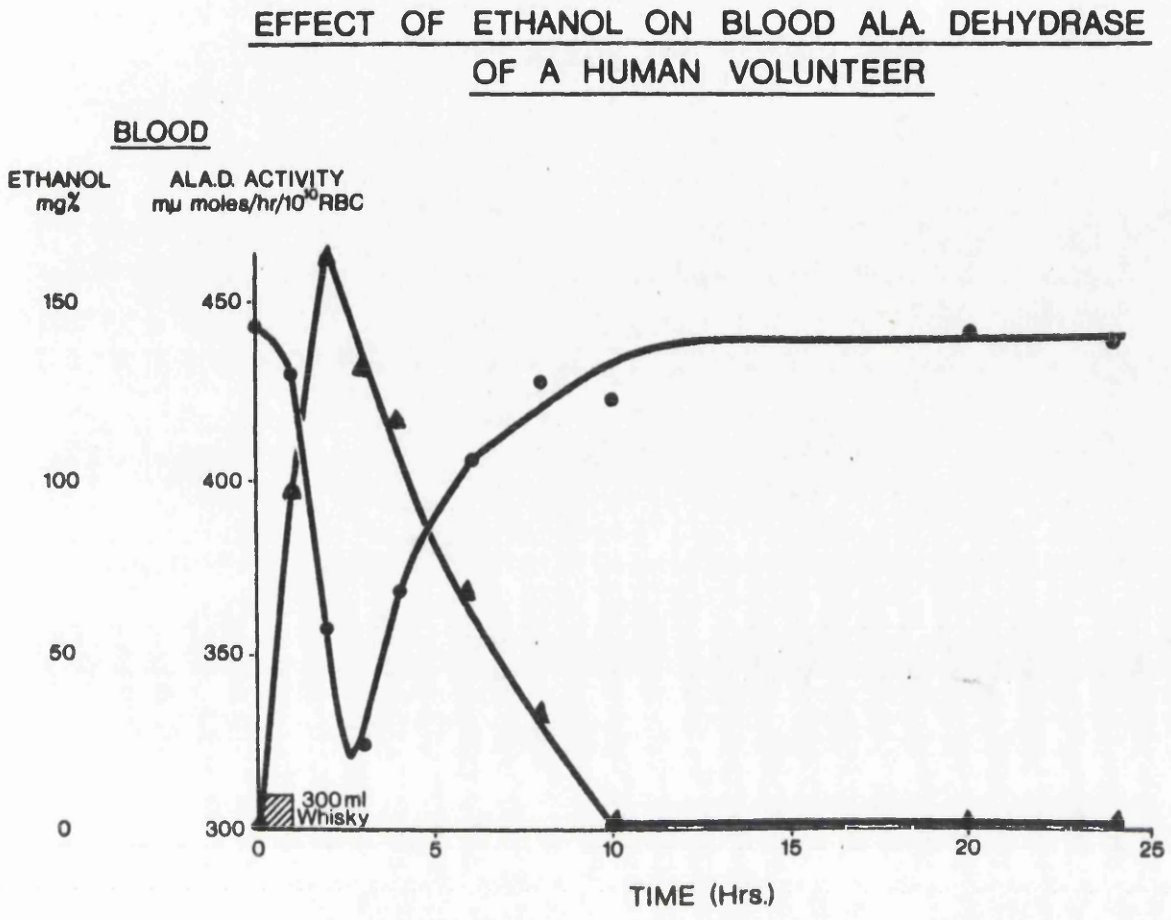


Figure 3.2: Effect of ethanol on blood ALA dehydrase of a human volunteer.

Dots● represent enzyme activity, triangles:▲ Blood concentration. These results show effects after ingestion of 300 ml. whisky over one hour at the commencement of the experiment.

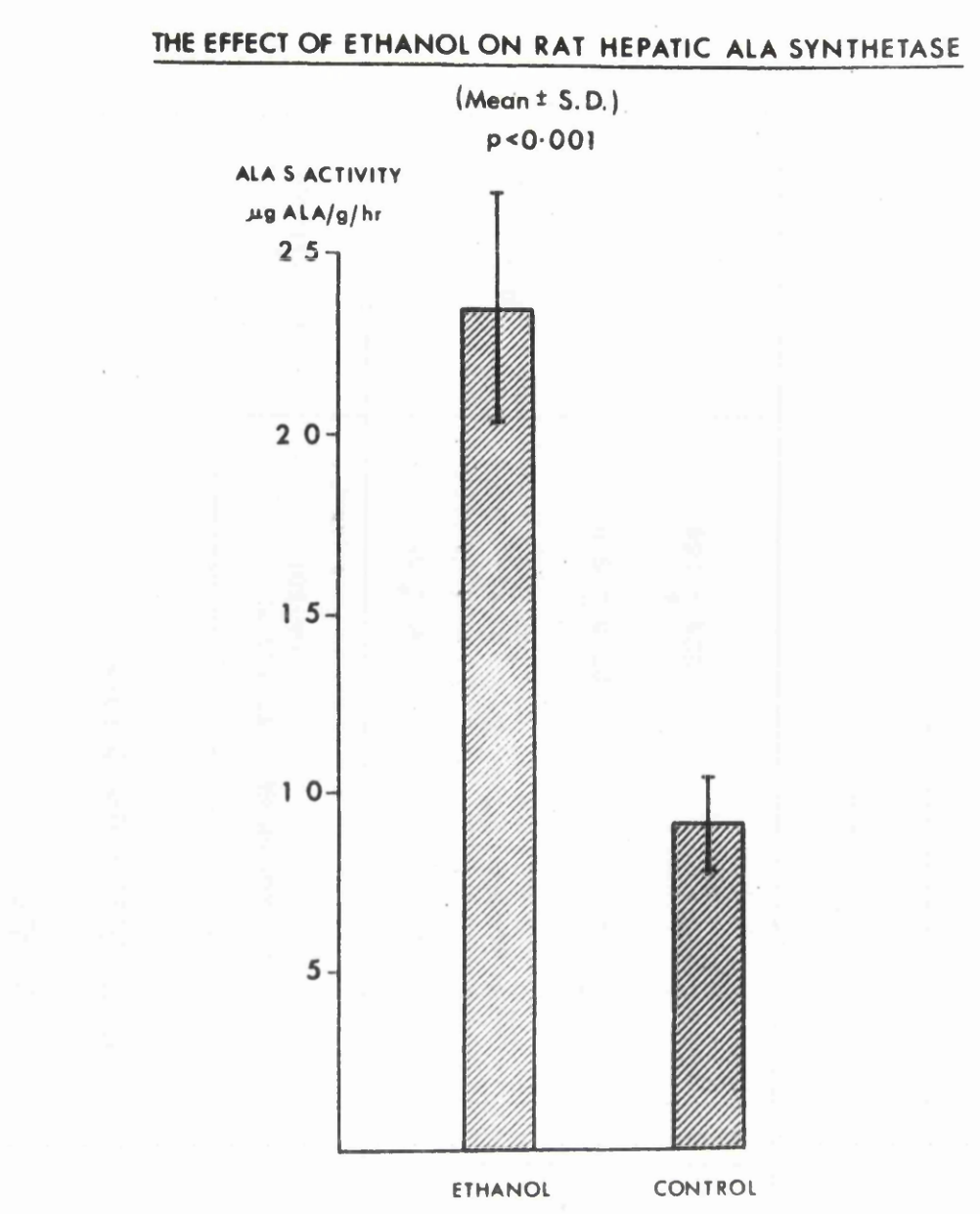


Fig. 3.3. The effect of ethanol, on rat hepatic ALA synthetase - each bar gives the mean ± the standard deviation.

TABLE 3.2.

TISSUE ALA DEHYDRASE ACTIVITIES IN RATS

Tissue	No. of Animals	ALA DEHYDRASE ACTIVITY * ETHANOL	CONTROL	Significance
LIVER	11	1241 ± 61	1426 ± 51	$P < 0.001$
KIDNEY	6	323 ± 52	404 ± 24	$P < 0.05$
HEART	8	97.5 ± 1.2	97.5 ± 6.9	N.S.
SPLEEN	6	206 ± 125	224 ± 164	N.S.

* n.mol PBG/g.wet wt. tissue/hr.

N.S. No significance.

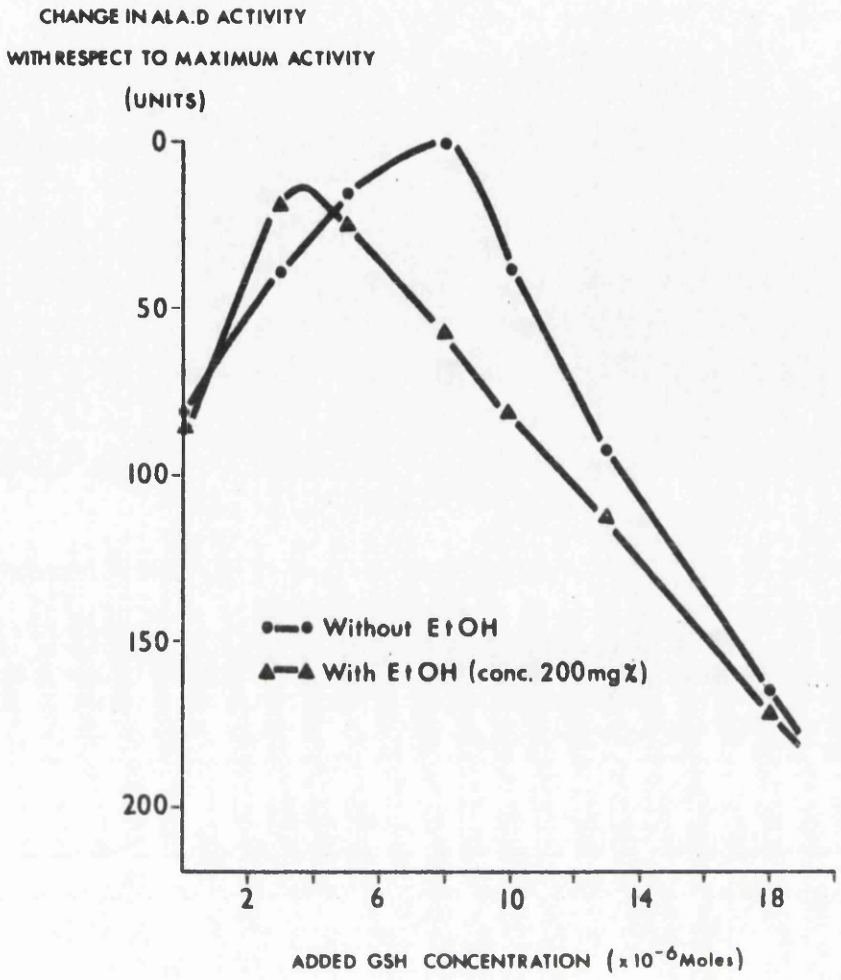


Fig. 3. 4. In vitro effect of variation of added reduced Glutathione (GSH) concentration with and without added ethanol (ETOH) on rat hepatic ALA dehydrase activity. The units of activity are nanomoles PBG produced/g. wet wt. tissue/hour.

coefficient of correlation r = -0.91 and equation y = 701.8 - 0.06 x.

Thus it would appear that this effect is manifested by the depression of the optimal exogenous activating thiol group concentration which could be explained by an increase in the concentration of endogenous thiol groups. When rat blood was assayed for free sulphydryl groups it was found that in animals treated with ethanol the concentration of blood GSH rose both in normal and fasted animals (Table 3.3).

In rats, ethanol is metabolised by the liver at a maximal rate of 10 - 20mg/100ml/hr. Using a rat liver homogenate system ALA dehydrase was assayed in the presence of ethanol concentration 10mg/100ml and in sequence, equimolar concentrations of acetaldehyde, acetate and acetyl CoA, the metabolites of ethanol. Of these, ethanol produced the maximal depression of activity, while acetaldehyde caused about half of this depression and acetate and acetyl CoA had no effect (Table 3.4).

TABLE 3. 3.

Effect of Ethanol (EtOH) on reduced glutathione concentration in rat blood*

	G.S.H. concentration (mg%)	Significance
Fasted Rats	33.5 ± 4.6	
		P < 0.02
Fasted Rats + EtOH	41.8 ± 5.3	
Normal Rats	36.3 ± 5.0	
		P < 0.005
Normal Rats + EtOH	48.3 ± 3.3	

* Results are mean ± S.D., results from 6 rats in each group

TABLE 3.4.

'IN VITRO' EFFECT OF ETHANOL AND METABOLITES ON HEPATIC ALA DEHYDRASE ACTIVITY

Additions *	Change in Hepatic ALA Dehydrase Activity + (% of Control)	Significance
ETHANOL	- 10.90 ± 0.50	$P < 0.001$
ACETALDEHYDE	- 6.33 ± 0.48	$P < 0.001$
ACETATE	- 0.65 ± 0.78	N.S.
ACETYL CoA	- 0.48 ± 0.50	N.S.

* All used in equimolar quantities (2.2×10^{-6} moles)

\+ Each result is the mean ± SD of 5 estimates

N.S. No significance

DISCUSSION

These results demonstrate a highly significant negative correlation between ethanol concentration in blood and tissues and ALA dehydrase activity in these tissues. It has also been shown that only in those organs which handle the ethanol by metabolism or excretion, the liver and kidney, is the activity lowered. The results on ALA synthetase corroborate the work of Shanley et.al. (1968, 1969). ALA dehydrase has been purified and characterised by others in several systems (Gibson, Neuberger and Scott, 1955; Nandi, Baker-Cohen and Shemin, 1968; Shetty and Miller, 1969). Nandi and Shemin (1968) showed the bacterial enzyme to have the properties of an allosteric enzyme (Monod, Wyman and Changeux, 1965) with an equilibrium mixture of monomer dimer and trimer activated by potassium ions which are an absolute requirement for activity at low substrate concentrations. (Burnham and Lascelles, 1963). The enzyme also required thiols for activation. Previously, Shanley et.al. (1968) had demonstrated an elevation of ALA synthetase, the initial enzyme of the haem biosynthetic pathway, by ethanol. This is the rate-controlling enzyme of the pathway (Granick and Urata, 1963) and there is evidence for transient porphyrinuria and potentiation of attacks of porphyria by ethanol (Goldberg and Rimington, 1962; Holmes and Barnes 1965). The effects of ethanol on various metabolic systems has been studied by others; (Isselbacher and Greenberger 1964: Scheig 1970, Porta et.al. 1970) it is oxidised through hepatic alcohol dehydrogenase and hepatic aldehyde dehydrogenase to acetate and then to Acetyl CoA, microsomal oxidation having no effect 'in vivo' (Tephly et.al. 1969). Since the increased redox potential (Lindros and Hillbom, 1969) blocks the citric acid cycle (Forsander and Himberg, 1969; Williamson et.al. 1969) ethanol is then metabolised extra-hepatically to form fatty acids and lipids (Viel, Donoso, Salcedo and Varela, 1967). Ethanol also affects many other metabolic

processes in the liver (Rubin and Lieber, 1968; Lieber and Rubin, 1969). It is well known that ethanol elevates the redox potential within the cell (Zakim, 1968) and Gajdos and Gajdos-Török (1968) have shown that where ALA synthetase is elevated so the NADH level is also raised. Shanley et.al. (1968) suggested that this elevation of ALA synthetase, a mitochondrial enzyme, by ethanol might be due to the altered redox state within the mitochondrion leading to a derepression of the ALA synthetase gene by substrate induction. This is not unreasonable since Lindros and Hillbom (1969) have shown that the NADH/NAD^+ ratio, raised in the cytosol by ethanol is paralleled by an equivalent rise within the mitochondrion possibly due to increased permeability of the mitochondrion (French 1969). These facts presume a subsequent rise in ALA dehydrase activity. That this is not so reflects either a secondary control mechanism within the biosynthetic pathway or a localised cellular effect possibly due to the changed redox potential in the cell.

The limiting step in the metabolism of ethanol is the initial oxidation of ethanol to acetaldehyde by alcohol dehydrogenase (Lundsgaard 1938; Theorell and Chance, 1951; and Westerfield et.al. 1943). This takes place in the cytoplasm of liver cells (Nyberg et.al. 1953, Buttner 1965). The oxidation then rapidly proceeds to Acetyl CoA with the production of two reducing equivalents of NADH (Lundquist et.al. 1969) this is shown diagrammatically in fig.3.5.

We have demonstrated that ALA dehydrase activity in the presence of ethanol needs a lower activating concentration of GSH and that in the absence of ethanol, ALA-dehydrase activity falls with increasing GSH concentration. Further, of four ethanol metabolites, only one, acetaldehyde, has any depressing effect on ALA dehydrase activity and this, like ethanol, is oxidised to acetate and produces one equivalent of NADH, the levels of

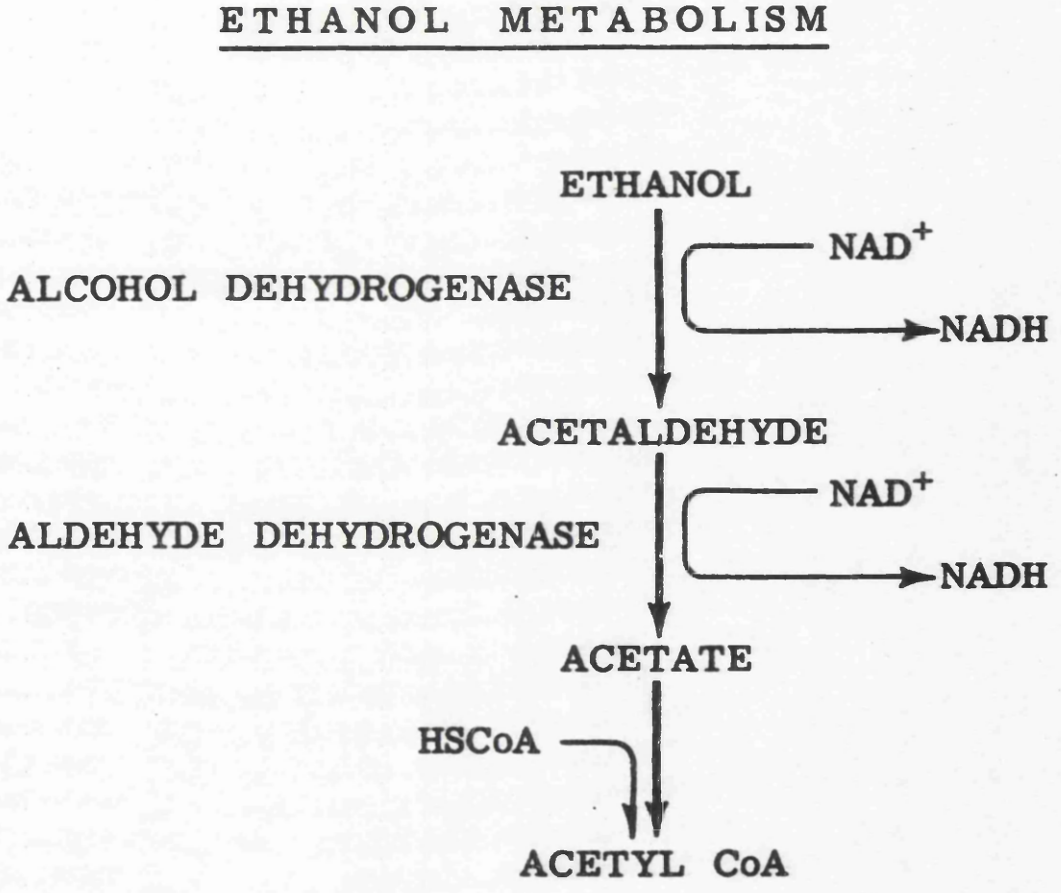


Fig. 3.5. Pathway of Ethanol oxidation in the liver.

acetaldehyde being independent of the ethanol concentration (Majchrowicz and Mendelson 1970). Thus since ethanol utilisation is independent of its concentration (Papenberg et.al. 1970) the ethanol effect on ALA dehydrase is limited to the initial stages of its oxidation and to the production of reducing equivalents. This increased redox potential within the cell will upset the balance of the redox state of other compounds within the cell. Specifically it may influence the equilibrium between the oxidised and reduced forms of sulphydryl compounds. If this is so the increased concentration of blood GSH that has been demonstrated 'in vivo' might inhibit the activity of the enzyme since as we have shown 'in vitro' the activation of ALA dehydrase by the sulphydryl groups of GSH reaches a maximum, after which, ALA dehydrase rapidly loses activity with increasing GSH concentration (fig.3.6). Consequently the effects of ethanol on ALA dehydrase will be localised to those organs known to metabolise or handle ethanol, since it is only in those organs that the redox potential will be sufficiently elevated to repress ALA dehydrase activity.

Thus we have shown that changes in the activity of a haem enzyme can be measured in the blood, and these measurements reflect the enzyme activity within parenchymal cells. This correlation in animals might also apply to man.

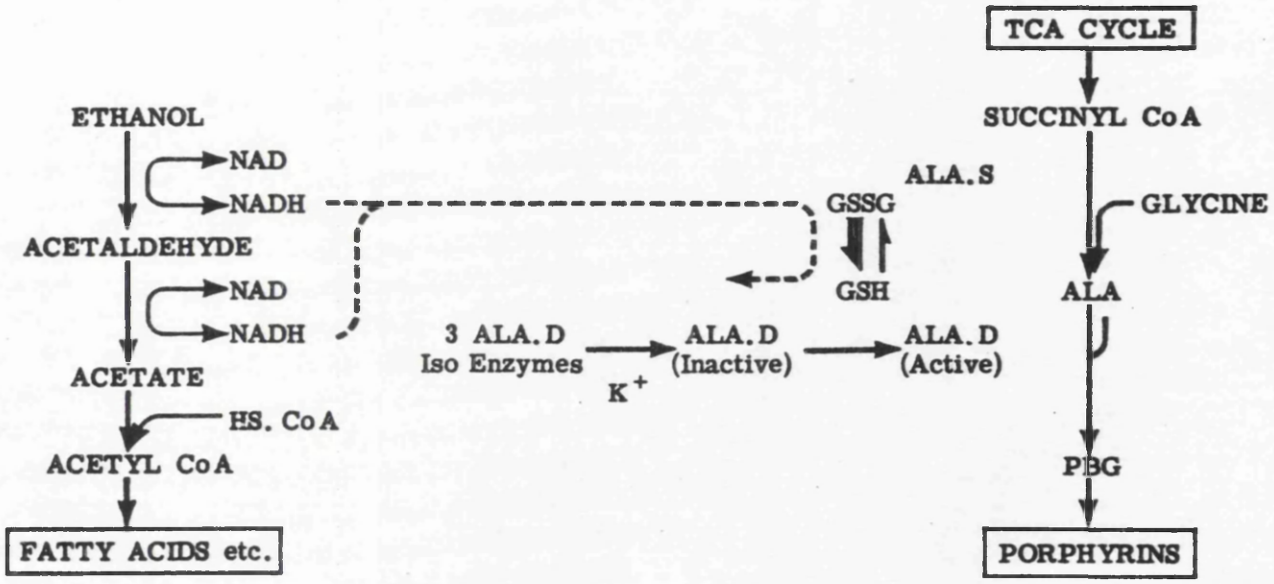


Fig. 3.6. Postulated mechanism of the ethanol effect on ALA dehydrase activity.

