STUDIES OF CONTROL OF THE LEVEL OF δ-AMINOLEVULINIC ACID SYNTHETASE IN MAMMALIAN LIVER

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of
Doctor of Philosophy

by

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SUMMARY

The work in this thesis was carried out with the initial aim of establishing an *in vitro* system derived from mammalian liver, in which control of the level of δ-aminolevulinic acid (ALA) synthetase could be investigated.

Preliminary studies suggested that isolated guinea pig liver cells prepared using collagenase might provide a useful model system, since addition of AIA plus hydrocortisone to cell suspensions caused a 4-fold induction of ALA synthetase over two hours. However, further studies with this system were deferred because of low yields obtained in preparation of isolated cells.

Attention was turned to ALA synthetase regulation in isolated intact liver: a rat liver perfusion system was set up and the effects of a number of agents on the levels of ALA synthetase over a three-hour perfusion investigated.

(1) Perfusion without added inducers (control perfusion) caused a three-fold induction of total ALA synthetase activity. Control perfusions of livers, preinduced *in vivo* with AIA, resulted in a further rapid increase in mitochondrial ALA synthetase activity to about double the preinduced level. Possible reasons for these increases are discussed.

(2) Perfusion (of livers from starved but otherwise untreated rats) with AIA caused a small increase in total

activity over that in controls, but overall (3-4 fold) induction in perfused liver was much less than observed over a comparable period *in vivo*. Evidence was obtained suggesting that AIA favoured redistribution of ALA synthetase activity from cytosol to mitochondria.

(3) Perfusion with lead acetate resulted in a small but significant increase in total ALA synthetase over that in controls (total 4-fold induction). The increase was prevented by cycloheximide or actinomycin D. Administration of lead acetate *in vivo* had little effect on ALA synthetase levels.

(4) Administration of progesterone *in vivo* or perfusion with progesterone resulted in a 4-5 fold induction of ALA synthetase over 3 hours. In perfused liver the increase was prevented by cycloheximide or actinomycin D. Perfusion with pregnanolone also caused 6-fold induction, but in perfusions with etiocholanolone changes in ALA synthetase were the same as in controls.

(5) Perfusion with the dibutyryl derivative of cyclic AMP resulted in a 5-fold induction which was prevented by cycloheximide and partly blocked by actinomycin D. Some evidence was obtained that glucose did not act direclty on liver to prevent induction of ALA synthetase and also that insulin partly prevented induction. It was suggested that repression by dietary glucose of drug-stimulated induction of ALA synthetase observed *in vivo* might involve an indirect

effect of glucose, possibly mediated by changes in hepatic cyclic AMP levels.

(6) Addition of hemin to the perfusate prevented induction by progesterone suggesting that heme acts direclty on the liver to repress ALA synthetase. Tentative evidence was obtained suggesting that heme reversed or blocked the effect of AIA on intracellular distribution of ALA synthetase.

STATEMENT

This thesis contains no material previously submitted for any other degree or diploma in this or any other University. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except where due reference is made in the text.

A.M. EDWARDS

ACKNOWLEDGEMENTS

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ABBREVIATIONS

AIA	allylisopropylacetamide
AIP	acute intermittent porphyria
ALA	δ-aminolevulinic acid
ALA-S	δ-aminolevulinic acid synthetase
ALA pyrrole	2-methyl-3-acetyl-4-propionic acid pyrrole
CoA	coenzyme A
COPRO	coproporphyrin
COPROGEN	coproporphyrinogen
cyclic AMP	adenosine-3',5'-monophosphate
dibutyryl cyclic AMP	$N^6,O^{2'}$-dibutyryl adenosine-3',5'-monophosphate
DDC	3,5-dicarbethoxydihydrocollidine
hemin	ferric protoporphyrin IX
MW	molecular weight
PCMB	*p*-chloromercuribenzoate
PBG	porphobilinogen
PROTO	protoporphyrin IX
Rh. spheroides	*Rhodopseudomonas spheroides*
SKF 525A	diethylaminoethyl-diphenyl-propylacetate
URO	uroporphyrin
UROGEN	uroporphyrinogen

DEFINITIONS

HEME has been used as a general term for iron protoporphyrin IX compounds;

INDUCTION has been used to mean an increase in amount of active enzyme dependent on continuing protein synthesis but with no further significance with respect to the mechanism involved.

CHAPTER I

INTRODUCTION

CHAPTER 1

INTRODUCTION

In 1788, King George III suffered an acute attack of an hereditary complaint involving abdominal pain, constipation, darkening of the urine, weakness of the limbs, a fast pulse and