NAD and NADH are respectively the oxidised and reduced forms of nicotinamide adenine dinucleotide. HS CoA represents coenzyme A. GSSG and GSH are the oxidised and reduced froms of glutathione and ALA.S., δ aminolaevulic acid synthetase and ALA D. δaminolaevulic acid dehydrase, the first and second enzymes of the haem biosynthetic pathway.

SECTION 4

STEROIDS

In the past there has been some association of the adrenal gland, steroids, and acute intermittent porphyria. Urquhart (1898) suggested that adrenal extracts be used in patients with porphyria since diseases of the adrenals had been recorded in this condition, and Campbell (1898) gave 'suprarenal tabloids' to a patient with porphyria without effect. More recently, in 1949, Prunty found evidence of adrenal cortical hyperplasia in 3 patients with porphyria, and Abrahams et al (1947) noted hyperplasia in one patient with acute porphyria. Adrenocorticotrophic hormone (ACTH) (Goldberg et al 1952) and cortisone (Watson 1954) have been used in the treatment of porphyria sometimes, but not normally, with benefit.

Granick (1966) using a chick embryo liver culture technique demonstrated that some steroids had porphyrinogenic activity and in subsequent papers (Granick and Kappas 1967 a and b) described more exhaustively the effects of a series of steroids. They found that the most potent inducers of porphyrin synthesis in chick embryo liver culture were C_{19} and C_{21} 5 βH steroids, these being metabolic products of the steroid hormones. They further showed that in the conjugated glucuronide form, these compounds did not induce porphyrin synthesis in culture. Kappas & Granick (1967) then showed that this induction of synthesis was abolished by Uridine diphosphoglucuronic acid and by inhibitors of nucleic acid and protein biosynthesis. In view of these results it was suggested that these steroids induced the 'de novo' synthesis of ALA synthetase in this system. These steroids were the first natural compounds to be implicated in porphyrinogenesis and its control.

Subsequently Levere et al (1967); Kappas et al (1968) and Kappas and Granick (1968b) showed that these steroids do induce ALA synthetase in erythroid cell culture and chick embryo liver culture, although they were unable to demonstrate any enhanced ALA synthetase formation in guineapig or rat liver by these steroids. Although these facts indicated that steroid imbalance might be

associated with porphyria, Tschudy (1968) could find no evidence for elevated urinary 17 ketosteroids in patients with hereditary hepatic porphyria. In the following year, however, Goldberg et al (1969) in a study on humans and rats, showed elevated fractions of some urinary 17 ketosteroids in the urine of patients with acute intermittent porphyria although the total 17 ketosteroid levels were within the normal range. It was further found that one of these steroids dehydroepiandrosterone (DHA) caused an elevation of ALA synthetase in rat liver. Necheles & Rai (1969) then showed that one of the 5 βH steroids, etiocholanolone, caused an increase in the uptake of ^{14}C glycine into haem in human bone marrow cells. This was abolished by actinomycin D and puromycin. It was then found that plasma from patients in attacks of A.I.P. was capable of inducing ALA synthetase in chick embryo liver culture and again nucleic acid and protein biosynthesis inhibitors abolished this effect. It was also shown that this effect was produced by the plasma of some patients receiving oral contraceptive steroids (Kappas et al, 1969). Finally, in mice Gorshein & Gardner (1970) showed that these 5 βH steroids stimulated ^{59}Fe incorporation into erythrocytes.

As Kappas et al (1969) showed, there is some association of oral contraception with porphyria, yet the role of oral contraception, and oestrogens in porphyria is difficult to define. In the past there have been conflicting reports on the effects of these compounds on A.I.P. and other porphyrins. In 1960 Watson described cases of patients treated with oestrogens for carcinoma of the breast and prostate who showed photocutaneous manifestations of hepatic porphyria. He then found increased PBG and uroporphyrin in cases of latent porphyria treated with stilboestrol (Watson et al, 1962). Redecker (1963) studied patients with A.I.P. and found increased urinary PBG excretion after treatment with oestrogens and progestrogen. There has been suggested relationships between A.I.P. and contraceptives (Wetterberg, 1964), and Koskelo et al (1966) showed elevated urinary ALA after contraceptives

and similar results were reported for ethinyl oestradiol (Welland et al 1964) whilst Becker (1965) described three cases of porphyria cutanea tarda induced by stilboestrol.

Despite this, combined contraceptive preparations have been used to alleviate the symptoms of porphyria in some female patients in whom the attacks of porphyria could be associated with pregnancy or onset of menses (Welland et al 1964; Haeger-Aronsen, 1963; Perlroth et al 1965). Copeman et al (1966) on the contrary found no change in porphyrin excretion after oestrogens.

In a study on rats Tschudy et al (1967) found that oestradiol caused oscillations in the hepatic levels of ALA synthetase and ALA dehydrase. They suggested that this was due to a perturbation in the negative feedback loop control of the pathway with any elevation of these enzymes merely being a rebound after a decline in activity.

Finally, Rifkind et al (1970) have examined this induction of ALA synthetase by these contraceptive steroids, in chick embryo liver culture. They found that the active component of these steroids was the progestogen whilst no oestrogen significantly altered the level of enzyme on its own. The normal processes of C_{18} C_{19} and C_{21} steroid biosynthesis are shown on fig. 4.7. In this study the effects of a number of these steroids have been examined on an 'in vivo' rat system.

METHODS

The nomenclature used in this section for these steroids is based on the numbering system shown for the steroid nucleus in fig. 4.1. In the series of steroids studied some are unsaturated about positions 4 and 5, and 5 and 6.

STEROID STRUCTURE

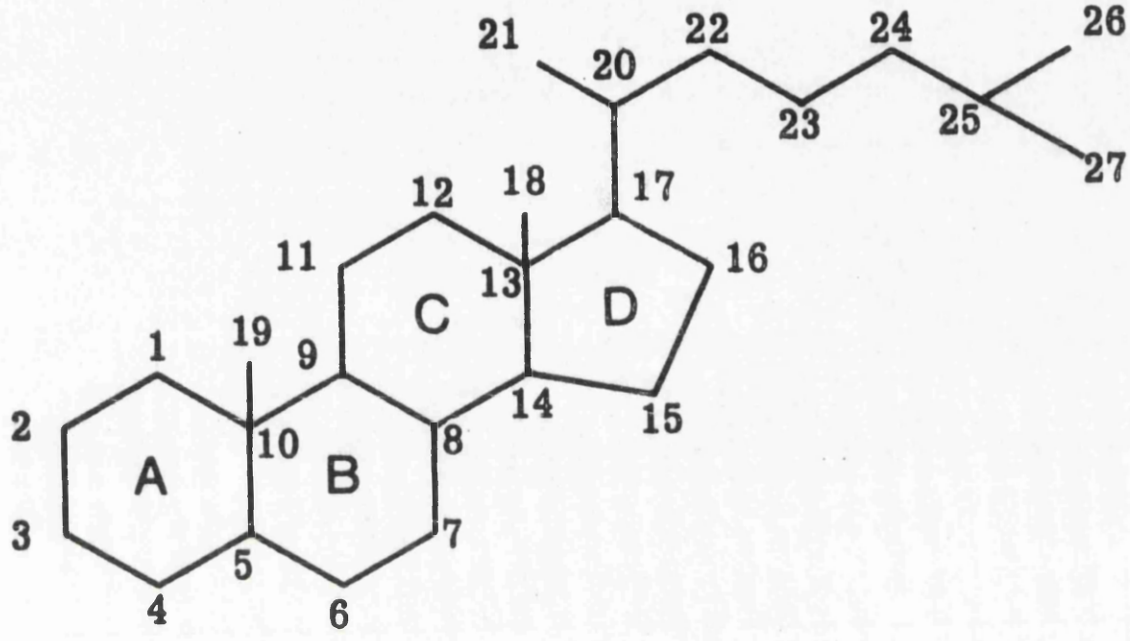


Fig. 4.1. The numbering system of the steroid nucleus and the nomenclature of its rings.

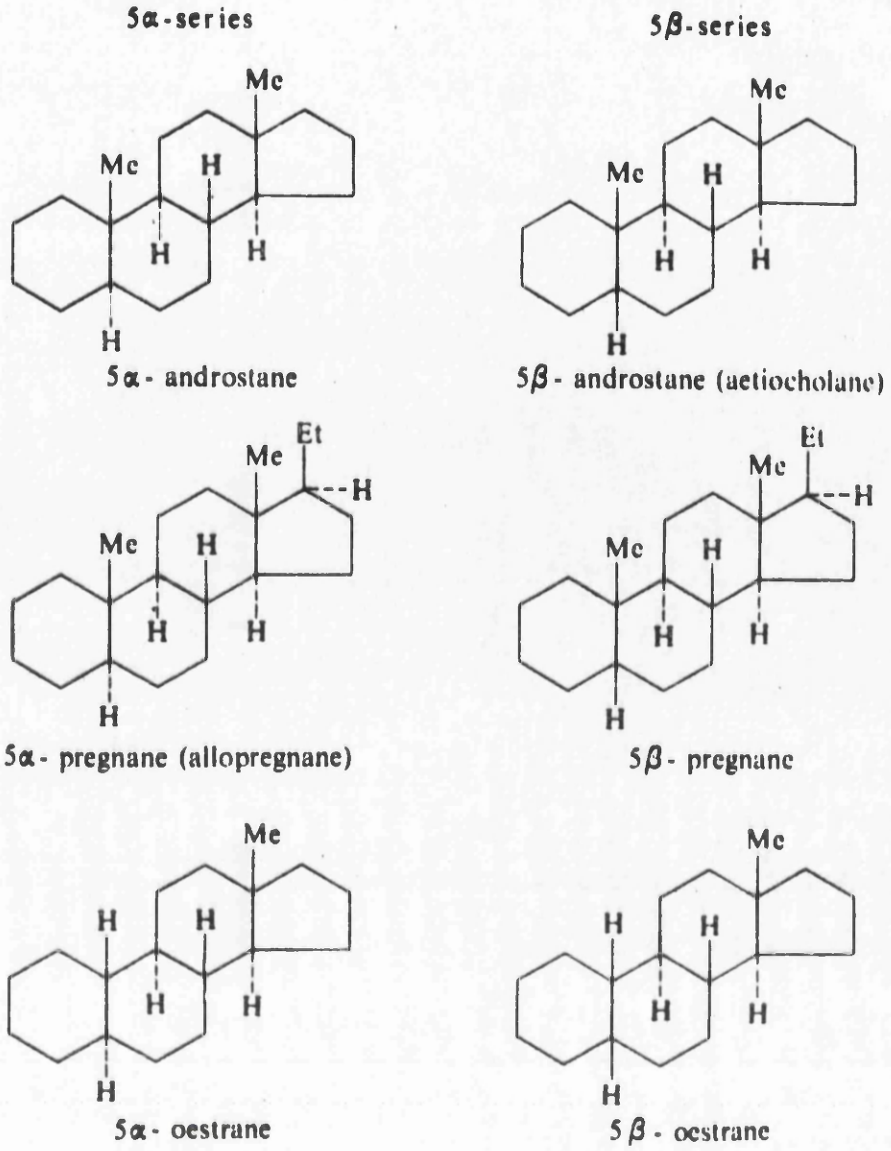


Fig. 4.2 The names and formulae of the C_{19}, C_{21} and C_{18} steroid parent compounds (Me - Methyl; Et - Ethyl)

These double bonds are designated respectively $\Delta 4$ or 4 and $\Delta 5$ or 5. Figure 4.2, shows the C_{19} C_{21} and C_{18} steroids respectively. These are described, when fully saturated according to the conformation of the hydrogen at carbon 5. When it sticks above the ring it is β, and below the ring α, giving bent and planar structures respectively (fig. 4.3). In both of the 4 and 5 unsaturated cases the rings are bent whilst the fully unsaturated aromatic oestrogen ring is again planar. (Fig. 4.3).

During these experiments unless otherwise indicated these steroids were administered at a dosage of 1 mg/kg to the rats, as an intraperitoneal injection of a concentrated ethanol solution (250 mg/ml) or if insoluble as an aqueous suspension of the same strength. Steroids were administered every 24 hours until the termination of the experiment. In all cases an equivalent number of matched control animals were treated and killed at the same time as the test animals. The animal experimental plan for the antibiotics experiments is shown in fig. 4.4. In this, the animals were treated for three days. The figures refer to the time in hours of the experiment. Cycloheximide was administered 4 hours, and actinomycin-D, 7 hours before death to allow sufficient time for their effects to take place.

There is a daily rhythm in the metabolism of metopirone by rats (Szeberenyi et al 1969) with a trough in activity at 10.00am and peak at 10.00 p.m. In our experiments the substance was administered at 10.00 a.m. to allow for a lower toxicity of this substance at this time since it has been shown to be more toxic in mice in the evening (Ertel et al 1963).

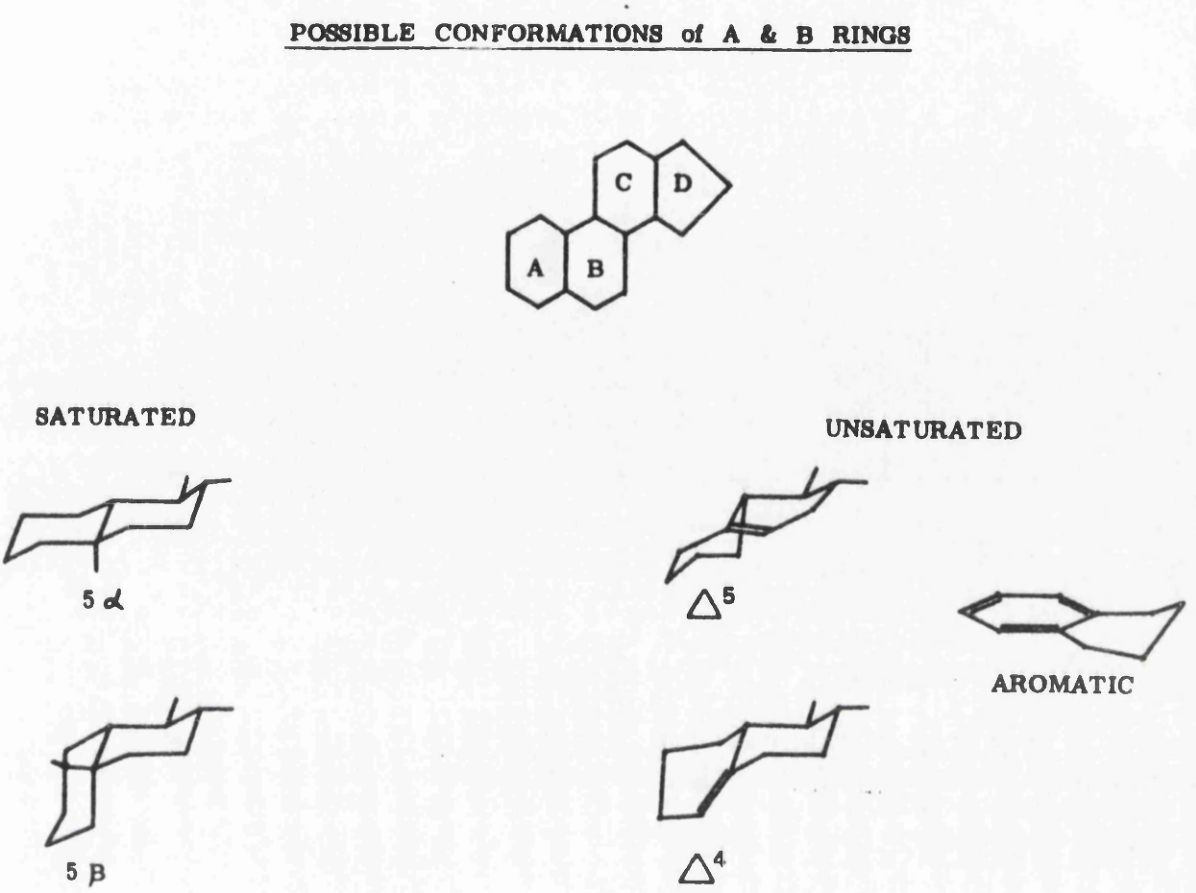


Fig. 4.3 Possible Conformations of the A & B rings of the steroid nucleus.

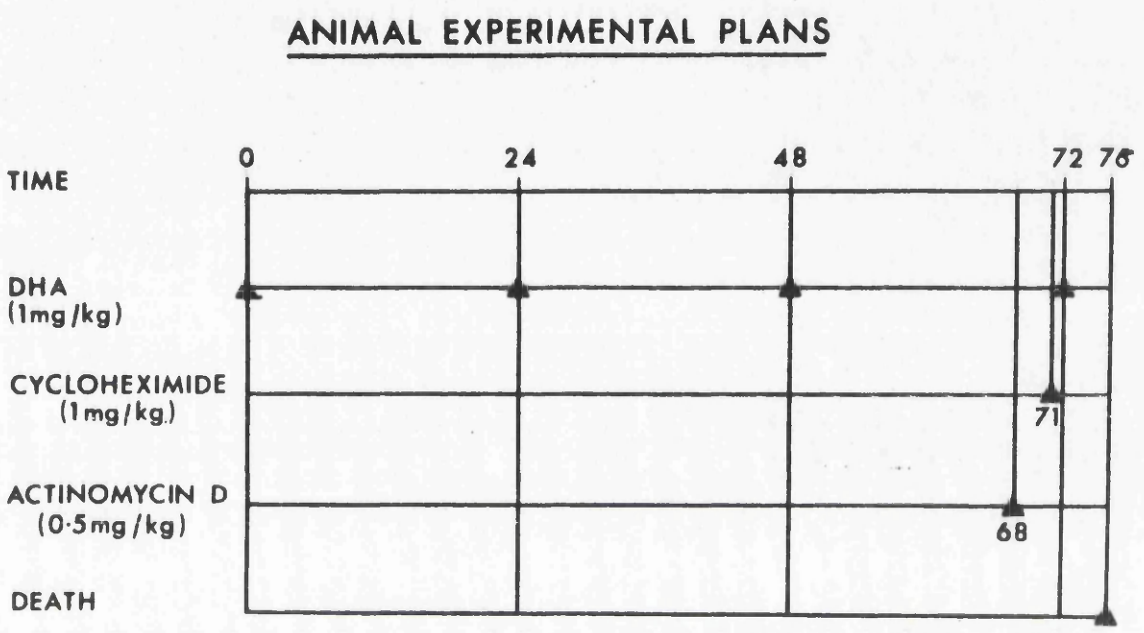


Fig. 4.4. Animal Experimental Plan, Antibiotics experiment. Figures give time of dosage and death.

RESULTS

1) General Studies

Table 4.1. showed the series of steroids studied in these experiments. The nomenclature is as explained before and they are numbered to facilitate their identification in subsequent tables. These steroids fall into three groups according to the number of carbon atoms in their structure, that is C_{21}, C_{19} and C_{18}.

Table 4.2 shows the effects of the C_{21} steroids. Significant elevations ($p < 0.001$) of hepatic ALA synthetase were found in rats.treated with 17 hydroxpregnenolone. No other figures were significantly elevated. Table 4.3 shows the effects of C_{19} steroids. Of these androstenedione and dehydroepiandrosterone significantly elevated ALA synthetase activity ($p < 0.001$) whilst androstenediol and etiocholanolone also elevated ALA.S activity at a lower level of significance. ($p < 0.02$). None of the C_{18} steroids elevated the activity of ALA.S. (Table 4.4) whilst the activity of ALA dehydrase was unaffected by any of these steroids.

2) Studies on Dehydroepiandrosterone(DHA)

Significant elevations ($p < 0.01$) of hepatic ALA.S levels were found within 24 hours after a daily intraperitoneal injection of 0.5mg free DHA reaching maximal levels at about 72 hours. (fig.4.5). Injection of DHA sulphate also caused a significant elevation of hepatic ALA.S in rats ($p < 0.001$) whilst DHA acetate and DHA with equimolar amounts uridine diphospho-glucuronic acid had no significant effect on activity over the control activity (Table 4.5).

This elevation of ALA.S by DHA was followed in a time course experiment and studied using the antibiotics cycloheximide and actinomycin D.

Table 4.1. STEROIDS STUDIED

No. of Carbons	SYSTEMATIC NOMENCLATURE			NUMBER	COMMON NAME
21	4	Pregnen	3.20 Dione	1	Progesterone
	5	Pregnen	3 β ol 20 One	2	Pregnenolone
	5	Pregnen	3 β 17 α diol 20 one	3	17 Hydroxy Pregnenolone.
	5β	Pregnan	3α 20 α Diol	4	Pregnandiol
	5β	Pregnan	3α 17α 20α Triol	5	Pregnantriol
19	4	Androsten	3, 17 Dione	6	Androstenedione
	4	Androsten	17 βol 3 One	7	Testosterone
	4	Androsten	3, 11, 17 Trione	8	Androstenetrione
	5	Androsten	3 βol 17 One	9	Dehydro Epi Androsterone (DHA)
	5	Androsten	3 β 17 β Diol	10	Androstene Diol
	5β	Androstan	3, 17 Dione	11	Etiocholan Dione
	5β	Androstan	3α OL 11,17 Dione	12	11 Keto Etio Cholanolone
	5β	Androstan	3α 17 β Diol	13	Etiocholandiol
	5β	Androstan	3α OL 17 One	14	Etiocholanolone
	5	Androstan	3α OL 17 One	15	Androsterone
	5	Androstan	3 βOL 17 One	16	Epi Androsterone
	5	Androstan	3, 17 Dione	17	Androstan Dione
18	1,3,5 Oestratrien		3,17 β Diol	18	Oestradiol
	1,3,5 Oestratrien		3,16α, 17β Triol	19	Oestriol
	1,3,5 Oestratrien		3OL 17 One	20	Oestrone

TABLE 4.2.

THE EFFECT OF CERTAIN C21 STEROIDS ON RAT HEPATIC

ALA SYNTHETASE & ALA DEHYDRASE ACTIVITIES

No.	Steroid	No of Rats	ALA S. Activity+	ALA D Activity x
1.	Progesterone	5	6.92 ± 2.05	1137 ± 112
2	Pregnenolone	5	8.57 ± 1.45	1005 ± 108
3	17 Hydroxy-pregnenolone	6	10.64 ± 1.61*	1117 ± 89
4	Pregnan diol	5	7.52 ± 1.82	1135 ± 126
5	Pregnan triol	6	7.26 ± 1.48	1096 ± 111
	Control	50	7.20 ± 1.53	1080 ± 164

\+ µg ALA/ wet weight liver/hr

x n mol PBG/g wet wt liver/hr

* statistically significant elevation $P < 0.002$

TABLE 4.3

THE EFFECT OF CERTAIN C19 STEROIDS ON RAT HEPATIC ALA SYNTHETASE & ALA DEHYDRASE ACTIVITIES

No.	Steroid	No. of Rats	ALA S Activity +	ALA D Activity x
6	Androstenedione	6	12.26 ± 1.41 *	1010 ± 200
7	Testosterone	5	8.25 ± 1.92	995 ± 238
8	Androstenetrione	6	7.70 ± 3.25	1180 ± 119
9	Dehydroepiandrosterone	12	14.42 ± 1.28 *	1053 ± 100
10	Androstenediol	5	9.80 ± 1.02 x	1075 ± 147
11	Etiocholandione	5	8.65 ± 1.56	1125 ± 118
12	11 Keto Etiocholanolone	5	8.35 ± 1.43	1120 ± 137
13	Etiocholandiol	6	7.50 ± 2.25	1082 ± 321
14	Etiocholanolone	5	10.40 ± 3.02 x	1008 ± 213
15	Androsterone	6	6.50 ± 2.25	1030 ± 221
16	Epiandrosterone	6	7.09 ± 1.58	1112 ± 230
17	Androstandione	5	6.00 ± 2.22	1025 ± 150

Statistically significant * $p < 0.001$ x $p < 0.02$

TABLE 4.4.

THE EFFECT OF CERTAIN C18 STEROIDS ON RAT

HEPATIC ALA SYNTHETASE & ALA DEHYDRASE ACTIVITIES

No.	Steroid	No. of rats	ALA S Activity	ALA D Activity
18	Oestradiol	6	6.75 ± 1.18	1145 ± 230
19	Oestriol	6	6.92 ± 1.46	1115 ± 198
20	Oestrone	5	7.16 ± 1.26	1078 ± 113
	Control	50	7.20 ± 1.53	1080 ± 164

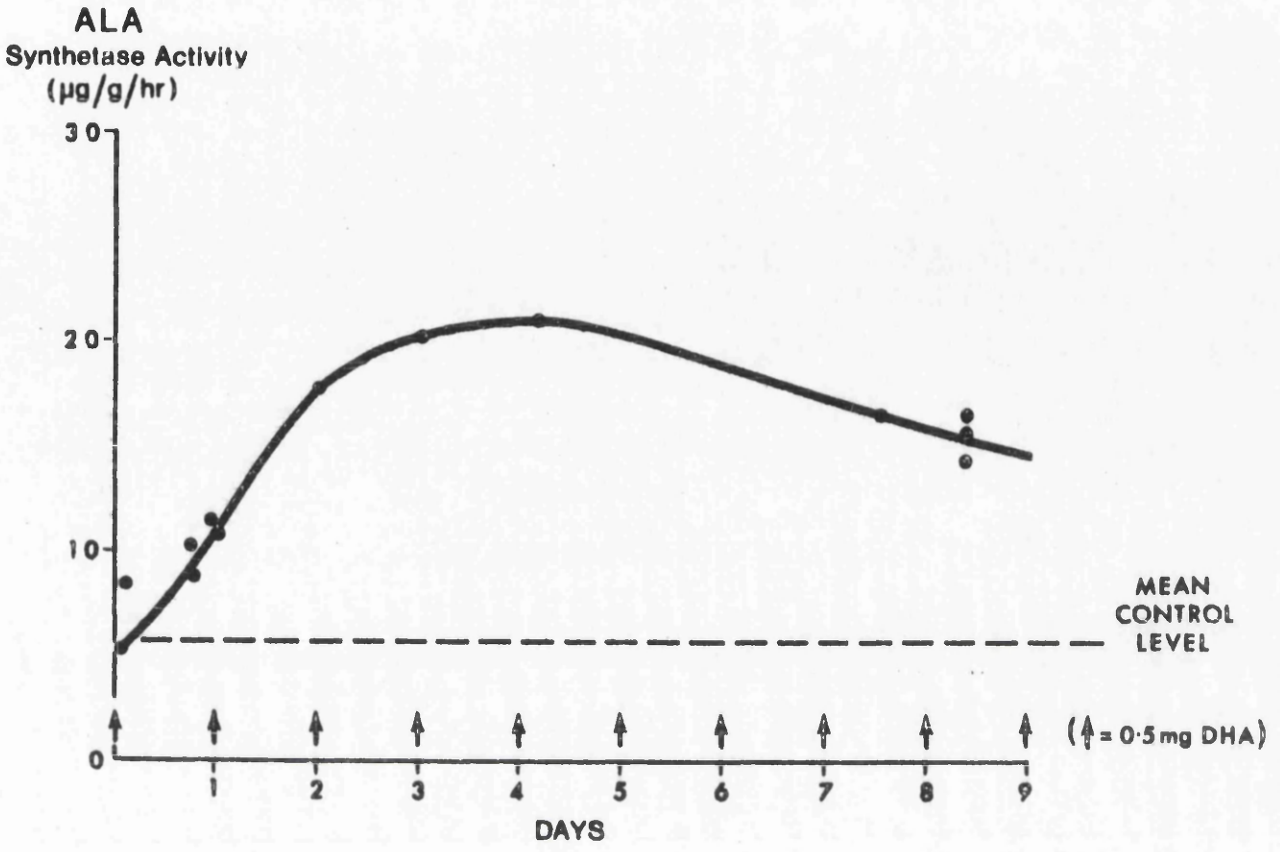


Fig. 4.5. The effect of daily intraperitoneal injections of DHA on hepatic ALA. synthetase activity in rats.
Each point represents one animal, the mean control level is the mean hepatic ALA.S. activity in 25 rats.

TABLE 4.5

THE EFFECTS OF DHA ON ALA SYNTHETASE ACTIVITY

Treatment	No. of animals	ALA.S. Activity	Significance with respect to control
Control	25	6.00 $\pm$ 2.22	-
DHA (1 mg/kg)	12	14.42 $\pm$ 1.28	$P < 0.001$
DHA Sulphate (1 mg/kg)	6	13.39 $\pm$ 2.71	$P < 0.001$
DHA Acetate (1 mg/kg)	6	7.21 $\pm$ 1.28	N.S.
DHA (1 mg/kg) + Equimolar UDP glucuronic acid	6	8.30 $\pm$ 1.22	N.S.

(3 days dosage)

The results are shown in figure 4.6. The time course of elevation followed a pattern similar to the previous time course experiment (fig.4.5.) Each point on the figure represents one animal, the arrows indicate the time of dosage of DHA (1 mg/kg); as can be seen both actinomycin D and cycloheximide abolished the DHA elevation of ALA.S. activity; indeed they abolished all activity in about 8 and 10 hours respectively. All three hepatic porphyrins were significantly elevated by DHA (Table 4.6).

3) Steroid antagonists.

In this section the effects of the compounds metopirone, and dexamethasone were investigated (fig.4.7). Metopirone significantly elevated ALA.S. activity ($p < 0.001$) and depressed ALA.D activity ($p < 0.001$) (Table 4.7). In animals treated with AIA (300 mg/kg) dexamethasone had no effect on the enzyme levels of the rats although in the animals treated with both AIA and dexamethasone, there was a large improvement in the condition of the animals (Table 4.8.).

DISCUSSION.

The various pathways of steroid metabolism are shown in fig.4.8. These are collated from Lorraine & Bell (1966); Cantarow & Schepartz (1967); Cameron et al (1969) and Payne and Mason (1969). Of the steroids shown in this scheme only those ones marked with an asterisk, that is 17α hydroxypregnenolone, DHA, DHA sulphate, androstenedione and etiocholanolone have been shown to have some porphyrinogenic activity in the present system. Of these, the most potent inducer was DHA, and this was picked out for further study. These results are to some extent in agreement with the work of Granick & Kappas (1967) who showed that the most potent inducers of porphyrin fluorescence in chick embryo liver

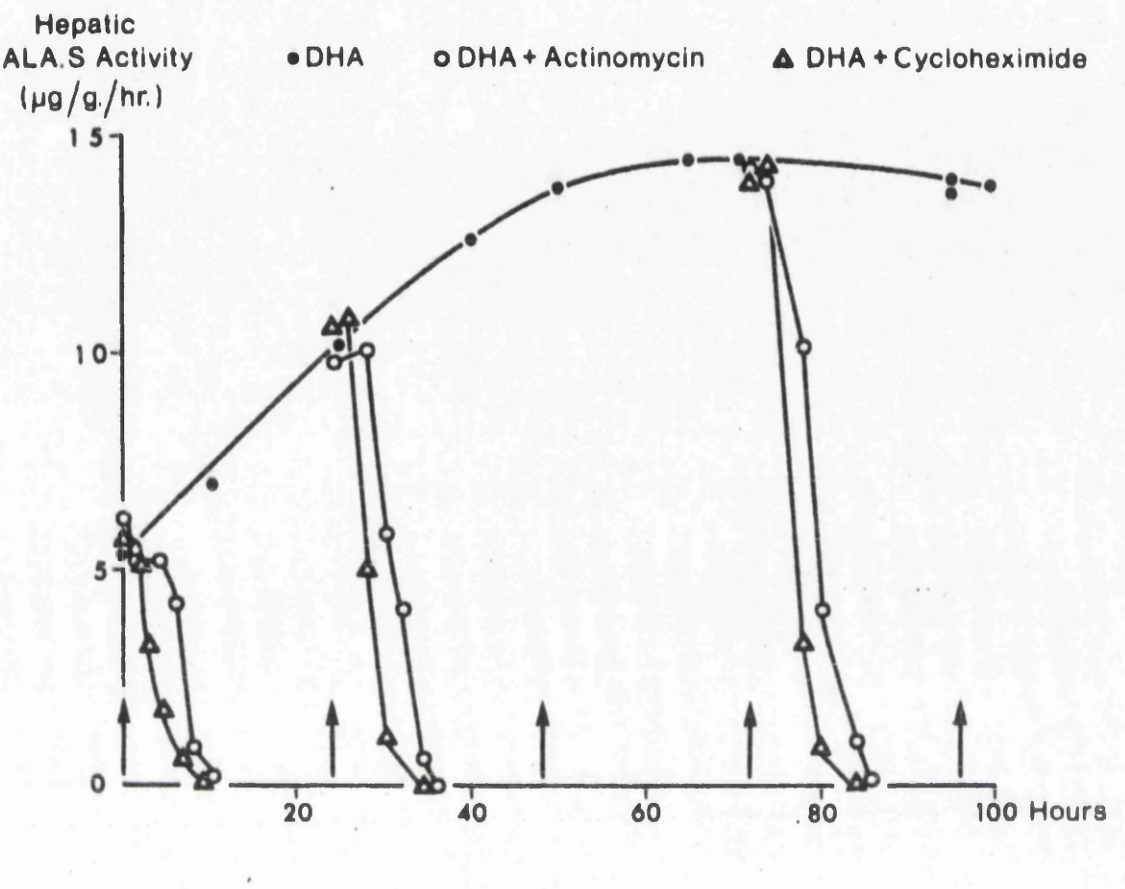


Fig. 4.6. The effect of intraperitoneal injections of DHA and Actinomycin D and Cycloheximide on the activity of rat hepatic ALA Synthetase.

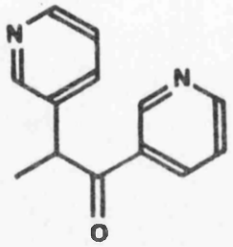
Each point represents one animal. Circles represent animals treated with actinomycin D; triangles, cycloheximide.

The arrows represent the times of dosage.

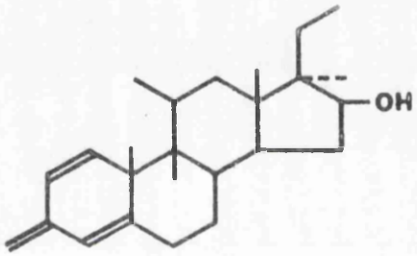
TABLE 4.6.

THE EFFECT OF DHA ON HEPATIC PORPHYRINS IN RATS

Treatment	Porphyrins (µg/g wet wt.) Uro	Copro	Proto
Control	0.03 ± 0.02	0.12 ± 0.10	0.10 ± 0.09
DHA (1mg/kg) for 3 days	0.08 ± 0.02	0.29 ± 0.09	0.30 ± 0.08
Significance P<	0.01	0.02	0.001



METOPIRONE

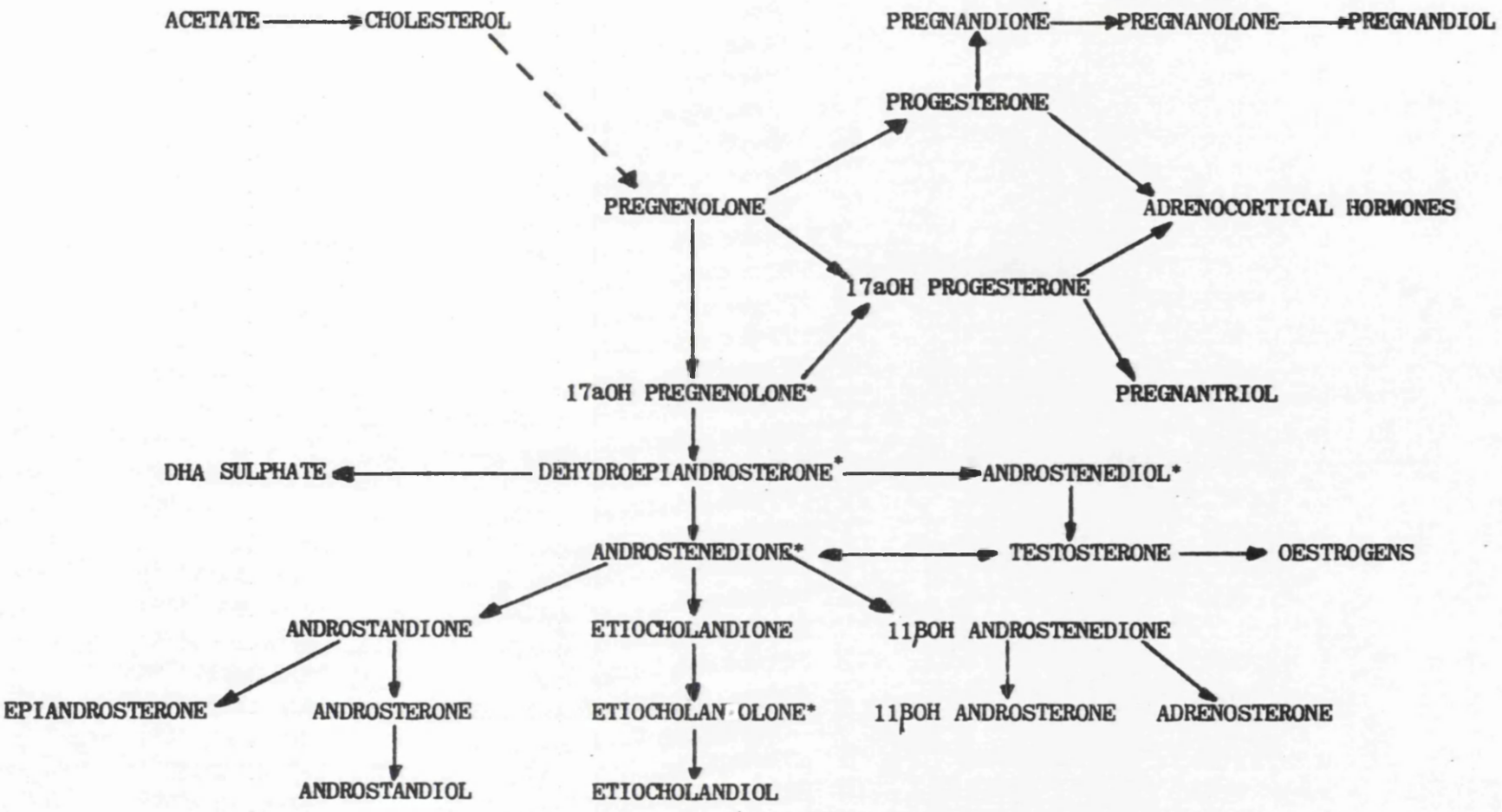


DEXAMETHASONE

Fig. 4.7. Structures of metopirone and dexamethasone

FIGURE 4.8.

STEROID METABOLISM



(* :- Porphyrinogenic)
at dosage used

TABLE 4.7.

THE EFFECT OF METOPIRONE ON BODY AND LIVER WEIGHT AND HEPATIC ALA S AND ALA D ACTIVITIES IN RATS

Treatment	No. of rats	Increase in body wt. (g)	Liver wt. (g/ 100g. body wt.	ALA S. activity	ALA D. activity
Control	6	20 ± 11	4.02 ± 0.52	6.05 ± 3.22	1100 ± 125
Metopirone (200 mg/kg)	6	25 ± 12	4.59 ± 0.61	18.67 ± 3.93	788 ± 84

TABLE 4.8.

THE EFFECTS OF DEXAMETHASONE AND ALLYISOPROPYL ACETAMIDE

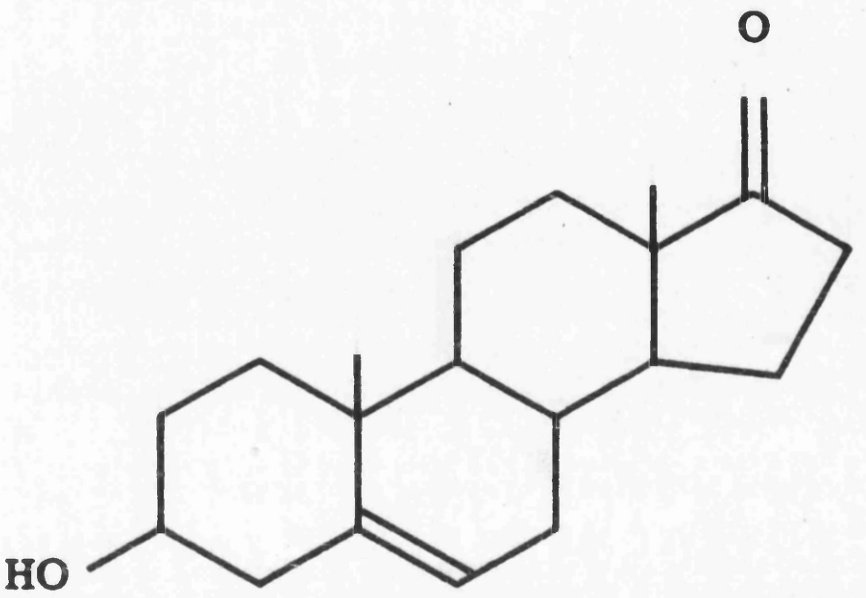
ON RAT HEPATIC ALA SYNTHETASE

Treatment	No. of rats	ALA S. Activity	CONDITION
Control	6	9.05 ± 2.25	Normal - lively & bright eyed Livers: bright red & firm.
Dexamethasone (6 mg/kg)	6	8.25 ± 2.42	" "
A.I.A. (300 mg/kg)	6	84.23 ± 9.56	Ill - Lethargic - porphyrin excretion from Harder's Gland Livers: Vacuolar & necrosed, brown.
A.I.A. (300 mg/kg) Dexamethasone (6 mg/kg)	6	78.69 ± 15.23	Almost normal. Quiet but eyes normal, livers fairly firm, red.

culture were the 5 βH steroids. Their system did not implicate the unsaturated steroids to any great extent. This may be due to species differences in the metabolism of steroids since it has been shown that there are differences in the incorporation of steroids into mammalian and avian erythrocytes (Ohtsuka & Koide, 1969) which suggested a variation in the species metabolism of these compounds which would probably apply to other cells.

The effects of dehydroepiandrosterone (DHA) whose structure is shown opposite (Fig. 4.9) were examined in greater detail. It significantly elevates ALA synthetase activity as does its sulphate, yet it does not effect ALA dehydrase. Both actinomycin D and cycloheximide abolish this elevation of ALA.S by DHA and this suggests that the elevation of activity thus produced is due to the 'de novo' synthesis of ALA synthetase. It was further found that the glucuronide and acetate conjugates of these steroids were ineffective as inducers of the enzyme. Again these results are consistent with the work of Kappas & Granick (1968). It was also found that hepatic porphyrins are elevated as would be expected with a highly elevated ALA synthetase activity.

In previous work on human patients with acute intermittent porphyria (Goldberg et al, 1969) it was shown that of the fractionated 17 ketosteroids in urine, DHA was one of the most highly elevated steroids in several patients in attack. Jones & Griffiths (1968) showed that in the adrenals, DHA sulphate is produced in the zona reticularis by its sulphokinase. For this reason it was decided to study the effects of dexamethasone, used to suppress adrenal function, on acute intermittent porphyria. Dexamethasone causes a decrease in the urinary excretion of DHA and other 17 hydroxycorticosteroids (Lorraine & Bell, 1966). It was found that in A.I.P. dexamethasone caused a significant reduction in the urinary steroids measured, especially DHA and in addition urinary porphyrins and precursors were significantly depressed (Moore et al 1969) fig. 4.10. In animal studies there was no change in hepatic enzyme levels or porphyrins during treatment of AIA induced porphyria with dexamethasone although there was some



DEHYDRO-EPIANDROSTERONE (DHA)

(5 Androstene 3 β ol 17 One)

Fig. 4.9. The structure of Dehydroepiandrosterone (DHA)

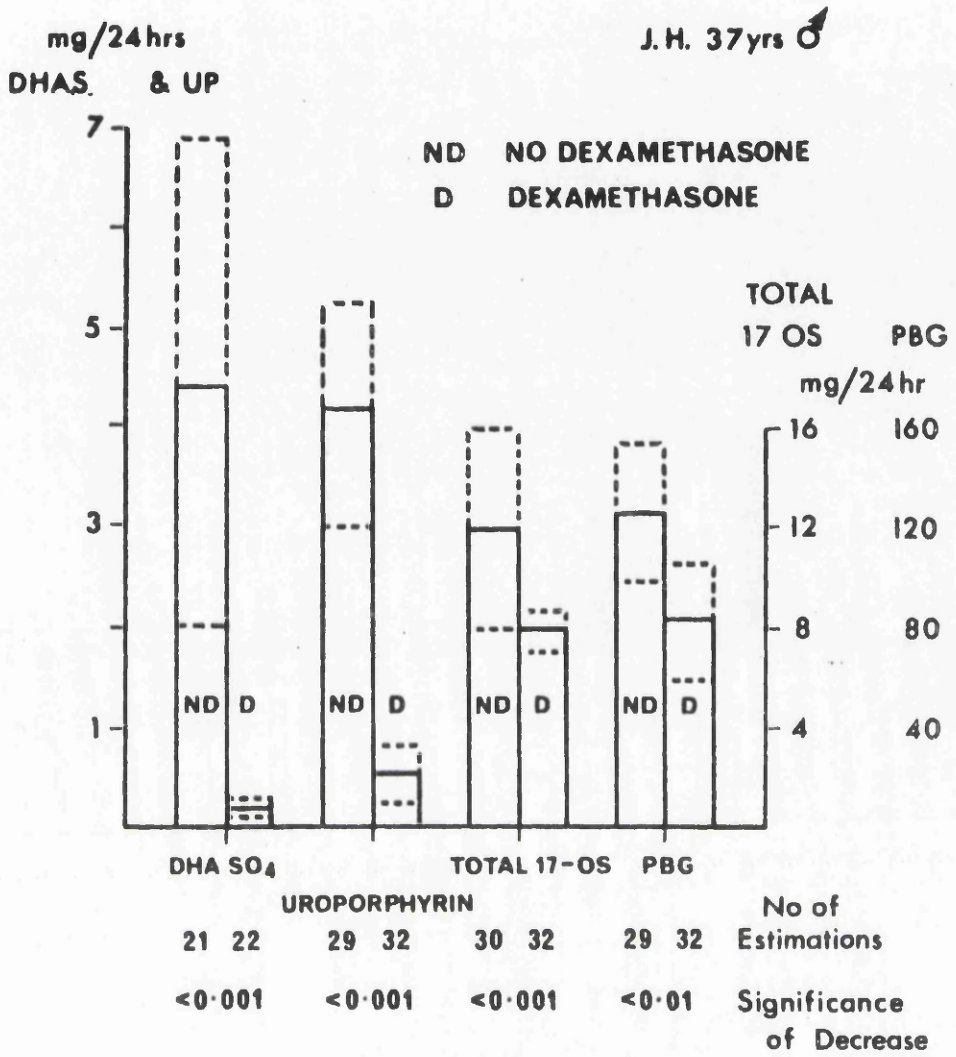


Fig. 4.10 The Effect of dexamethasone on the urinary excretion of Porphyrins and 17 ketosteroids in a human male suffering from Acute Intermittent Porphyria.

physiological improvement in the animals condition. The lack of change may be because in the hypothetical scheme of control AIA does not act through the steroids but rather acts on its own against the repressor mechanism. Its effect in humans is probably related to suppression of ACTH release (Sirett & Gibbs, 1969) but may also be due to other factors in this system, since it has been shown to lead to a significant depression of citric acid levels in humans (Wajchenberg et al, 1969). This depression of citrate could lead to a depression of succinate with a subsequent decrease in the entry of succinyl CoA substrate into the biosynthetic pathway.

The effect of metopirone (2 methyl, 1, 2 bis (3' pyridyl) 1 propanone) and its assessment is less easy. It is known to inhibit various adrenal mitochondrial steroid hydroxylases (Liddle et al, 1958; Sanzari & Peron, 1966) and since these are similar to drug metabolising enzyme systems in the liver mediated by cytochrome P.450 as the terminal oxidase (Estabrook et al 1963; Omura et al, 1965) it also interferes with drug hydroxylation (Leibman 1969). In addition Kahl & Netter (1970) have shown that it also inhibits respiration of liver slices and leads to a change in the redox state of the cell and an increase in aerobic glycolysis. In these experiments it was used with a view to blocking the formation of cortisol and corticosterone with a subsequent rise in 17 hydroxycorticosteroids induced by elevated ACTH (Lorraine & Bell 1966). To this end it may be that the elevation of ALA synthetase is related to the rise in these steroids but in view of the other cellular effects of this compound, it would be unwise to ascribe totally this rise to the rise in these steroids. The depression of ALA dehydrase activity may be due to the altered redox state of the cell (Section 3.)

The case for or against the use of oral contraceptives in porphyria is complicated. It seems certain that the progestogen content of 'the pill' is porphyrinogenic and that the oestrogen content has no effect on the haem

biosynthetic pathway (Rifkind et al 1970) as would appear to be further corroborated by the results found in this study which show oestrogens to be ineffective in raising ALA.S activity, as are synthetic oestrogens like mestranol (Aitken and Moore 1970), whilst some androgens and one progestogen are effective. In opposition to the hypothesis that DHA is the effective substance in this system, Bulbrook & Hayward (1969) have shown that oral contraceptives lower the urinary excretion of DHA and of other 17 OH corticosteroids and 11 deoxy 17 oxosteroids. In women, in general, fluctuations of the urinary total 17 oxosteroids may be observed during the menstrual cycle and these steroids increase in amount during pregnancy (Lorraine & Bell 1966). Longhino et al (1968) measured the urinary excretion of certain of these steroids during the menstrual cycle and showed that there was some fluctuation in these levels, with both androstenedione and DHA elevated during the luteal phase of the cycle. It is now known that there is a sex/age correlation between the incidence of acute intermittent porphyria and urinary 17 oxosteroid excretion. Hamburger (1948) showed that the levels of urinary 17 ketosteroid excretion in men reached a peak in the fourth decade of life whilst in women it was greatest in the third decade. (Fig. 4.11). The most frequent age of onset of symptoms of A.I.P. in males is again in the fourth decade of life and in females in the third decade (fig. 4.12) (Goldberg & Rimington, 1962). Thus there is a direct correlation between these two factors in acute porphyria.

URINARY 17 KETOSTEROID EXCRETION in NORMAL HUMANS

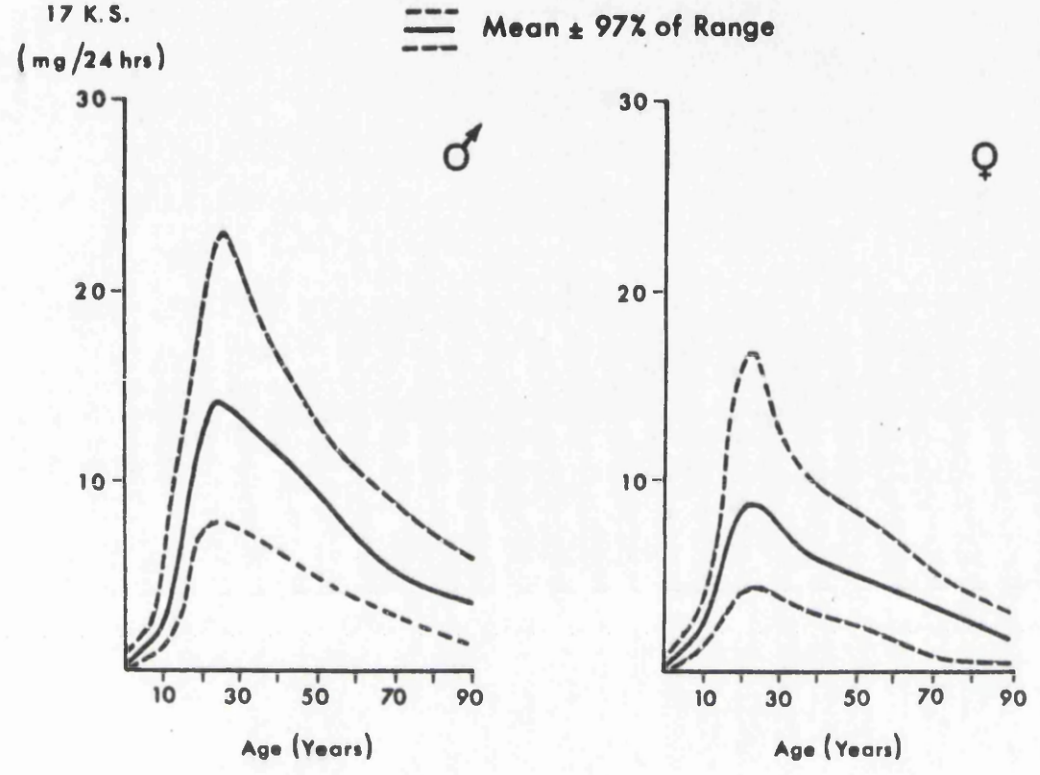


Fig. 4.11.

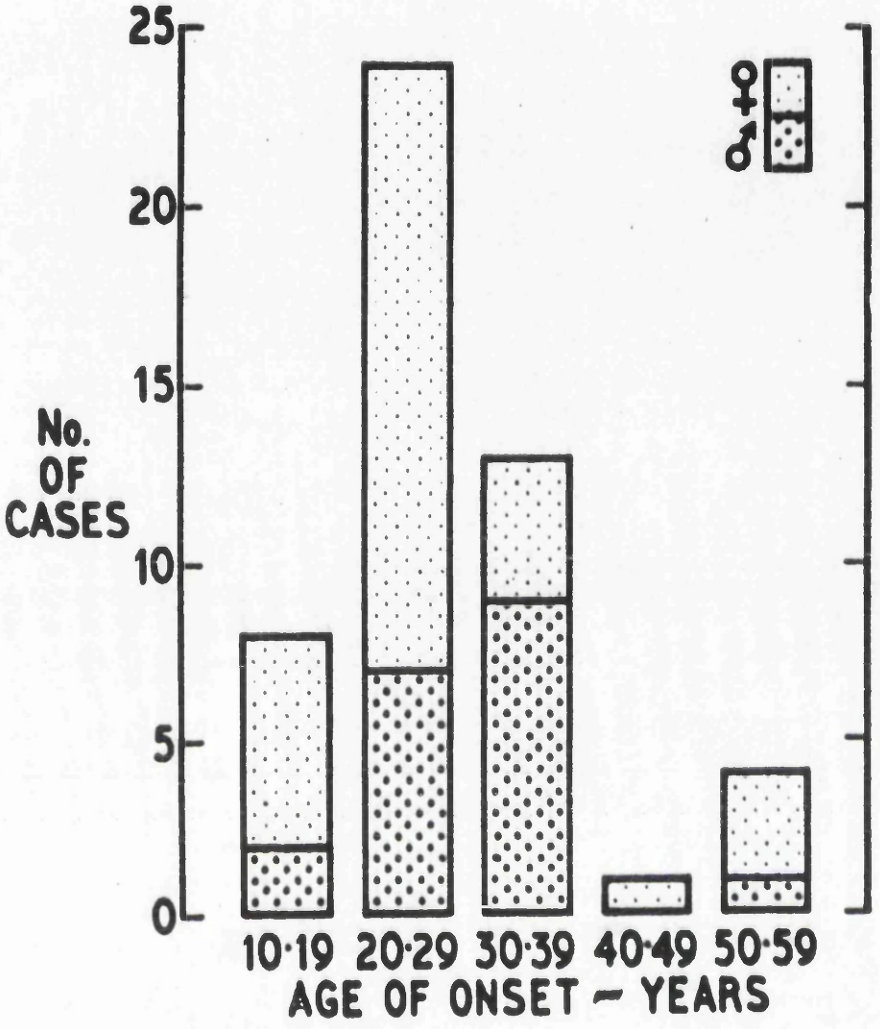


Fig. 4.12. The Age of onset of attacks of porphyria in human males and females.

SECTION 5

STUDIES IN HEREDITARY COPROPORPHYRIA

INTRODUCTION

The history of porphyria, as such, in humans only commenced less than one hundred years ago. (Goldberg & Rimington, 1962). There are basically two types of porphyria, erthropoietic and hepatic porphyria . Hereditary coproporphyria is a hepatic porphyria which is characterised from the other hepatic porphyrias by the excessive excretion of coproporphyrin in faeces and urine at all times, accompanied by an elevation in porphyrin precursor excretion in acute attacks.

There are four commoner forms of hepatic porphyria; acute intermittent porphyria; variegate porphyria; porphyria cutanea tarda and symptomatic porphyria, of which, the first three are inherited as Mendelian autosomal dominants and the last is acquired, usually through alcoholic excess. There is also the less common hereditary coproporphyria which is also inherited as a Mendelian autosomal dominant.

Tschudy et.al (1965) produced the first communication on elevated hepatic ALA.Synthetase activity (ALA.S) in porphyria in a patient with Acute Intermittent Porphyria (A.I.P.,) who died and from whom tissue was obtained shortly after death. The second communication was from Nakao et.al (1966) who also reported that ALA.S was elevated in another patient with A.I.P. The tissue in this case was obtained surgically. Dowdle et.al (1967) reported elevated ALA.S. activity in six patients with porphyria, one with A.I.P., two with variegate porphyria and 3 with symptomatic porphyria. In addition two normal human values were given. In these studies the samples were obtained by needle biopsy. In 1966 Levere reported a case of stilboestrol-induced hepatic porphyria with elevated ALA.S. activity and in addition two normal values. In a larger study on twelve subjects with

symptomatic porphyria Zail & Joubert (1968) showed that all of the patients with porphyria had high levels of ALA synthetase activity. Finally in 1969 Masuya published a paper dealing with both hepatic and erythropoietic porphyrias in which he quoted figures for elevated ALA synthetase activity in 2 patients with A.I.P., 1 with variegate porphyria and 1 with cutaneous porphyria.

There have been many other papers dealing with the metabolism of haem in porphyria which have not measured ALA synthetase activity directly but have indicated that this enzyme is raised. Dowdle et.al (1968) measured the distribution of ^{14}C labelled ALA in patients with porphyria, and concluded that the excessive haem precursor production in A.I.P., variegate porphyria and symptomatic porphyria cannot be explained by a block in any alternative pathways of ALA metabolism. They suggested that in each of these hepatic porphyrias the cause was an initial elevation of ALA synthetase activity although there would appear to be other defects in both A.I.P. and symptomatic porphyria. None of these previous authors quote any work on hereditary coproporphyria. This section deals with the activities of hepatic ALA synthetase and blood ALA dehydrase in this porphyria both in attack and remission, in addition to urinary and faecal porphyrin levels and levels of urinary 17 oxosteroids.

MATERIALS AND METHODS

Human Studies.

Levels of urinary 17 oxosteroids, uroporphyrin, coproporphyrin PBG, ALA were measured in 4 patients with hereditary coproporphyria of whom two were in attack (L.L. and A.B.) In addition measurements were made of blood ALA dehydrase in all four patients and hepatic ALA synthetase in two (A.B. and J.McS.).

CASE REPORTS

CASE 1.

In June 1969 A.B., a 30-year old housewife was admitted to hospital for hysterectomy and appendectomy. Following surgery she complained of persistent lower abdominal pain, anorexia, constipation and symptoms of cystitis. She had difficulty in sleeping and became increasingly agitated and depressed. She had always been a nervous person and at the age of 21 years anorexia nervosa had been diagnosed. 3 years later she had become depressed and needed in-patient care. Following hysterectomy post operative management included treatment with a proprietary mixture of butobarbitone and promethazine, papaveretum and several antibiotics. She was discharged on July 14, but her mental state became worse and psychiatric advice was sought. On July 22 a diagnosis of agitated depression with hysterical features was made and the patient admitted for psychiatric care. She was given amylobarbitone and imipramine. In spite of this treatment the patient's condition deteriorated with the appearance of confusion and paranoia. Electro-convulsive therapy was started. The depression improved but periods of acute restlessness and confusion occurred frequently. Her gait became

unsteady and there was a coarse tremor of both arms and legs. Her urine had been noted to be dark during her surgical admission but the significance of this finding was not appreciated until August 7, when PBG was found in the urine and a diagnosis of acute intermittent porphyria made. Therapy with barbiturates and other drugs was stopped and treatment with chlorpromazine instituted. Abdominal pain, which had been a persistent feature cleared within 24 hours but her mental state remained abnormal. She was transferred to a general hospital. On physical examination her blood pressure was normal but her pulse rate was invariably above 90 per minute. Neurological examination revealed no objective weakness, or no sensory disturbance and there was no reduction in vital capacity. However her knee jerks became progressively more difficultto elicit, although there was no other evidence of peripheral neurological disease. Her mental state gradually improved and she was discharged at the end of September.

This patient is a member of a large family of whom 2, mother and sister, have been found to have a latent form of the disease with elevated coproporphyrin in urine and faeces.

This case history is reproduced by kind permission of Dr. McIntyre, Royal Free Hospital, London, in association with whom this work was carried out.

CASE 2.

This patient (L.L.) - a 17 year old girl - was admitted in April 1968 to a surgical ward in Victoria Hospital, Kirkcaldy. She gave a five day history of colicky abdominal pain and diarrhoea. She had been previously healthy and there was no family history of disease. On physical examination considerable tenderness was noted in the right iliac fossa, and appendicectomy was carried out using thiopentone sodium to induce anaesthesia. The appendix was of normal appearance. Post-operatively she vomited considerably and developed a mild pyrexia. Her blood pressure was found to be 170/100 mm Hg. At this stage it was noted that she was two months pregnant. One month post operatively she became slightly icteric with a bilirubin of 3.8 mg/100 ml. and raised transaminases. Three weeks after the onset of the icterus she developed a bullous eruption on the light exposed parts of her body. She was then found to be excreting huge amounts of coproporphyrin in her urine and faeces. The rash subsided after one week and thereafter she remained well until November 1968 when she was delivered of a still-born child.

Her second pregnancy was started in March 1969 and this proceeded uneventfully to term when a normal healthy child was born. One week after delivery she became acutely ill with abdominal pain and vomiting. Her blood pressure rose to 220/130 mm Hg and she suffered several epileptic seizures. This episode was accompanied by the urinary excretion of large amounts of ALA and PBG. After 10 days she began to improve rapidly and has had no symptoms since that time. Porphyrin analysis of her family revealed three examples of the disorder in latent form.

CASE 3.

This patient, J.McS, a 48 year old man, first presented in 1966 with a history of nervousness for a number of years. He had been drinking heavily to relieve the symptoms. There was no history of abdominal pain, vomiting or constipation. His urine had never been dark. On examination no abnormal findings were made. When his urine and stool were examined however, they showed grossly elevated levels of coproporphyrin. He has been seen regularly since then with no change in his clinical state.

CASE 4.

This patient, I.K., a 26 years old housewife, presented in 1966 during her second pregnancy with a urinary tract infection. Her Urine was noted to be dark after a short course of sulphonamides and was found to contain increased amounts of PBG; the only other symptom was depression of 2 years duration. Quantitative porphyrin analysis showed large amounts of coproporphyrin in both urine and faeces. The pregnancy ended with a neonatal death, the cause of which uncertain. Her next presentation in 1968 was with a further attack of depression during the early stages of her third pregnancy. This settled quickly and she proceeded to give birth to a normal live child. Since that time she has remained in remission. There is no evidence for other cases of this disorder in other members of her family.

METHODS:

The analysis of the steroids was carried out in the Department of Steroid Biochemistry, Royal Infirmary, Glasgow by J. Paxton, by whose permission I reproduce these steroid results. Individual urinary 17 oxosteroids were estimated by the method of O'Kelly (1968) which permits measurement of individual 17 oxosteroids as the separated steroid glucuronide and sulphate fractions of 24 hour urine specimens. The steroid conjugates are split, the sulphates by solvolysis and the glucuronides by β. glucuronidase. The free steroids are extracted, separated into groups by T.L.C. and measured as their trimethyl silyl ethers by gas liquid chromatography. Hepatic biopsies were obtained by needle biopsy, other methods are as in the methods section.

RESULTS:

Table 5.1 shows the normal levels of porphyrin excretion in urine and faeces. These are taken from Goldberg (1966) except for the asterisked values which are given by Moore (1970).

The results of the measurement of urinary porphyrins and precursors and faecal porphyrins are shown in Table 5.2. For all four of these patients the values are markedly elevated above the normal levels, and both patients in attack show much higher levels of urinary excretion of these substances than the patients in remission. The faecal protoporphyrin values are low. These figures are typical of hereditary coproporphyria (Berger & Goldberg, 1955).

At the same time as these collections were made, blood was withdrawn for ALA dehydrase assay and, in two of the patients, a needle biopsy sample was obtained for ALA synthetase assay. It was found that in these patients, only the two in attack showed any elevation of enzyme activity, both showing elevated blood ALA.D and in one a highly elevated hepatic ALA.S. activity. These values

TABLE 5.1.

SUMMARY OF NORMAL LEVELS OF URINARY PORPHYRINS & PRECURSORS, FAECAL PORPHYRINS & BLOOD PORPHYRINS IN HUMANS

Sample		No. of Cases	MEANS ± SD. or Range of MEANS	Range
URINE				
Uroporphyrin	(µg/24hr.)*	210	6 - 20	0 - 41
"	(µg/litre)	262	2.1 ± 3.8	0 - 18
Coproporphyrin	(µg/24hr.)*	231	46 - 163	1 - 283
"	(µg/litre)	262	60.2 ± 37.2	0 - 149
δAminolaevulic Acid	(mg/24hr.)	100	1.94 ± 1.18	0 - 5.30
"	(mg%)*	100	0.29 ± 0.14	0 - 0.57
Porphobilinogen	(mg/24hr.)	120	0.92 ± 0.93	0 - 3.60
"	(mg%)*	100	0.10 ± 0.05	0 - 0.57
FAECES				
Coproporphyrin	(µg/g dry weight)*	236	2 - 15	0 - 50
Protoporphyrin	(µg/g dry weight)*	236	15 - 28	0 - 113
BLOOD (Erythrocyte)				
Coproporphyrin	(µg/100ml RBC.)	178	1.78 ± 1.31	0 - 4.2
Protoporphyrin	(µg/100ml RBC.)	178	24.03 ± 12.83	0 - 37

* From Goldberg (1966) the rest from Moore (1970)

TABLE 5.2.

PORPHYRINS AND PRECURSORS IN URINE AND FAECES OF PATIENTS WITH HEREDITARY COPROPORPHYRIA.

PATIENT	URINE				FAECES	
	ALA (mg/100ml.)	PBG	Proto (µg/litre)	Copro	Copro (µg/g. dry wt.)	Proto
AB	0.98	0.97	540	4200	3200	63
LL	1.36	2.88	777	6554	11,194	101
JMcS	0.45	0.40	50	613	3954	92
IK	0.62	0.45	380	1105	3483	114

were normal in remission (Table 5.3)

When the fractionated urinary 17 ketosteroids were measured in these patients it was again found that only in the patients in attack were any steroid levels elevated, these values being normal in states of remission, whilst at the same time it was noted that all of the 24 hour urinary excretions of porphyrins and precursors were highly elevated in the patients in attack (Table 5.4).

DISCUSSION

The figures in Table 5.2 support the diagnosis of hereditary coproporphyria in these patients. The typical presentation of hereditary coproporphyria is highly elevated coproporphyrin in stool and urine associated with elevated porphyrin precursors in attack. In hereditary coproporphyria, the main permanent defect is elevated urinary and faecal coproporphyrin excretion in remission as well as in attack; this may be associated with a deficiency in coproporphyrinogen oxidase activity. In acute attack the extra factor which comes into play is thought to be an elevated activity of ALA. synthetase which is associated with raised urinary ALA and PBG excretion in addition to an elevated excretion of coproporphyrin.

These results have shown that ALA synthetase is elevated in one patient with hereditary coproporphyria in attack, as it is in other hepatic porphyrias. This is the first report of elevated ALA.S in hereditary corproporphyria. In the other case measured in these studies the level was normal but in this case the patient had always been in remission. In both cases in attack erythrocyte ALA dehydrase activity was elevated and in cases

TABLE 5.3

HAEM ENZYMES IN HEREDITARY COPROPORPHYRIA

Patient	Age	Sex	Clinical State	Hepatic ALA.S nMoles/g/hr.	Blood ALA.D nMoles/$_{10}$10 RBC/hr.
AB	30	F	Attack	234	994
LL	19	F	Attack	-	1429
JMcS	47	M	Remission	3.44	294
IK	30	F	Remission	-	619
NORMAL RANGE				< 5	439 - 796

TABLE 5.4

URINARY 17 OXOSTEROIDS (HEREDITARY COPROPORPHYRIA) (mg/24 hr.)

Patient	Sex	Age	No. of collections	SULPHATES		GLUCURONIDES	
				Dehydroepiandrosterone	Epiandrosterone	11 hydroxy aetiocholanolone	11-oxy-androsterone
AB	F	30	1	0.059	0.017	12.463*	0
LL	F	19	7	0.163 ± 0.152	1.051 ± 0.606^{+}	0.445 ± 0.148	1.088 ± 0.664^{x}
JMcS	M	47	1	+1.458	0.524	0.366	0.095
IK	F	30	1	0.073	0.052	0	0.075
NORMALS	M	18-42	17	1.150 ± 1.486	0.299 ± 0.199	0.977 ± 0.444	0.281 ± 0.109
	F	19-40	17	0.276 ± 0.292	0.100 ± 0.210	0.544 ± 0.334	0.170 ± 0.063

Significance * Elevated

$^{+}$ $P < 0.02$

^{x} $P < 0.001$

in remission the activity of this enzyme was normal or low. In the case of J.McS, the ALA dehydrase activity was low. This might be attributed to alcohol ingestion (Section 3).

In all of these patients the levels of porphyrin excretion are high although they are higher in the patients in attack. This elevation of these porphyrins can be related to the elevation of some urinary steroid levels in these patients in attack. This corresponds to the observation in previous work that the levels of certain urinary 17 oxosteroids are elevated in A.I.P., this always being the case in patients in attack (Goldberg et al, 1969). It is interesting to note that in all of these cases compounds that have been investigated previously in this thesis are implicated in the exacerbation of this disease in humans. The principal offenders are the barbiturates which have clearly been shown to be porphyrinogenic, both in this work and from previous work. The second offender is ethanol and the last the sulphonamides, which were first implicated by Rimington and Hemmings (1939) and more recently shown to raise hepatic ALA synthetase activity in rats (Beattie et al, 1970). Thus the elevation of hepatic haem enzyme levels and urinary porphyrins, precursors and steroid levels in hereditary copro-porphyria can be related to the clinical severity of the disease. These results lend strong support to the hypothesis that in hepatic porphyrias in attack, the accumulation of haem precursors is due primarily to the excessive activity of the initial and rate limiting enzyme of haem biosynthesis, ALA synthetase.

GENERAL DISCUSSION

In these experiments the effects of various compounds on the ability of rat liver to synthesise δaminolaevulic acid and hence Haem have been examined. This ability to synthesise haem is a biochemical function common to all aerobic cells, bacterial, plant and animal, since as well as being the prosthetic group of haemoglobin, haem is also required in various cytochromes, catalases, peroxidases and other haemoproteins as well as being used in the magnesium rather than iron porphyrin complex in chlorophyll. The fact that these cells are able to synthesise haem testifies to the existence in these cells of all the factors necessary to carry the reaction through from the ubiquitously distributed precursors, glycine and succinyl CoA to haem, although, obviously there is a variation between cells in the amounts of haem they are able to produce. This implies a fine regulation of the biosynthetic pathway under physiological conditions such that there is a close coupling of haem biosynthesis with the synthesis of other compounds such as globin, which combine or conjugate with haem. Within the complex of the haem biosynthetic pathway there are various possible sites for its control. The initial and terminal stages of the pathway take place in the mitochondrion (Granick & Mauzerall, 1960; Sano & Granick, 1961) and the intermediate steps take place in the cytoplasm. The variety of enzymes catalysing these reactions and the compartmentalisation offer a number of sites for controlling the pathway.

The availability of glycine, succinate and/or succinyl CoA could potentially limit the rate at which haem is formed and some evidence for this has been advanced (De Matteis, 1967). This would seem unlikely however, since probably less than 1% of all succinyl CoA formed in the cell enters this pathway, and in consequence alterations of the levels of succinyl CoA would represent an extremely inefficient method of control. Similarly the availability of both glycine and succinate in the cell is so large that alterations in the levels of either or both of these would result in a gross metabolic derangement in the cell.

The same applies to pyridoxal phosphate deficiency, although Elder & Mergel (1966) have shown that induced pyridoxine deficiency lowers porphyrin precursor excretion in patients with A.I.P. In these patients however, the pathway was already working at such a high level, as it is in all hepatic porphyrias, that it is much more vulnerable to attack in this direction.

The pathway could also be controlled by a separate metabolic sequence or cycle which breaks down ALA. This postulated cycle, the 'Shemin cycle', has already been discussed and has been shown to have no appreciable effect on this system.

Control of the activity of any of the other enzymes within the pathway could also theoretically take place, and actually occurs in pathological situations. In lead intoxication both ALA dehydrase and haem synthetase are inhibited (Chisholm, 1964), to such an extent that in severe lead poisoning there is a large build-up of both ALA and coproporphyrin in the urine. These facts have been used in the diagnosis of lead poisoning (Goldberg, 1968c).

In the porphyrias, the various patterns of porphyrin excretion can be explained by variations in the levels of enzymes within the pathway leading to different handling of excess ALA produced by ALA synthetase. In the erythropoietic porphyrias, the series I isomers of uroporphyrin and coproporphyrin are excreted in the urine. This can be explained by a low activity of the uroporphyrinogen synthetase (Booij & Rimington, 1957; Rimington & Booij, 1957), thus limiting the production of series III isomer. In the hepatic porphyrias there are other variations. In A.I.P. mainly porphyrin precursors are excreted whilst in variegate porphyria, coproporphyrin III and

protoporphyrin are excreted (Goldberg & Rimington, 1962). Again the cause probably lies in the fact that for normal concentrations of precursors the enzyme levels are adequate, but when challenged with increased levels of precursors, these activities become limiting within the pathway leading to a build-up of certain constituents. Thus there are three points which would suggest that in the normal metabolic situation, none of these three steps are rate limiting. Firstly there is no build-up of any intermediates in the pathway; secondly there is a high preponderance of the physiological type III porphyrin isomer over the type I isomer, showing the isomerisation stage to be non-limiting and finally most cellular homogenates can metabolise exogenous ALA to porphyrins but cannot convert glycine or succinyl CoA to porphyrins to any great extent (Granick & Urata, 1963; Saillen, 1963; Sardesai et al 1964; Levere & Granick 1967). In addition, in the normal situation the pathway operates very efficiently both in humans (fig.17) and in rats (fig.1.3) This could only be attained by a very closely linked series of controls and is made all the more sensitive by the short half life of ALA synthetase at the primary control point of the pathway (Granick & Urata, 1963). In acute porphyria the balance becomes very much less efficient and about 40% of the intermediates are lost by excretion. (fig.18). From this figure it can be seen that in this particular hepatic porphyria, A.I.P. the greatest excretion is at the precursor level showing that these are being produced in great excess. Thus it is clear that the rate of formation of ALA, and the activity of ALA synthetase are prime determinants of the rate at which the haem biosynthetic pathway operates.

Since ALA synthetase is mitochondrial, it has been suggested that mitochondrial DNA rather than nuclear DNA codes for this protein. This would seem to be excluded in mammals, at least, by the autosomal dominant pattern of inheritance in the hereditary hepatic porphyrias. If it were coded for by

BALANCE SHEET FOR THE HAEM. BIOSYNTHETIC PATHWAY IN HUMANS.

(70 kg. MALE)

PRODUCTION

There is a turnover of 320 mg. Haem/24hr.

∴ Production is 518.62 μ moles/24hr.

EXCRETION

Excreted Product	μ Mol Excreted/24hr.	Haem. Equivalents Excreted/24hr (μ Mol)
URINE		
A.L.A.	14.81	1.851
P.B.G.	4.07	1.017
UROPORPHYRIN	0.016	0.016
COPROPORPHYRIN	0.157	0.157
FAECES		
COPROPORPHYRIN	0.482	0.482
PROTOPORPHYRIN	1.308	1.308
	TOTAL EXCRETION	4.831

THIS REPRESENTS AN 0.9% LOSS OF HAEM. PRECURSORS.

Fig. 17.

BALANCE SHEET OF HAEM. PRODUCTION IN ACUTE PORPHYRIA.

PRODUCTION

Estimated production of Haem. in 70kg. man in 24hr. in acute attack of

ACUTE INTERMITTENT PORPHYRIA

518·6 μ moles

EXCRETION	μ Moles Excreted/24hr.	Haem. Equivalents (μ Moles)
URINE		
A.L.A.	483·96	60.49
P.B.G.	472.56	118.14
UROPORPHYRIN	10.19	10.19
COPROPORPHYRIN	1.05	1.05
FAECES		
COPROPORPHYRIN	1.87	1.87
PROTOPORPHYRIN	3.74	3.74
	TOTAL EXCRETION	195.49

THIS REPRESENTS A 37.7% LOSS OF PRECURSORS.

Fig. 18.

mitochondrial DNA the pattern of inheritance would be maternal since sperm mitochondria cannot be transmitted to the ovum. The probability from recent work (Scholnick et al, 1969) is that like cytochrome C (Gonzales - Cadavid & Campbell, 1967; Kadenbach, 1967) ALA synthetase is synthesised in the cytoplasm and transported to the mitochondrion, since the majority of protein synthesis within the mitochondrion is structural rather than functional.

As shown previously ALA synthetase activity may be controlled by either end product inhibition or repression in bacteria. The normal low levels of enzyme together with its very much higher levels in porphyria and easy inducability indicate that in normal circumstances the enzyme is highly repressed. By the operon concept of Jacob & Monod (1961), as postulated for bacteria, it is suggested that a similar method of control applies to eucaryotic cells, using the repressor/operator mechanism whereby a defect in either the operator or regulator gene leads to a derepression or activation of the structural gene for ALA synthetase which leads to an overproduction of porphyrins and their precursors (fig. 19)

The effect of various compounds such as the barbiturates on ALA synthetase are mediated by the 'de novo' formation of ALA synthetase rather than the activation of a 'latent' form of the enzyme or by the depression of its rate of degradation, as evidenced by work using antibiotics. It has always been indicated that ALA synthetase is the rate controlling factor in the biosynthesis of haem and that its synthesis can be regulated by these substances. The work using the antibiotics cycloheximide and actinomycin D to block protein synthesis show that to induce ALA synthetase, an intact protein synthesising system is required. This system commences at the level of DNA. This nucleic acid codes for a messenger RNA. In collaborative work at present in progress it has been shown that in chemically porphyric states, an excess of this messenger RNA is produced.

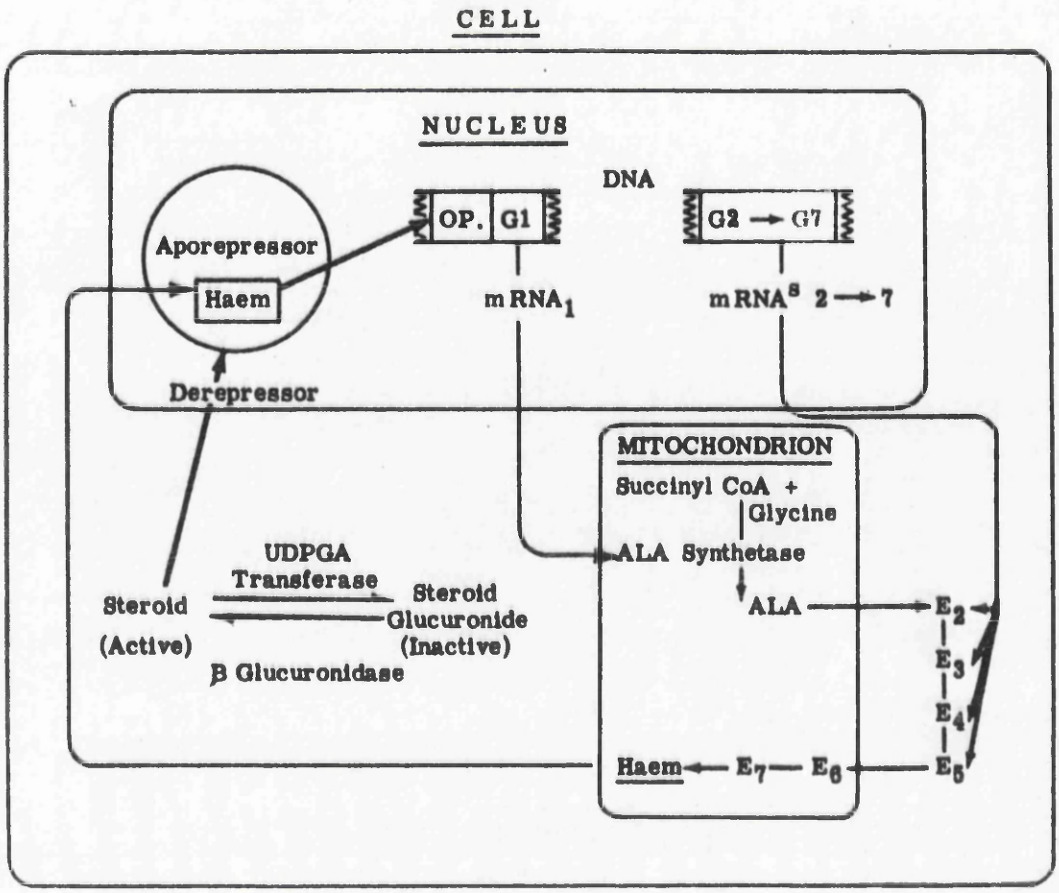


Fig. 19

Possible Scheme of control of the Haem Biosynthetic Pathway. OP. represents the operator gene and G1, G2 → G7 the Structural Genes. $mRNA_1$ (messenger RNA) codes for ALA synthetase whilst $mRNA^S$ 2 → 7 code for the subsequent enzymes (E_2 → E_7) of the biosynthetic pathway.

Haem is postulated to combine with the Aporepressor to form the repressor which functions at the level of the operator gene.

In this work (Skea et al 1970) it has been shown that RNA extracted from the liver of a rat made porphyric with AIA with a resultant 10-fold increase in ALA synthetase activity (fig. 20) significantly elevates the activity of ALA synthetase in chick embryo liver culture. This elevation rises to a peak in about 4 hours and has disappeared after 8 hours (fig. 21). This activity appears to reside mainly in the lower molecular weight fractions of the RNA (fig. 22) and these effects were abolished by cycloheximide and by incubation of the RNA with ribonuclease prior to putting it into the culture medium. These results follow on from the work of Hickman et al (1967) who showed elevated porphyrins after 18 hours in a simpler culture system, although this work has not been able to be duplicated (Moore et al, 1969b).

This messenger RNA produces ALA synthetase which through the rest of the biosynthetic pathway produces Haem. Haem in this model acts as a repressor, combining with the aporepressor molecule to form an active repressor which acts on the operator gene to prevent the function of the structural gene. It is at this level that the various drugs and chemicals are postulated to act. If these compounds combine with the aporepressor then, either by blocking the binding site, or by alteration of the molecular configuration, this inactive molecule can be formed. In this case the structural gene is derepressed and there is an uncontrolled formation of ALA synthetase, and in consequence an overproduction of the components of the pathway. The same effect would be produced if these compounds acted on the regulator gene, which produces the aporepressor, and lowered production of this aporepressor. Alternatively this effect could be produced by a depression of the levels of haem available for combination with the aporepressor molecule. In the case of the barbiturates, it is known that they elevate the levels of cytochrome P.450 (Conney, 1967) and by this means stimulate both their metabolism and the metabolism of other drugs. It is possible that by depressing haem levels within the cell by their utilisation for cytochrome P.450 production, ALA synthetase is in fact induced, by the depressed levels of active repressor.

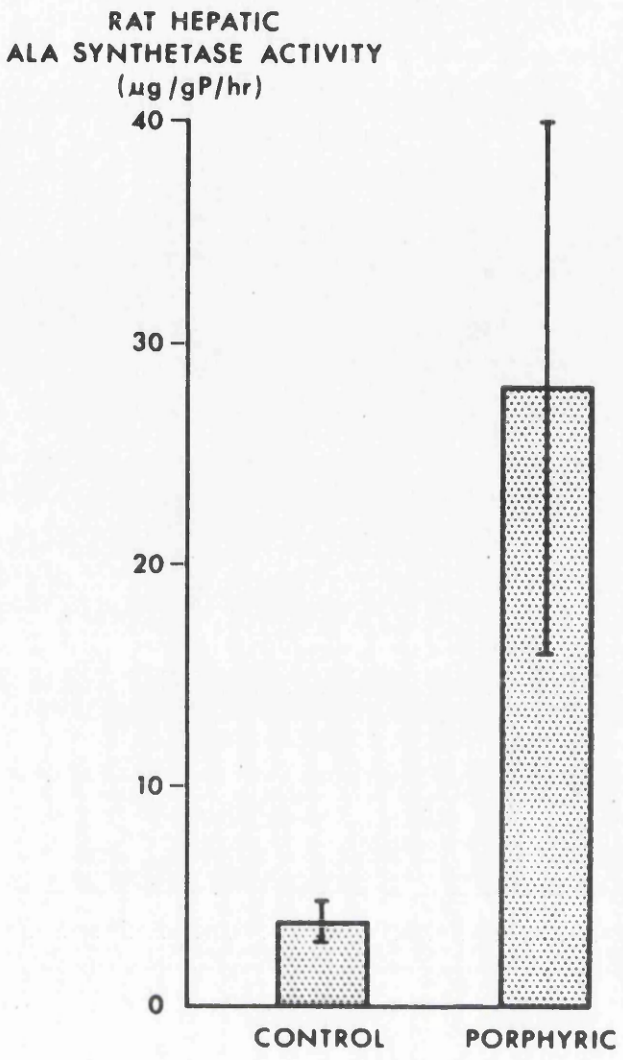


Fig. 20 The effect of intraperitoneal Allylisopropyl acetamide (AIA) on rat hepatic ALA synthetase activity. Bars represent mean ± standard deviation. Activity is expressed as µg ALA produced per g Protein per hour.

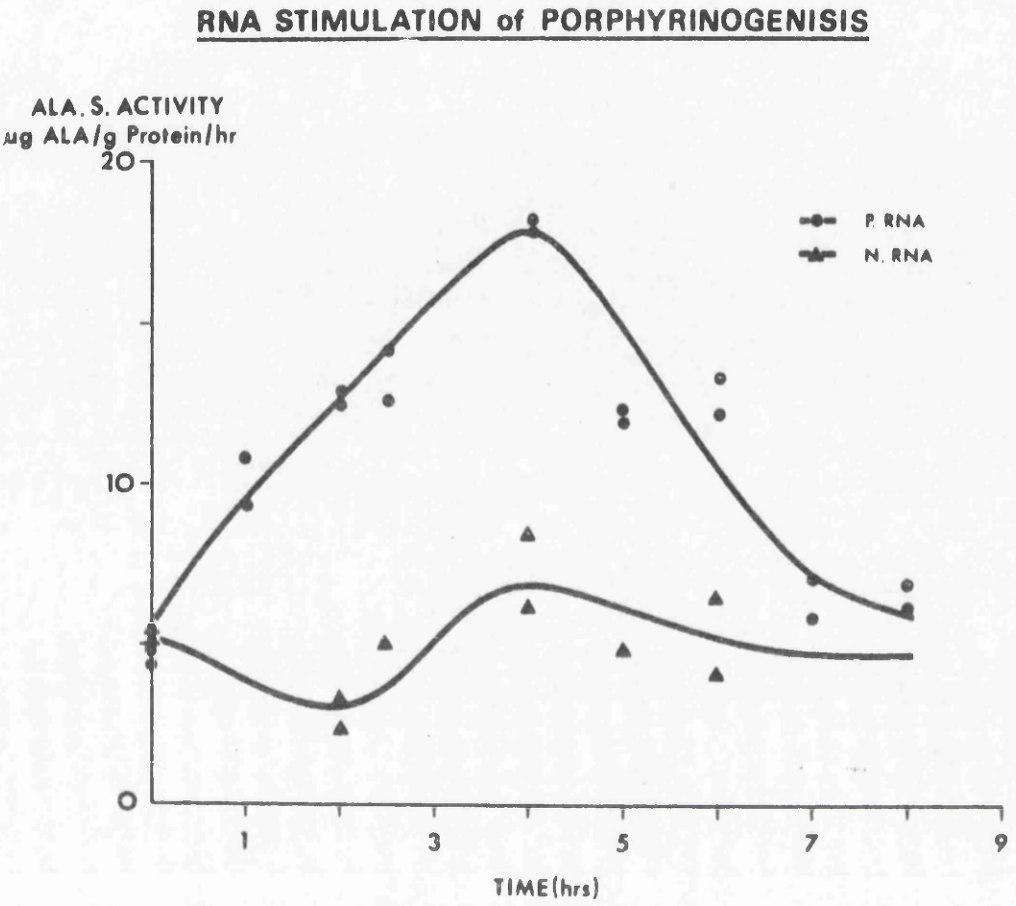


Fig. 21. Porphyric Rat Hepatic RNA stimulation of ALA synthetase activity in chick embryo liver culture. Each point represents one culture. PRNA: Porphyric RNA, N. RNA Normal RNA.

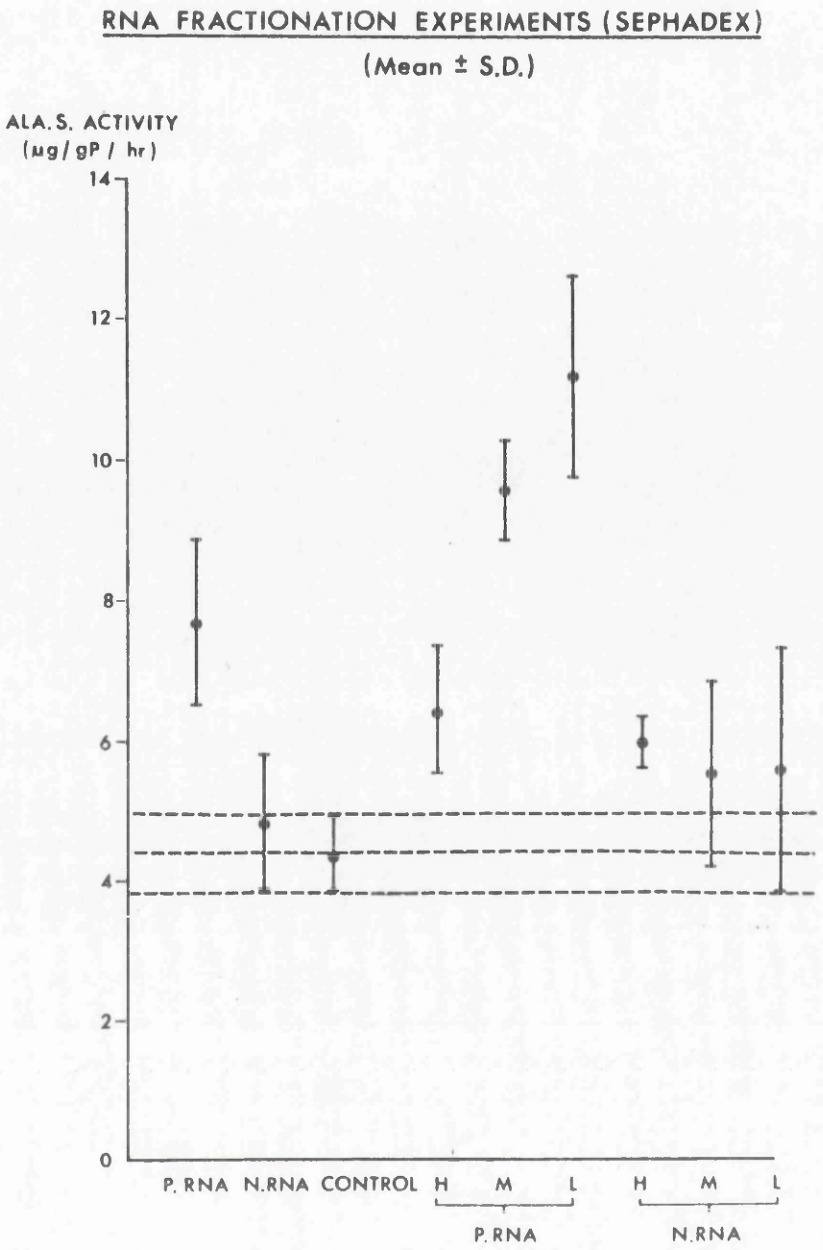


Fig. 22. The effect of rat hepatic RNA on ALA synthetase activity of chick embryo liver culture. P. RNA (Porphyric RNA) N. RNA (Normal RNA) and control (untreated) culture activities after preincubation of the cultives for 18 hours and treatment for 3 hours are shown in the first three bars. The subsequent 6 bars show the effect of the P.RNA and N.RNA after fractionation on Sephadex. G.100 (H - Heavy, M - Medium and L - Light fractions). The bars represent the mean ± SD.

Alternatively of course, the barbiturates could act in one of the other ways, raise ALA synthetase and hence haem, and by this means raise cytochrome P.450 levels.

Another method of detoxication other than oxidation is by the formation of soluble conjugates. It has been shown that steroid glucuronides are ineffective as inducers of ALA synthetase while the free steroids are active as inducers. Within the body, steroids and other compounds are detoxicated by glucuronide formation from UDP glucuronic acid and by the enzyme UDP glucuronyl transferase.

In the hepatic porphyrias a high basal level of ALA synthetase is genetically determined and little is required to elevate these levels even higher into an attack. This would suggest in addition some deficiency in the normal repressor control of enzyme activity. In the first sections barbiturates, ethanol and some steroids were all implicated in the elevation of rat hepatic ALA synthetase by the 'de novo' production of this enzyme. In the last section all attacks of hereditary co proporphyria were precipitated by one of these compounds, by the barbiturates for sedation and anaesthesia, sulphonamides for infection and ethanol. Also those patients in attack had elevated levels of steroids, and although these particular steroids have not been implicated in the steroids section as porphyrinogenic they could be metabolites of the porphyrinogenic steroids.

Steroids would seem to play an especially important role in the porphyrias. It is possible to relate these steroid effects to two of the factors involved in porphyrinogenesis. Ethanol has been shown to change the redox state of steroid sulphates, to raise androstenediol sulphate and lower DHA sulphate in human plasma (Cronholm & Sjovall, 1970). Diet also affects steroid levels. As shown earlier, diet plays a large part in

porphyrinogenesis, with starvation raising the levels of ALA synthetase. Hendrikx et al (1968) showed that starvation lowered DHA excretion; this could be related to an elevated level of DHA in the plasma which as has been shown earlier could increase hepatic ALA synthetase activity. The change in this system is an increase in the half life of DHA sulphate and these observations correspond well with the observation that ALA and PBG and porphyrin excretion levels rise during starvation in animals (Beattie, 1970).

Since steroids are the first endogenous factor to be implicated in the patho-genesis of the porphyrias, determinants such as impaired detoxication by glucuronide formation could represent an important ancillary mechanism in the exacerbation of the disorder 'in vivo' and possibly even play a more important role in the acquired porphyrias where the general mechanisms of detoxication are upset by foreign porphyrinogenic compounds. Thus a linking factor in the porphyrias could be the role played by steroids in their patho-genesis.

APPENDIX

APPENDIX - INDEX

1) METHODS

2) PORPHYRINOGENIC DRUGS

(1) METHODS.

ALA Synthetase Assay (micro) Assay 1.

Ref: Dowdle, et al (1967) as modified by M.R. Moore.

Reagents

1) Homogenisation Buffer 0.02M Na EDTA
   0.15M tris
   pH to 7.2 with HCl
2) Incubation Buffer 0.2M glycine
   0.1M Na citrate
   pH 7.4
3) 0.3M T.C.A.
4) 0.6M Na acetate
5) Acetyl acetone
6) Special Ehrlichs Reagent.

METHOD

Remove liver rapidly from animal and chill in ice-cold homogenisation buffer immediately, rinsing to remove as much blood as possible. Take tissue, blot on clean adsorbent paper and dry, cut off aliquot, and weigh into a homogenisation vessel. Make 1 in 4 homogenate accurately (if working to weight reference) using piston homogeniser in cold (ten rapid strokes). Into micro polyethylene test tubes put 100µl incubation buffer and 100µl homogenate. Use three tubes per assay, incubated thus, stoppered in a covered water bath at 37°C for 1 hour, one stopped immediately by the addition of 200µl TCA and mixed. After 1 hour remove tubes, unstopper and stop reaction with 200µl TCA, mix and stand for 10 minutes at 0°C. Take tubes and spin in microfuge for 3 mins. From each tube, using a constriction pipette, remove a 200µl aliquot of supernatant solution and put/

put this into a boiling tube containing 200µl H_2O, 50µl Na Acetate and 20µl acetyl acetone. Mix thoroughly and place in boiling water bath for 10 mins. Remove, allow to stand to cool and add 50µl H_2O and 500µl fresh cold Special Ehrlichs Reagent. Stand for 15 minutes and read at 552mµ in microcells. Read ALA content from standard graph.

A.L.A. Synthetase - Method for whole liver homogenates. Assay 2.

This is a modification of the method of Marver et al, 1966.

Reagents.

(1) Homogenisation Buffer: 0.9% NaCl (Saline) pH 7.4
0.5 mM EDTA (0.1461g/litre)
10.0 mM Tris (1.2114g/litre)

(2) Incubation Mixture: 100 mM glycine (7.505g/500ml) pH 7.2
50 mM Tris (6.0570g/500ml)
6.6 mM EDTA (1.9289g/500ml)

2N HCl
2N NaOH
0.5 M Phosphate Buffer pH 7.0
0.1 M HCl/4M NaCl
0.2 M HCl/4M NaCl

Acetyl Acetone

Methanol: glacial acetic acid (2:1) (MeOH/HAC)

0.01 M ammonia in n. Butanol (BUOH/NH_4OH)

Dowex 2 x 8 Cl^-

Special Ehrlich's Reagent

METHOD.

Remove liver rapidly from the rat, chill in ice cold homogenisation buffer, blot, and weigh out 2 - 3 g. aliquot for homogenisation. Make an accurate 3 ♯ 1 homogenate with homogenisation buffer using 10 strokes of a Potter homogeniser, keeping cold all the time. Take 3ml. incubation mixture, 1ml. homogenate in a 25 ml. conical flask and incubate at 37°C for one hour in a rapidly shaking water bath. This stage should be carried out with 3 flasks, 1 zero time and 2 one hour samples. Stop the reaction by transferring the contents of the flask into a centrifuge tube containing 2 ml 2M HCl. Stand for 15 minutes at room temperature and then centrifuge for 4 minutes at 2000 g. Decant into fresh tubes containing 1.55 ml 2M NaOH and 0.4 ml 0.5 M Phosphate Buffer. Wash ppt. once/

once with distilled water and transfer the washings to the tube. Adjust the pH of this solution to 6-6.5 using 2M NaOH, add 0.1 ml acetyl acetone, shake and place in boiling water bath for 1-minutes. Cool the tubes and add 2 ml 0.2MH HCl/4M NaCl.

This solution is then put on to a column prepared thus:-
Take columns, 20 cm x 1 cm., pack base with cotton wool plug and put in a slurry of Dowex 2 x 8 200 - 400 mesh to give 1 cm. packed resin, wash this, then add 5 ml 0.1M HCl/4M NaCl followed by a wash with 5 ml distilled water. The column is now ready for use. When the solution has been put on to the column, the amino-acetone pyrole is eluted with 2ml BuOH/NH_4OH followed by a wash with 5 ml of the same. The ALA pyrole is then eluted with 1.5 ml MeOH/HAC then 0.4 ml the same into 5 ml stoppered measuring cylinders. This solution is made to 2 ml, 1 ml special Ehrlich's reagent added, shaken thoroughly and allowed to stand for 15 minutes before reading at 555mμ. The readings are converted from a standard graph.

ALA Dehydrase - Method

Solutions

0.15M KCl
0.6 M KCl

Phosphate Buffer pH 6.8

0.0307 g glutathione in 20ml buffer (GSH)
0.0168 g ALA in 1ml H_2O

0.3M TCA

Special Ehrlich's reagent

METHOD

Take 10 ml fresh blood and put into a heparinised bottle, shake, then spin blood at 2,500rpm for 20 minutes at 4°C. Remove the serum and wash cells with 0.15M KCl, spin at 2,500rpm for 10 minutes at 4°C and remove KCl wash.

Take 2 ml packed R.B.C., make up to 10ml with 0.15M KCl, shake well to mix and place 1 ml sample in a tube for an R.B.C. count. Spin again at 2,500 r.p.m. for 10 minutes and remove KCl. To the packed cells add 5.4 ml distilled water and leave for 20 minutes on ice to Haemolyse. Add 1.8 ml 0.6M KCl, mix and put in bath at 37°C. *Take Thunberg tube and add 2 ml G.S.H. solution and 0.1 ml ALA solution, and equilibrate in bath.

Heat at 37°C for ½ hour to equilibrate temperatures, pipette 1 ml haemolysate into Thunberg tubes, then evacuate at the water pump and mix the tube contents. The 0 time samples are put into 2 ml 0.3M TCA immediately to stop the reaction. The other tubes are heated at 37°C shaking for 1 hour, and then poured into 2ml/

* For tissues use a whole homogenate of tissue in 0.15 MKC1 and start here

2ml 0.3M TCA to stop the reaction. The solution was spun to remove precipitate, the supernatant decanted, and again spun to remove further precipitate. Pipette off 2 ml and to this add 2 ml special Ehrlichs reagent. Spin to remove white ppt and read after 15 minutes at 553 mμ. This can be done up to ¾ of an hour.

NOTE: Times and temperature are important and all glassware must be metal ion free, i.e. acid washed.

Calculation

$$\frac{OD \times 1.47}{\text{cell count}} = \text{units ALA dehydrase}/10^{10}\text{RBC/hr}$$

(n moles PBG)

RNA. DNA (Ceriotti 1953, 1955) and PROTEIN (Lowry et al 1951) - METHOD FOR LIVER.

Reagents

2% Na_2CO_3 in 1 N NaOH :- A

0.5% $CuSO_45H_2O$ in 1% Na K Tartrate :- B

Solution C :- 50:1 A:B

Solution E: Folin Ciocalteau Reagent 1M

0.6M $HClO_4$

0.2M $HClO_4$

0.3M KOH

0.04% Indole aqueous solution

Conc. HCl

Bench grade Chloroform

METHOD

Take 1 lobe of liver, weigh and make a 1/20 homogenate in ice cold water. Take an aliquot of this homogenate and dilute it to a 1/50 homogenate for protein estimation, which is done by taking a 1 ml sample, adding 5 ml reagent C and standing for 10 minutes. Take this and add 0.5ml reagent E mixing immediately and thoroughly. Stand this for at least 30 minutes and read the samples against a blank at 750mμ. This gives protein, from a standard graph.

For nucleic acids, take 5 ml 1/20 homogenate, add 2.5ml 0.6N $HClO_4$, mix and stand for 10 minutes at 0°C. Centrifuge this in the cold at 2000g for 5 minutes, discard supernatant, and wash precipitate twice with 0.2N_4HClO . Drain the tubes and add 4 ml 0.3N KOH and incubate the tube at 37° shaking for one hour, ensuring that the precipitate is dissolved. After incubation cool and add 5 ml 0.6N $HClO_4$ and stand for 10 minutes in the cold. Centrifuge at 2000g for 10 minutes and remove supernatant to 100 ml. cylinder for RNA estimation, wash precipitate twice with 5 ml 0.2N $HClO_4$ keeping washings for RNA estimation. For RNA estimation, take

supernatant and washings, and make up to 100 ml. This solution is then read at 260 mμ for RNA.

For DNA estimation, redissolve the precipitate in 5 ml 0.3M KOH, transfer to a 25 ml cylinder, add a further 3 ml 0.3 M KOH and make up to 25 ml with H_2O. Take 2 ml of this solution add 1 ml indole solution, 1 ml conc HCl and put in boiling water bath for 10 minutes. Cool and extract this solution with chloroform until no more pink colour can be extracted. Read the OD of the upper layer at 490 mμ for DNA from standard graph.

Reduced Glutathione in Blood.

Ref: Beutler et al (1962)

Reagents

1) Precipitating solution
   1.67g metaphosphoric acid
   0.2g disodium EDTA
   30.0g NaCl

   All made up to 100ml in distilled water.
   Store cold.

2) Phosphate Solution — 0.3M Na_2HPO_4 in distilled water.

3) DTNB Reagent (0.040g 55' dithiobis 2 nitrobenzoic acid made up to 100mls in 1% Sodium Citrate.)

Procedure.

Put 0.2ml blood into 1.8ml distilled water and then add 3ml precipitating solution to the haemolysate. Allow to stand for 5 mins. and centrifuge at 1000g for 10 mins. in cold.

Take 2ml supernatant and add to 8ml phosphate solution. Add 1ml DTNB reagent, mix well and read at 412 mμ within 5 mins.

For standards do not use a GSH concentration of greater than 100mg%.

Dissolve GSH in 2 : 3 , H_2O:ppt. soln.

ALA AND PBG TECHNIQUE

Ref: Mauzerall and Granick (1956) as described by Haeger Aronsen (1960)

PRINCIPLE - In contrast to ALA, PBG is retained by the anion-exchanger Dowex 2 in the acetate form. ALA, however, is retained by the cation-exchanger Dowex 50 in the hydrogen form.

After elution from the ion-exchangers, PBG and ALA are demonstrated by means of p-dimethylaminobenzaldehyde (DMAB), the former substance directly; the latter after quantitative conversion to the 3-acetyl-2-methylpyrrole 4-(3') propionic acid by heating with acetylacetone. With DMAB in acid solution, PBG as well as the pyrrole obtained on condensation of ALA and acetylacetone, forms coloured complexes, which are spectrophotometrically determined.

REAGENTS

Dowex 2 - x 8, 200 - 400 mesh - Preparation: The ionexchanger is placed in water and allowed to sediment after which the water is sucked off. The washing is repeated until the supernatant is clear. The exchanger is then prepared on a column, first by conversion to the acetate form by washing with 3 M sodium acetate until the eluate is chloride-free (as tested with silver nitrate). It is then washed with water until the washings are neutral (as tested with litmus paper). The ion-exchanger is stored in about twice its volume of water in a covered vessel. Stored in this way it will keep for at least 3 - 4 months at room temperature.

Dowex 50 - x 8, 200 - 400 mesh. - Preparation: The finest particles are separated off by repeated suspension and sedimentation in water. The ion-exchanger is converted to the sodium form during about 20 hours' storage in twice its volume of 2 N sodium hydroxide, after which it is washed with water until the washings are neutral. It is then reconverted to the acid form by treating alternately with about 1 volume of 4 N hydrochloric acid and 6 volumes of 2 N hydrochloric acid. The exchanger is stored in twice its volume of 1 N hydrochloric acid in a covered vessel. It will then keep for at least 3 - 4 months at room temperature.

Acetic acid 1 N and 0.2 N

Sodium acetate - M

Acetate buffer, pH 4.6 - 57 ml of glacial acetic acid and 136 g. of sodium acetate trihydrate are diluted with water to 100 ml.

Acetylacetone.

Ehrlich's reagent I. - 2 g. of p-dimethylaminobenzaldehyde and 100 ml. of 6 N hydrochloric acid.

Ehrlich's reagent II. - 1 g. of p-dimethylaminobenzaldehyde is dissolved in about 30 ml of glacial acetic acid and 8 ml. of 70% perchloride acid S.G. 1.7 in a 50 ml volumetric flask, after which the solution is diluted to the 50 ml. mark with glacial acetic acid (N.B. Will keep only for about 6 hours).

ALA AND PBG TECHNIQUE

COLLECTION OF SAMPLE - PBG is labile and is therefore preferably determined on freshly voided urine. However, if it is necessary to store the urine before analysis, it should be done at a pH of about 7 - 8 (alkalinised with sodium carbonate) and a temperature of about+ 4°C., in the dark. ALA on the other hand, will keep for at least 20 days at pH 4 - 6 and a temperature of about + 4°C.

When added to the Dowex 2 column, the pH of the urine should be 4 - 7.

ANALYSIS - Chromatographic columns, about 1 by 10 cm. are used for these analyses. Cotton wool plugs are placed in the bottom. The columns are then packed by sedimentation of the ion-exchangers to a level of 2 - 3 cm. Filter papers are placed at the top. Each PBG determination requires 1 Dowex 2 column; each ALA determination 1 Dowex 2 and 1 Dowex 50 column. Before the columns are used, they are rinsed in water, Dowex 2 with about 5 ml and Dowex 50 with about 25 ml.

Exactly 1 ml. of urine is placed on a Dowex 2 column. After urine has passed through the column at a flow rate of about 6 drops a minute into a test tube (A), the column is washed twice, each time with 2 ml of water. The washings are collected together with the urine in A, which is then set aside.

PBG is eluted from the Dowex 2 column with 2 ml of 1 N acetic acid and after this has passed through, with 2 ml. of 0.2 N acetic acid. The combined eluates are collected in a 10 ml. volumetric flask and diluted to the mark with water.

2 ml of Ehrlich's reagent I is added to a 2 ml. aliquot of the well mixed PBG solution. If the result is positive, a pink to cherry-red colour rapidly develops. The extinction is read after exactly 5 minutes in a spectrophotometer, at 555 mμ and a 1 cm. cuvette. An equal volume of Ehrlich's reagent I and water is used as a blank.

The contents of A are quantitatively transferred to a Dowex 50 column. The urea is removed from the ion-exchanger with about 30 ml. of water. (If the eluate contains urea, a bright lemon-yellow colour will develop on mixture with an equal volume of Ehrlich's reagent I). The ion-exchanger is then washed with 3 ml. of 0.5 M sodium acetate. After draining, a 10 ml. volumetric flask is placed under the column, after which a further 7 ml. of M sodium acetate is allowed to pass. 0.2ml. of acetylacetone is added directly to the flask and the mixture is shaken thoroughly. The solution is afterwards diluted with acetate buffer, pH 4.6 up to the mark. The entire quantity of fluid is transferred to a 15 ml. glass-stoppered test tube, which is then placed in boiling water for 10 minutes and subsequently cooled to room temperature.

To 2 ml. of this solution is added 2 ml. of Ehrlich's reagent II. If the test is positive, a pink to cherry-red colour will successively develop. The extinction is read after exactly 5 minutes in a spectrophotometer, at 553 mμ and a 1 cm cuvette. The blank consists of 7 ml. of 0.5 M sodium acetate, treated in the same manner as the Dowex 50 eluate.

Porphyrins Ref: Rimington (1961)

URINARY COPROPORPHYRIN AND UROPORPHYRIN

Principle.

Coproporphyrin and any coproporphyrinogen are first extracted by ether containing acetic acid. Coproporphyrinogen is oxidised to porphyrin by shaking the solution with dilute iodine. The total coproporphyrin is then transferred to 5% hydrochloric acid and determined spectrophotometrically using a formula to correct for absorbing impurities. Uroporphyrin is removed from the residual urine, plus washings of the ether phase, by adjusting to pH. 1.5 and shaking with cyclohexanone. After addition of ether it is transferred to 5% nydrochloric acid and determined spectrophotometrically using a correction formula as in the case of coproporphyrin (Dresel, Rimington and Tooth, 1956).

Materials required.

Ion-free distilled water for preparing reagents and for all manipulations.

Acetic acid (glacial).

Ether: anaesthetic grade, free from peroxides. Less pure ether may be stored in dark bottles over powdered ferrous sulphate with which it is occasionally shaken vigorously, When required, the decanted ether is washed twice with distilled water (about $\frac{1}{6}$ of its volume each time) and used without drying.

Cyclohexanone: technical grade redistilled *in vacuo* to obtain colourless fraction with constant B.P.

Dilute sodium acetate solution: 30 g. of sodium acetate hydrated (ANALAR) dissolved in water and made up to 1 l., solution filtered.

Dilute iodine solution, 0.005%. This is prepared freshly for each day's use by diluting 1 ml. of a stock solution of 1 g. iodine (resublimed) in 100 ml. of absolute ethanol with distilled water to make 200 ml. The stock solution is stable if kept at 5°C. in the dark.

Hydrochloric acid (approx. 5 g/100 ml.) : 120 ml. concentrated acid made up to 1l. with distilled water.

0.1M Hydrochloric acid.

METHOD

The concentration of porphyrin in the fresh urine specimen is first ascertained roughly by the qualitative test described in Broadsheet No. 20. The volume taken for the determination is based upon this result. Of an apparently normal urine, take 25 ml.; if fluorescence is very intense, 2-5 ml., diluted to 25 ml. with distilled water, will suffice. The sample is accurately pipetted into a separating funnel, water added to make 25 ml., if necessary, and 2.5 ml. of glacial acetic acid is added followed by 50 ml. of ether. The mixture is shaken vigorously (i.e. about 200 times) for 1 min. and after the phases have separated, the lower (aqueous) phase is run into a second separating funnel and shaken similarly with 50 ml. of ether. This ether extract is combined with the first and the coproporphyrin-free aqueous phase (U) retained for determination of uroporphyrin.

The combined ether extracts are washed in the separating funnel by shaking for 1 min, as follows:-

1. Successively with 20 ml. portions of dilute sodium acetate solution until the aqueous phase no longer shows red fluorescence. Normally, two shakings should suffice. These washings are combined, twice shaken for 1 min. with an equal volume of fresh ether to recapture any coproporphyrin which may have been carried over (this ether is added to main bulk) and then added to urine residue, U.
2. Once with 50 ml. of 0.005% dilute iodine solution.
3. Once with 25 ml. of distilled water.

Uroporphyrin, present in U, the urine residue plus washings of the ether phase, is recovered by adding conc. hydrochloric acid to pH 1.5 (pH meter). Measure the volume and shake for 2 min. thoroughly but not too vigorously with two successive portions (each V/2) of cyclohexanone, allowing *good separation* of the phases. To the combined cyclohexanone extracts add a volume (2V) of ether and mix. Occasionally a small quantity of aqueous phase separates at this stage but it should not be removed from the funnel. The uroporphyrin is extracted by shaking with successive small volumes (1.5-2ml.) of 5% hydrochloric acid until the extract no longer shows red fluorescence under ultra-violet light. The extracts are conveniently collected in a graduated measuring cylinder, mixed and their total volume recorded. This solution is filtered or centrifuged and the optical density is measured at 430 mμ, 380 mμ, and at the peak of the Soret band (about 405 mμ) and uroporphyrin content calculated as follows:-

Calculation.

If V = vol. of urine taken,

v = vol. of acid extract,

D. 430 = optical density at 430 mμ,

D 380 = optical density at 380 mμ,

D max. = optical density at the peak of the Soret band,

then $\left[2D\ max. - (D\ 430 + D\ 380)\right] \times 832 \times \frac{v}{V} = \mu g$, uroporphyrin/1.

Coproporphyrin is now extracted by shaking the ethereal solution with successive 2 ml. portions of 5% hydrochloric acid until the extract no longer shows red fluorescence under ultra-violet light when viewed in a thin-walled tube. The extracts are conveniently collected in a graduated measuring cylinder, mixed, and their total volume recorded. This solution is filtered or centrifuged and the optical density is measured in a 1 cm. cell at 430 mμ, 380 mμ and at the peak of the Soret band (about 401 mu), and coproporphyrin content calculated as follows:-

Calculation:

For derivation of the formula used to correct for impurities having spectral absorption in the Soret band region, see Rimington and Sveinsson (1950); Holti, Rimington, Tate and Thomas (1958).

If V = vol. of urine taken,

v = vol. of acid extract,

D 430 = optical density at 430 mμ

D 380 = optical density at 380 mμ

Dmax. = optical density at peak of Soret band,

then $\left[2D\ max. - (D\ 430 + D\ 380)\right] \times 837 \times \frac{v}{V}$ = μg. coproporphyrin/1.

FAECAL COPROPORPHYRIN AND PROTOPORPHYRIN

Principle

Ether soluble porphyrins and porphyrinogens are extracted by ether containing acetic acid. Porphyrinogen is oxidised to porphyrin by shaking with a dilute solution of iodine. Coproporphyrin is then transferred to 0.1 N hydrochloric acid and protoporphyrin to 5% hydrochloric acid. Pigments derived from chlorophyll remain in the ether phase.

METHOD

The sample is well mixed with a glass rod or otherwise and two portions each of about 0.5 g. weighed accurately into the tared weighing bottle and stoppered tube respectively. The moisture content of the sample is determined by heating the weighing bottle and contents at 105^{o} until constant in weight (about 4 hours).

To the boiling tube is added about 2 ml. acetic acid, and the sample disintegrated by shaking and stirring, if necessary, with the glass rod. About 20 ml. of ether is added and the mixture shaken vigorously for 1 min. The extract is cleared by light centrifugation (about 2000 r.p.m. for 3 mins.) and transferred to a large measuring cylinder (250 ml.).

The faecal residue is extracted repeatedly with acetic acid and ether in exactly the same manner until the extract no longer shows fluorescence under ultra-violet light. The volume of the combined ether extracts is recorded and either the whole or an aliquot portion (depending upon the intensity of red fluorescence is transferred to a separatory funnel. It is washed twice by shaking for 1 min. with 25 ml. of dilute sodium acetate solution. The combined washings are twice shaken for 1 min. with 25 ml. of fresh ether to recapture any porphyrin which may have been carried over (this ether is added to the main bulk) and are then reserved for examination for uroporphyrin. The ether is then shaken with about one-fifth of its volume of dilute iodine solution and finally with 25 ml. of water.

Coproporphyrin is now extracted by shaking with successive small quantities (2 ml.) of 0.1 N hydrochloric acid until the extract no longer shows red fluorescence in ultra-violet light. The extracts are conveniently collected in a graduated measuring cylinder, mixed, and their total volume recorded. Optical density of this solution is measured at 430 mμ, 380 mμ, and at the peak of the Soret band (about 401 mμ) and coproporphyrin content calculated as follows:-

Calculation.

If A = weight of faeces taken for extraction,

W = wet weight taken for moisture determination,

w = dry weight found in moisture determination,

V = volume of acid extract,

D 430 = optical density at 430 mμ,

D 380 = optical density at 380 mμ,

D max. = optical density at peak of Soret band,

then $\left[2D\ max. - (D430 + D380)\right] \times 0.730 \times \frac{V}{A} \times \frac{W}{w}$

= μg. coproporphyrin/g. dry weight.

Protoporphyrin is extracted from the etheral solution after all coproporphyrin has been removed from the latter. This is done by shaking with successive small quantities (2ml.) of 5% hydrochloric acid until the extract no longer shows red fluorescence in ultra-violet light. Any fluorescence persisting in the ether layer is due to pigments derived from chlorophyll. The acid extracts are conveniently collected in a graduated measuring cylinder, mixed and their volume recorded. Optical density of this solution is measured at 430 mμ, 380 mμ/

380 mμ„ and at the peak of the Soret band (about 407 mμ) and protoporphyrin content calculated as follows:-

Calculation.

If A = weight of faeces taken for extraction,

W = wet weight taken for moisture determination,

w = dry weight found in moisture determination,

V = volume of acid extract,

D 430 = optical density at 430 mμ,

D 380 = optical density at 380 mμ,

D max. = optical density at peak of Soret band,

then $[2D\ max. - (D430 + D380)] \times 1.226 \times \frac{V}{A} \times \frac{W}{w}$

= μg. protoporphyrin/g. dry weight.

TISSUE UROPORPHYRIN, COPROPORPHYRIN AND PROTOPORPHYRIN

Tissue porphyrins are measured as per faecal porphyrins, the tissue sample is taken weighed and homogenised in glacial acetic acid in a piston homogeniser, ether added, homogenised again and the method continued as given before. For the uroporphyrin content the remaining tissue pellet after centrifugation is homogenised in 0.1M NH_4 OH. to which extract the sodium acetate washings are added from the previous ether treatment. This solution is used in the method described for urine. For tissues wet weight/dry weight ratio is taken as 1 and the results calculated as µg/gwet wt. tissue.

SPECIAL EHRLICHS REAGENT

For 100ml.

2.5g p. Dimethylaminobenzaldehyde (pDMAB)

0.5g HgC12 Dissolved in loml glacial acetic acid.

Perchloric Acid S.G.1.7.

Glacial Acetic Acid.

METHOD

Dissolve pDMAB in about 50ml glacial acetic acid, add 24.5ml Perchloric acid, and 4ml $HgCl_2$ solution. Mix, cool and make up to 100ml with glacial acetic acid in a volumetric flask.* Store in a dark bottle at $0^{o}C$.

* If at this stage any brown colouration appears, the reagent must be discarded.

(2) PORPHYRINOGENIC DRUGS.

DRUGS BELIEVED TO PRECIPITATE PORPHYRIA

1: Clinical Evaluation

Barbiturates (1)
Sulphonamides (1)
Sulphonal (1)
Apronal (Sedormid (1)
Sex Hormones (1)
Oral Contraceptives (1)
Phenytoin & Other Hydantoins (1)
Methsuximide & Other Sucinimides (1)
Tolbutamide (1)
Griseofulvin (1)
Aminopyrine (1)
Chlordiazepoxide (Librium) (1)
Dichloralphenazone (Welldorm) (1)
Meprobamate (Equanil) (1)
MethylDOPA (Aldomet) (3)
Ethanol (4)
Imipramine (Tofranil) (10)
Ergot preparations (6)
Hexachlorobenzene (1)
Chlorpropamide (7)
Chloroquine (1)

2: 'In Vitro' &'In Vivo' Evidence

Barbiturates (12)
Sulphonamides (11)
Methyprylone (Nolundar) (2)
Glutethimide (Doriden) (2,14)
Carbromal (1)
Chloramphenicol (2)
Pyrazinamide (Anti T.B.) (2)
Troxidone (2)
Nikethamide (1)
Bemegride (8)
Theophylline (2)
Caffeine (2)
Metyrapone (1,14)
Menthol (2)
Pentazocine (Fortral) (14)
Hydrocortisone NaSuccinate (Efcortelan) (14)
Dichloralphenazone (Welldorm) (11)
MethylDOPA (11)
Ethanol (14)
Steroids C19 & C21 (13,14)

DRUGS BELIEVED SAFE IN PORPHYRIA

1: Clinical Evaluation

Pethidine (9)
Morphine (9)
Mefenamic acid (9)
Asprin (6)
Chlorpromazine (9)
Chloral Hydrate (8)
Penicillin (6)
Propoxyphene (6)

2: 'In Vitro' & 'In Vivo' Evidence

Phenylbutazone (2)
Reserpine (2)
Dicoumarol (2)
Morphine (11)
Pethidine (11)
Dexamethasone (Decadron) (14)

References

1. DeMatteis F. 1967
2. Granick S. 1966
3. Goldberg A. 1968b
4. Goldberg A. 1959
5. Shanley B.C. et.al. 1968
6. Tschudy D.P. 1969
7. Zarowitz H. & Newhouse S. 1965
8. Chisolm J.J. 1969
9. Dean G. 1970
10. Goldberg A. et.al. 1967
11. Beattie A.D. et.al. 1970
12. Moore M.R. et.al. 1970
13. Goldberg A. et.al. 1969
14. This Thesis

REFERENCES

Abrahams, A., Gavey, C.J. and MacLagan, N.F. (1947)

Brit. med. J. 2, 327.

Aitken, J.M., Moore, M.R. (1970)

Unpublished observations.

Bacchetti, S., Whitmore, G.F. (1969)

Biophys. J. *9*, 1427

Bagdasarian, M. (1958)

Nature *181*, 1399

Baliga, B.S., Pronczuk, A.W., Munro, H.N. (1969)

J. biol. Chem. *244*, 4480

Barnes, C.D., Eltherington, L.G. (1964)

in 'Drug dosage in Laboratory Animals' Berkeley, Los Angeles

Batlle, A.M. del C., Benson, A., Rimington, C. (1965)

Biochem. J. *97*, 731

Batlle, A.M. del C., Ferramola, A.M., Grinstein, M. (1967)

Biochem. J. *104*, 244

Battistini, V., Morrow, J.J., Thompson, G.G., Ginsburg, D., Moore, M.R., Goldberg, A. (1970)

Brit. J. Haemat. In press

Beattie, A.D. (1970)

Unpublished observations

Beattie, A.D., Moore, M.R., Goldberg, A. (1970)

8th International Congress of Haematology, Abstracts p.37

Becker, F.T. (1965)

Arch. Derm. *92*, 252

Bennett, L.L., Smithers, D., Ward, C.T. (1964)

Biochim. biophys. Acta. *87*, 60

Berger, H., Goldberg, A. (1955)

Brit. med. J. 2, 85

Beutler, E., Duron, O., Kelly, B.M. (1963)

J. Lab. clin. Med. *61*, 882

Bogorad, L. (1958a)

J. biol. Chem. *233*, 501

Bogorad, L. (1958b)

J. biol. Chem. *233*, 510

Bogorad, L. (1960)

in 'Comparative Biochemistry of Photoreactive systems'

p. 257. Ed. M.B. Allen, Academic Press, New York

Bonsignore, D. (1966)

Med. d Lavoro *67*, 647

Bonzanio, A., Meo, G., Pisani, W. (1965)

Boll Soc. ital. Biol. sper. *41* 854

Booij, M.L., Rimington, C. (1957)

Biochem. J. *65*, 4p

Bottomley, S.S., Smithee, G.A. (1968)

Biochim. biophys. Acta. *159*, 27

Bottomley, S.S., Smithee, G.A. (1969)

J. Lab. clin. Med. *74*, 445

Braunstein, A.E., Poznanskaya, A.A., Spryshkova, R.A., Gnuchev, N.V. (1964)

Dokl. Akad. Nauk SSSR. *157*, 982

Brown, E.G. (1958a)

Nature. *182*, 313

Brown, E.G. (1958b)

Biochem. J. *70*, 313

Bruni, A., Luciani, S., Contessa A.R. (1964)

Nature. *201*, 1219

Bulbrook, R.D., Hayward, J.L. (1969)

Lancet *2*, 1033

Burka, E.R. (1968)

Science *162*, 1287

Burnham, B.F., Lascelles, J. (1963)

Biochem. J. *87*, 462

Buttner, H. (1965)

Biochem. Z. *341*, 300

Calissano, P., Bonsignore, D., Cartasegna, C. (1966)

Biochem. J. *101*, 550

Cameron, E.H.D., Jones, T., Jones, D., Anderson, A.B.M., Griffiths, K. (1969)

J. Endocrin. *45*, 215

Campbell, K. (1898)

J. ment. Sci. *44*, 305

Cantarow, A., Schepartz, B. (1967)

in 'Biochemistry', Saunders, Philadelphia and London

Cartasegna, C., Vergnano, C., Ardonio., V. (1969)

Boll. Soc. Ital. Biol. sper. *45*, 313

Ceriotti, G. (1952)

J. biol. Chem. *198*, 297

Ceriotti, G. (1955)

J. biol. Chem. *214*, 59

Chisholm, J.J. (1964)

J. Pediat. *64*, 174

Chisholm, J.J. (1969)

5th. med. J. 2, 1927

Coleman, D.L. (1966)

J. biol. Chem. *241*, 5511

Conney, A.H. (1967)

Pharmacol. Rev. *19*, 817

Cookson, G.H., Rimington, C. (1953)

Nature. *171*, 875

Cookson, G.H., Rimington, C. (1954)

Biochem. J. *57*, 476

Copeman, P.W.M., Cripps, D.J., Summerly, R. (1966)

Brit. med. J. *1*, 461

Cornford, P. (1964)

Biochem, J. *91*, 64

Cowger, M.L., Labbe, R.F. (1967)

Biochem. Pharmacol. *16*, 2189

Cronholm, T., Sjovall, J. (1970)

Europ. J. Biochem. *13*, 124

Culp, W., McKeehan, W., Hardesty, B. (1969)

Proc. nat. Acad. Sci. *64*, 388

Dale, R.A. (1969)

Biochem. J. *114*, 499

Dean, G. (1970)

S. Afr. med. J. *41*, 927

De Barreiro, O.C. (1967)

Biochim. biophys. Acta. *139*, 479

De Barreiro, O.C. (1969)

Biochem. Pharmacol. *18*, 2267

De Bruin, A. (1968)

Med. d. Lavoro, *59*, 11

Delena S., Brown N. (1969)

Metabolism, 18, 901

De Matteis, F. (1964)

Biochim. biophys. Acta. *82*, 641

De Matteis, F. (1967)

Pharmacol. Rev. *19*, 523

De Matteis, F. (1968)

Biochem. J. *109*, 775

De Matteis, F. (1970)

F.E.B.S. Letters. *6*, 243

De Matteis, F., Rimington, C. (1962)

Lancet. *1*, 1332

Dent, C.E., Rimington, C. (1947)

Biochem. J. *41*, 253

Dowdle, E.B., Mustard, P., Eales, L. (1967)

S. Afr. med. J. *41*, 1093

Dowdle, E.B., Mustard, P., Spong, N., Eales, L. (1968)

Clin. Sci. *34*, 233

Dowdle, E.B., Sweeney, G.D., Saunders, S.J., Eales, L. (1963)

National Conference on Nuclear Energy, p.339

Doyle, D., Schimke, R.T. (1969)

J. biol. Chem. *244*, 5449

Dresel, E.I.B., Falk, J.E. (1953)

Nature, *172*, 1185

Dresel, E.I.B., Falk, J.E. (1956a)

Biochem. J. *63*, 80

Dresel, E.I.B., Falk, J.E. (1956b)

Biochem. J. *63*, 388

Dresel, E.I.B., Rimington, C., Tooth, B.E. (1956)

Scand. J. clin. Lab. Invest. *8*, 73

Druyan, R., Debernard, B., Rabinowitz, M. (1969)

J. biol. Chem. *244*, 5874

Elder, T.D., Mergel, C.E., (1966)

Amer, J. Med. *41*, 369

Elkind, M.M. (1969)

in Radiation Research'. p. 558.

Ed. G. Silini, North Holland, Amsterdam

Elkind, M.M., Sakamoto, K., Kamper, C. (1968)

Cell and Tissue Kinetics. *1*, 209

Ennis, H.L., Lubin, M. (1964)

Science, *146*, 1474

Ertel, R.J., Ungar, F., Halberg, E. (1963)

Fed. Proc. *22*, 211

Estabrook, R.W., Cooper, D.Y., Rosenthal, D. (1963)

Biochem. Z. *338*, 741

Feldman, M., Yagil, G. (1969)

Biochem. biophys. Res. Commun. *37*, 198

Felsher, B.F., Redeker, A.G. (1966)

Medicine, *45*, 575

Ferretti, J.J., Gray, E.D. (1968)

J. Bact. *95*, 1400

Forsander, O.A., Himberg, J.J. (1969)

Metabolism, *18*, 776

French, S.W. (1969)

Gastroenterology, *54*, 1106

Fukumara, H. (1965)

Biochem. biophys. Res. Commun. *18*, 297

Gajdos, A., Gajdos-Török, M. (1961)

Lancet, *2*, 175

Gajdos, A., Gajdos-Török, M. (1963)

S.Afr. J. Lab. clin. Med. *9*, 232

Gajdos, A., Gajdos-Török, M. (1968a)

Bull. Soc. Chim. Biol. *50*, 1809

Gajdos, A., Gajdos-Török, M. (1968b)

Symp. Biochem. Soc. *28*, 45

Gajdos, A., Gajdos-Török, M. (1969)

Biochemical Med. *2*, 372

Gajdos, A., Gajdos-Török, M., Palma Carlos, A., Palma Carlos, M.L. (1966)

Bull. Soc. Chim. biol. *48*, 803

Gajdos, A., Gajdos-Török, M., Struma, M.V. (1969)

C.R. Soc. Biol. *163*, 1343

Gibson, K.D., Laver, W.G., Neuberger, A. (1958)

Biochem. J. *70*, 71

Gibson, K.D., Matthey, M., Neuberger, A., Tait, G.H. (1961)

Nature. *192*, 204

Gibson, K.D., Neuberger, A., Scott, J.J. (1955)

Biochem. J. *61*, 618

Gibson, S.L.M., Goldberg, A. (1970)

Clin. Sci. *38*, 63

Goldberg, A. (1953)

4th Congress of the European Society of Haematology

Abstracts p. 27

Goldberg, A. (1954)

Biochem. J. *57*, 55

Goldberg, A. (1959)

Quart. J. Med. *28*, 183

Goldberg, A. (1966)

in 'Modern Trends in Dermatology'

Ed. R.M.B. McKenna, Butterworth, London.

Goldberg, A. (1968a)

Symp. Biochem. Soc. *28*, 35

Goldberg, A. (1968b)

Proc. roy. Soc. Med. *61*, 193

Goldberg, A. (1968c)

Seminars in Haematology *5*, 424

Goldberg, A. (1970)

in ' Recent Advances in Haematology'

Ed. A. Goldberg and Brain. In press

Goldberg, A., Ashenbrucker, H., Cartwright, G.E., Wintrobe, M.M.

(1956)

Blood, *11*, 821

Goldberg, A., MacDonald, A.C., Rimington, C. (1952)

Brit. med. J. *2*, 1174

Goldberg, A., Moore, M.R., Beattie, A.D., Hall, P.E., McCallum, J., Grant, J.K. (1969a)
Lancet *1*, 115

Goldberg, A., Moore, M.R., Beattie, A.D., Hall, P.E., Mccallum, J., Grant, J.K. (1969b)
Scot. med. J. *14*, 97

Goldberg, A., Rimington, C. (1954)
Proc. roy. Soc. B. *143*, 257

Goldberg, A., Rimington, C. (1962)
'Diseases of Porphyrin Metabolism'
Thomas, Springifield, Illinois.

Goldberg, A., Rimington, C., Lochhead, A.C. (1967)
Lancet *1*, 632

Gonzales-Cadavid, N.F., Campbell, P.N. (1967)
Biochem. J. *105*, 443

Gordon, A.S. (1970)
Proc. 8th International Congress of Haematology. p.7.

Gorshein, D., Gardner, F.H. (1970)
Proc. nat. Acad. Sci. *65*, 564

Gorski, J., Axman, M.C. (1964)
Arch. Biochem. Biophys. *105*, 517

Granick, S. (1965)
Ann. N.Y. Acad, Sci. *123*, 188

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

Granick, S., Kappas, A. (1967a)

Proc. nat. Acad. Sci. *57*, 1463

Granick, S., Kappas, A. (1967b)

J. biol. Chem. *242*, 4587

Granick, S., Levere, R.D. (1964)

in 'Progress in Haematology' Vol. IV. p.1; Grune & Stratton

Granick, S., Mauzerall, D. (1958)

J. biol. Chem. *232*, 1119

Granick, S., Mauzerall, D. (1960)

in 'Pathways of Chemical Metabolism. Vol. II'. p.526

Ed. D. Greenberg, Academic Press, New York

Granick, S., Urata, G. (1963)

J. biol. Chem *238*, 821

Haeger-Aronsen, B. (1960)

Scand. J. clin. Lab. Invest. *12*, Suppl. 47

Haeger-Aronsen, B. (1963)

S. Afr. J. Lab. clin. Med. *9*, 288

Hamburger, C. (1948)

Acta endocr. (Kbh) *1*, 19

Hayashi, N., Yoda, B., Kikuchi, G. (1968)

J. Biochem. *63*, 446

Heikel, T., Lockwood, W.H., Rimington C. (1958)

Nature. *182*, 313

Hendrikx, A., Heyns, W., DeMoor, P. (1968)

J. clin. Endocr. *28*, 1525

Hernberg, S., Nikkanen, J. (1970)

Lancet. *1*, 63

Hickman, R., Saunders, S.J., Dowdle, E.B., Eales, L. (1967)

Lancet *2*, 656

Higuchi, M., Goto, K., Fujimoto, M., Namiki, O., Kikuchi, G. (1965)

Biochim. biophys. Acta *95*, 94

Higuchi, M., Ohba, T., Sakai, H., Kurashima, Y., Kikuchi, G. (1968)

J. Biochem. *64*, 795

Holmes, J.G., Barnes, H.D., (1965)

Trans. St. John's Hosp. derm. Soc. *51*, 54

Holti, G., Rimington, C., Tate, B.C., Thomas, G. (1958)

Quart. J. Med. *27*, 1.

Isselbacher, K.J., Greenberger, N.J. (1964)

New. Engl. J. Med. *270*, 351

Jacob, F., Monod, J. (1961)

J. molec. Biol. *3*, 318

Jasinski, D.R., Martin, W.R., Hoeldtke, R.D. (1970)

Clin. Pharmacol. Ther. *11*, 385

Jones, T., Griffiths, K. (1968)

J. Endocr. *42*, 559

Kadenbach, B. (1967)

Biochim. biophys. Acta *138*, 651

Kahl, G.F., Netter, K.J. (1970)
Biochem. Pharmacol. *19*, 27

Kappas, A., Granick, S. (1968a)
J. biol. Chem. *243*, 346

Kappas, A., Granick, S. (1968b)
Proc. nat. Acad. Sci. *151*, 842

Kappas, A., Song, C.S., Levere, R.D., Sachson, R.A., Granick, S. (1968)
Proc. nat. Acad. Sci. *61*, 509

Kappas, A., Song, C.S., Sassa, S., Levere, R.D., Granick, S. (1969)
Proc. nat. Acad. Sci. *64*, 557

Kerridge, D. (1958)
J. gen. Microbiol. *19*, 497

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

Koskelo, P., Eisalo, A., Toivonen, I. (1966)
Brit. med. J. *1*, 652

Kosower, N.S., Kosower, E.M., Zinn, A.B., Carraway, R. (1969)
Biochem. Med. *2*, 389

Kowalski, E., Dancewicz, A.M., Szot, Z. (1957)
Bull. Acad. pol. Sci. (Cl.2.) *5*, 223

Kyogoku, Y., Lord, R.C., Rich, A. (1968)
Nature, *218*, 69

Labbe, R.F., Hubbard, N. (1960)
Biochim. biophys. Acta. *41*, 185

Labbe, R.F., Kurumada, T., Onisawa, J. (1965)

Biochim. biophys. Acta *111*, 403

Labbe, R.F., Talman, E.L., Aldrich, R.A. (1954)

Biochim. biophys. Acta *15*, 590

Laroche, C., Remy, J.H. (1970)

Thérapie *25*, 73

Lascelles, J. (1959)

Biochem. J. *72*, 508

Lascelles, J. (1960)

J. gen. Microbiol. *23*, 487

Lascelles, J. (1968)

Symp. Biochem. Soc. *28*, 49

Leibman, K.C. (1969)

Molec. Pharmacol. *5*, 1

Levere, R.D. (1966)

Blood *28*, 569

Levere, R.D., Granick, S. (1967)

J. biol. Chem. *242*, 1903

Levere, R.D., Kappas, A., Granick, S. (1967a)

Proc. nat. Acad. Sci. *58*, 985

Levere, R.D., Kappas, A., Granick, S. (1967b)

J. clin. Invest. *46*, 1084

Levrat, M., Tissot, A., Mouliner, B., Tete, R. (1963)

Lyon Med. *94*, 37

Lichtman, H.C., Feldman, F. (1963)

J. clin. Invest. *42*, 830

Liddle, G.W., Island, D., Ester, H., Tomkins, G.M. (1958)

J. clin. Invest. *37*, 912

Lieber, C.S., Rubin, E. (1969)

Gastroenterology *54*, 642

Lin, S.Y., Mosteller, R.D., Hardesty, B. (1966)

J. molec. Biol. *21*, 51

Lindros, K.O., Hillbom, M.E. (1969)

Life Sciences *8*, 1017

Lockwood, W.H. (1953)

Aust. J. exp. Biol. med. Sci. *31*, 453

Longhino, N., Tajic, M., Vedris, M., Jankovic, D., Drobryak, P. (1968)

Acta endocr. (Buc) *59*, 644

Loraine, J.A., Bell, E.T. (1966)

Hormone Assays and their Clinical Application,

Livingstone, Edinburgh

Loriaux, D.L., Delena, S., Brown, H. (1969)

Metabolism *18*, 860

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. (1951)

J. biol. Chem. *193*, 265

Ludwig, G.D., Scott, R.M., Chaykin, L. (1965)

Fed. Proc. *24*, 223

Lundquist, F., Fugmann, U., Klaning, E., Rasmussen, H. (1959)

Biochem. J. *72*, 409

Lundsgaard, E. (1938)

Bull Johns Hopk. Hosp. *63*, 15

Magasanik, B. (1961)

Symp. Quant. Biol. *26*, 249

Majchrowicz, E., Mendelson, J.H. (1970)

Science *168*, 1100

Marriot, J. (1968)

Symp. Biochem. Soc. *28*, 61

Marriot, J., Neuberger, A., Tait, G.H. (1969)

Biochem. J. *111*, 385

Marver, H.S., Tschudy, D.P., Perlroth, M.G., Collins, A. (1966)

J. biol. Chem. *241*, 2803

Marver, H.S., Collins, A., Tschudy, D.P., Rechcigl, M. (1966)

J. biol. Chem. *241*, 4323

Masuya, T. (1969)

Acta haemat. jap. *32*, 465

Mathews-Roth, M.M., Pathak, M.A., Fitzpatrick, T.B., Harber, L.C., Kass, E.H. (1970)

New Engl. J. Med. *282*, 1231

Mauzerall, D., Granick, S. (1956)

J. biol. Chem. *219*, 435

Mauzerall, D., Granick, S. (1958)

J. biol. Chem. *232*, 1141

Menon, I.A., Shemin, D. (1967)
Arch. Biochem. Biophys. *121*, 304

Meo, G., Pisani, W., Bonzanio, L. (1965)
Boll. Soc. ital, Biol. sper. *41*, 851

Mitchison, J.M. (1969)
Science *165*, 657

Miyakoshi, T., Kikuchi, G. (1963)
Tohoku J. exp. Med. *79*, 199

Monod, J., Wyman, J., Changeux, J.P. (1965)
J. molec. Biol. *12*, 88

Moore, M.R., (1970)
Unpublished results

Moore, M.R., Battistini, V., Beattie, A.D., Goldberg, A. (1970)
Biochem. Pharmacol. *19*, 751

Moore, M.R., Beattie, A.D., Thompson, G.G., Goldberg, A. (1970a)
Clin. Sci. *38*, 33p

Moore, M.R., Beattie, A.D., Thompson, G.G., Goldberg, A. (1970b)
Clin. Sci. In press

Moore, M.R., Beattie, A.D., Hall, P.E., Grant, J.K., Goldberg, A. (1969)
Unpublished observations

Moore, M.R., Corrance, M., Davidson, J.N. (1969)
Unpublished observations

Moore, M.R., Goldberg, A. (1970)
Clin. Sci. *39*, 9p

Morrow, J.J., Urata, G., Goldberg, A. (1969)
Clin. Sci. *37*, 533

Murty, H.S., Nair, P.P. (1969)
Nature *223*, 200

McKay, R., Druyan, R., Rabinowitz, M. (1968)
Fed. Proc. *27*, 774

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

McKeehan, W., Hardesty, B. (1969)
Biochem. biophys. Res. Commun. *36*, 625

Nakao, K., Wada, O., Kitamura, T., Uono, K., Urata, G. (1966)
Nature *210*, 838

Nakao, K., Wada, O., Yano, Y. (1968)
Clin. chim. Acta. *19*, 319

Nandi, D.L., Baker-Cohen, K.F., Shemin, D. (1968)
J. biol. Chem. *243*, 1224

Nandi, D.L., Shemin, D. (1968a)
J. biol. Chem. *243*, 1231

Nandi, D.L., Shemin, D. (1968b)
J. biol. Chem. *243*, 1236

Nandi, D.L., Waygood, E.R. (1967)
Canad. J. Biochem. *45*, 327

Narisawa, K., Kikuchi, G. (1966)
Biochem. biophys. Acta *123*, 596

Necheles, T.F., Rai, U.S. (1969)

Blood *34*, 380

Nemeth, A.M., Russell, C.S., Shemin, D. (1957)

J. biol. Chem. *233*, 415

Neuberger, A., Turner, J.M. (1963)

Biochim. biophys. Acta *67*, 342

Neuwirt, J., Ponka, P., Borova, J. (1969)

Europ. J. Biochem. *9*, 36

Neuwirt, J., Ponka, P., Borova, J., Prchal, J.F. (1969)

Blut *19*, 17

Nyberg, A., Schuberth, J., Anggard, L. (1953)

Acta chem. scand. *7*, 1170

Oaks, W.W., Schultz, J., Fleischmajer, R. (1969)

Dermatologia *138*, 10

Ohtsuka, E., Koide, S.S. (1969)

Gen. comp. Endocr. *12*, 598

O'Kelly, D.A. (1968)

Ph.D. Thesis, University of Glasgow

Omura, T., Sato, R., Cooper, D.Y., Rosenthal, O., Estabrook, R.W. (1965)

Fed. Proc. *24*, 1181

Papenberg, J., Wartburg, J.P., Aebi, H. (1970)

Enzym. biol. clin. *11*, 237

Paul, J., Hunter, J.A. (1969)

J. molec. Biol. *42*, 31

Payne, A.H., Mason, M. (1969)

Steroids, *13*, 213

Paxton, J. (1970)

Personal communication

Perlroth, M.G., Marver, H.S., Tschudy, D.P. (1965)

J. Amer. med. Ass. *194*, 135

Perlroth, M.G., Tschudy, D.P., Ratner, A., Spaur, W., Rederer, A. (1968)

Metabolism, *17*, 571

Pisani, W., Bonzanio, A., Fiandaca, G. (1968)

Boll. Soc. ital. Biol. sperm. *44*, 2069

Pollock, M.R.

Biochim. biophys. Acta *76*, 80

Porra, R.J., Jones, O.T.G. (1963)

Biochem, J. *87*, 181

Porta, E.A., Koch, O.R., Hartroft, W.S. (1970)

Exp. molec. Path. *12*, 104

Prunty, F.T.G. (1949)

J. clin. Invest. *28*, 690

Redecker, A.G. (1964)

Lancet *2*, 1178

Reich, E., Goldberg, I.H. (1964)

Progress in Nucleic Acid Research and Molecular Biology *3*, 183

Rifkind, A.B., Gillette, P.N., Song, C.S., Kappas, A. (1970)

J. clin. Endocr. *30*, 330

Rimington, C. (1961)

Association of Clinical Pathologists. Broadsheet No.36

Rimington, C. (1966)

Relazione al 7 Congresso Internationale di Patologia Clinica, Rome

Rimington, C., Booij, H.L. (1957)

Biochem. J. *65*, 3p

Rimington, C., Hemmings, A.W. (1939)

Biochem. J. *33*, 960

Rimington, C., Sveinsson, S.L. (1950)

Scand. J. clin. Lab. Invest. *2*, 209

Rose, I.A., Hellman, E.S., Tschudy, D.P. (1961)

Metabolism *10*, 514

Rubin, E., Lieber, C.S. (1968)

Ann. intern. Med. *69*, 1063

Rubino, G. (1961)

Panminerva Med. *1*, 41

Rubino, G. (1965)

Minerva med. *1*, 50

Rubino, G.F., Pisani, W., Bonzanio, A., Rasetti, L. (1967)

Ital. J. Biochem. *16*, 367

Saillen, R. (1963)

Helv. med. Acta *30*, 208

Sano, S., Granick, S. (1961)

J. biol. Chem. *236*, 1173

Sanzari, N.P., Peron, F.G. (1966)

Steroids *8*, 929

Sardesai, V.M., Waldman, J., Orten, J.M. (1964)

Blood *24*, 178

Scheig, R. (1970)

Amer. J. clin. Nutr. *23*, 467

Schimke, R.T., Ganschow, R., Doyle, D., Arias, I.M. (1968)

Fed. Proc. *27*, 1223

Schmid, R., Marver, H.S., Hammaker, L. (1966)

Biochem. biophys. Res. Commun. *24*, 319

Schmid, R., Schwartz, S. (1952)

Proc. Soc. exp. Biol. *81*, 685

Schmid, R., Shemin, D. (1955)

J. Amer. chem. Soc. *77*, 506

Schneck, D.W., Racz, W.J., Hirsch, G.H., Bubbar, G.L., Marks, G.S. (1968)

Biochem. Pharmacol. *17*, 1385

Scholnick, P.L., Hammaker, L.E., Marver, H.S. (1969)

Proc. nat. Acad. Sci. *63*, 65

Schulman, M.P., Richert, D.A. (1956)

Fed. Proc. *15*, 349

Schwartz, S. (1953)

Vet. Admin. Tech. Bull. 10

Schwartz, S., Zagaria, R. (1951)

in 'Toxicology of Uranium'.

Ed. A. Tannenbaum, McGraw-Hill, New York

Schwochau, M.E., Hadwiger, L.A. (1968)

Arch. Biochem. Biophys. *126*, 731

Schwochau, M.E., Hadwiger, L.A. (1969)

Arch. Biochem. Biophys. *134*, 34

Shanley, B.C., Zail, S.S. Joubert, S.M. (1968)

Lancet *1*, 70

Shanley, B.C., Zail, S.S., Joubert, S.M. (1969)

Brit. J. Haemat. *17*, 389

Shemin, D. (1955)

CIBA Foundation; Symp. on Porphyrin biosynthesis and metabolism p.4.

Ed. G.W. Wolstenholme, Churchill, London.

Shemin, D. (1968)

Symp. Biochem. Soc. *28*, 75

Shemin, D., Kumin, S. (1952)

J. biol. Chem. *198*, 827

Shemin, D., Rittenberg, D. (1945)

J. biol, Chem. *159*, 567

Shemin, D., Rittenberg, D. (1946)

J. biol. Chem. *160*, 621

Shemin, D., Russell, C.S. (1953)

J. Amer. chem. Soc. *75*, 4873

Shetty, A.S., Miller, G.W. (1969)
Biochem. J. *114* 331

Shigesada, K., Ebisuno, T., Katsuki, H. (1970)
Biochem. biophys. Res. Commun. *39*, 135

Siegel, M.R., Sisler, H.D. (1964)
Biochim. biophys. Acta *87*, 83

Siegel, M.R., Sisler, H.D. (1965)
Biochim. Biophys. Acta *103*, 558

Sisler, H.D., Siegel, M.R. (1967)
in 'Antibiotics' V. p.283,
Ed. D. Gottlieb and P.D. Shaw, Springer, New York

Simons, J.A. (1970)
Science *167*, 1378

Sirett, N.E., Gibbs, F.P. (1969)
Endocrinology *85*, 355

Skea, B.R., Downie, E.D., Moore, M.R., Davidson, J.N. (1970)
Biochem J. In press

Song, C.S., Singer, J.W., Levere, R.D., Harris, D.F.,
Kappas, A. (1968)
J. Lab. Clin. Med. *72*, 1019

Stadtman, E.R., Overath, P., Eggerer, H., Lynen, F. (1960)
Biochem. biophys. Res. Commun. 2, 1

Stein, J.A., Tschudy, D.P., Corcoran, P.L., Collins, A. (1970)
J. biol. Chem. *245*, 2213

Stern, J.R., Coon, M.J., Delcampillo, A., Schneider, M.C. (1956)
J. biol. Chem. *221*, 15

Stokvis, B.J. (1895)

Z. klin. Med. *28*, 1

Szeberenyi, S., Szalay, K.S., Garattini, S. (1969)

Biochem. Pharmacol. *18*, 2767

Takaku, F., Wada, O., Sassa, S., Nakao, K. (1968)

Cancer Res. *28*, 1250

Tephly, T.R., Tinelli, F., Watkins, W.D. (1969)

Science *166*, 627

Theorell, H., Chance, B. (1951)

Acta chem. Scand. *5*, 1127

Tien, W., White, D.C. (1968)

Proc. nat. Acad. Sci. *61*, 1392

Tigier, H.A., Batlle, A.M., del C. Locascio, G. (1968)

Biochim. biophys. Acta *151*, 300

Tornheim, K., O'Dell, R.G., Prosky, Y. (1969)

Proc. Soc. exp. Biol. *131*, 601

Trakatellis, A.C., Montjar, M., Axelrod, A.E. (1965)

Biochemistry *4*, 2065

Tschudy, D.P. (1968)

Ann. N.Y. Acad. Sci. *151*, 850

Tschudy, D.P. (1969)

in 'Duncan's Diseases of Metabolism'. p.600

Ed. A. Bondy, Saunders, Philadelphia.

Tschudy, D.P., Perlroth, M.G., Marver, H.S., Collins, A., Hunter, G. Rechcigl, M. (1965)

Proc. nat. Acad. Sci. *53*, 841

Tschudy, D.P., Rose, J., Hellman, E., Collins, A., Rechcigl, M. (1962)

Metabolism *11*, 1287

Tschudy, D.P., Welland, F.H., Collins, A., Hunter, G. (1964)

Metabolism, *13*, 396

Tuboi, S., Kim, H.J., Kikuchi, G. (1969)

Arch. Biochem. Biophys. *130*, 92

Tuboi, S., Kim, H.J., Kikuchi, G. (1970a)

Arch. Biochem. Biophys. *138*, 147

Tuboi, S., Kim, H.J., Kikuchi, G. (1970b)

Arch. Biochem. Biophys. *138*, 155

Urata, G., Granick, S. (1963)

J. biol. Chem. *238*, 811

Urquhart, J. (1898)

J. ment. Sci. *44*, 313

Vavra, J.D. (1967)

J. clin. Invest. *46*, 1127

Viel, B., Donoso, S., Salcedo, D., Varela, A. (1967)

Rev. med. Chile *95*, 575

Wajchenberg, B.L., Leme, C.E., Kieffer, J., Pereira, V.G. (1969)

Clin. Sci. *36*, 15

Waldenstrom, J. (1937)

Acta med. Scand. Suppl. 82

Watson, C.J., (1954)

in Advances in Internal Medicine VI.

Ed. W. Dock, I. Snapper, Year Book Publishers Inc., Chicago.

Watson, C.J. (1960)

New Engl. J. Med. *263,* 1205

Watson, C.J., Runge, W., Bossenmaier, I. (1962)

Metabolism *11,* 1129

Waxman, A., Collins, A., Tschudy, D.P. (1966)

Biochem. biophys. Res. Commun. *24,* 675

Welland, F.H., Hellman, E.S., Collins, A., Hunter, G.W.,

Tschudy, D.P. (1964)

Metabolism *13,* 251

Welland, F.H., Hellman, E.S., Gaddis, E.M., Collins, A.,

Tschudy, D.P. (1964)

Metabolism *13,* 232

Welland, F.H., Schwartz, H.C. (1966)

Clin. Res. *14,* 142

Westall, R.G. (1952)

Nature *170,* 614

Westerfield, W.W., Stoltz, E., Berg, R.L. (1943)

J. biol. Chem. *149,* 237

Wetterberg, L. (1964)

Lancet 2, 1178

Wetterberg, L. (1970)

J. clin Pharmacol. *10*, 199

Widuczynski, I., Stoppani, A.O.H. (1965)

Biochim. biophys. Acta *104*, 413

Williamson, J.R., Scholz, R., Browning, E.T., Thurman, R.G., Fukami, M.H. (1969)

J. biol. Chem. *244*, 5044

Woods, J.S., Dixon, R.L. (1969)

Proc. West Pharmacol. Soc. *12*, 129

Woods, J.S., Dixon, R.L. (1970)

Life Sciences *9*, 711

Woods, J.S., Dixon, R.L. (1970)

Biochem. Pharmacol. *19*, 1951

Yeh, S.D.H., Shils, M.E. (1969)

Biochem. Pharmacol. *18*, 1919

Yoshida, T., Ishihara, N. (1969)

Tohoku J. exp. Med. *99*, 1.

Young, C.W., Robinson, P.F., Sacktor, B.

Biochem. Pharmacol. *12*, 885

Zail, S.S., Joubert, S.M. (1968)

Brit. J. Haemat. *15*, 123

Zakim, D. (1968)

Proc. Soc. exp. Biol. *129*, 393

Zarowitz, H., Newhouse, S. (1965)

N.Y. St.J. Med. *65*, 2385

Zieve, L., Hill, E., Schwartz, S., Watson, C.J. (1953)
J. Lab. clin. Med. *41*, 663

PAPERS

1) Excessive Urinary Excretion of Certain Porphyrinogenic Steroids in Human Acute Intermittent Porphyria.

A. Goldberg., M.R. Moore., A.D. Beattie., P.E. Hall., J. McCallum., J.K. Grant.

The Lancet (1969) i 115-118

2) The Effects of Certain Barbiturates on the Hepatic Porphyrin Metabolism of Rats.

M.R. Moore., V. Battistini., A.D. Beattie., A. Goldberg.

Biochemical Pharmacology (1970) 19 751-757

3) Erythrocyte Delta-Aminolaevulic Acid Dehydrase Activity in Anaemia.

V. Battistini., J.J. Morrow., D. Ginsburg ., G.G. Thompson., M.R. Moore., A. Goldberg.

British Journal of Haematology - In press.

4) Depression of δ-Aminolaevulic Acid Dehydrase Activity by Ethanol in Man and Rat.

M.R. Moore., A.D. Beattie., G.G. Thompson., A. Goldberg.

Clinical Science - In press. (1970) 40 81-88

5) Hereditary Coproporphyria; Haem Enzyme Levels and Urinary 17 Oxosteroid Excretion.

M.R. Moore., A. Goldberg., A.D. Beattie., J. Paxton., N. McIntyre., A.J.G. Pearson.

In Preparation.

COMMUNICATIONS TO SOCIETIES

1) Excessive urinary excretion of certain porphyrinogenic steroids in human acute intermittent porphyria.

Scottish Society for Experimental Medicine.

Goldberg, A., Moore, M.R., Beattie, A.D., Hall, P.E., McCallum, J., Grant, J.K.

Scot. med. J. 14 (1969) 97.

2) Negative correlation of blood ethanol and erythrocyte delta-aminolaevulic acid (ALA) dehydrase levels in Man.

Medical Research Society.

Moore, M.R., Beattie, A.D., Thompson, G.G., Goldberg, A.

Clin. Sci. 38 (1970) 33p.

3) Enzyme induction and Haem biosynthesis.

Medical Research Society.

Moore, M.R., Goldberg, A.

Clin. Sci. 39 (1970) 9p.

4) Induction of δ-Aminolaevulic acid Synthetase activity in cultured chick embryo liver cells by Ribonucleic acid.

Biochemical Society

Skea, B.R., Downie, E.D., Moore, M.R., Davidson, J.N.

Biochem.J. - 121 (1970) 25p.

5) The effect of certain drugs including ethanol on enzymes of the haem biosynthetic pathway.

Beattie, A.D. Moore, M.R., Goldberg, A.

8th International Congress of Haematology 1970, abstracts p37.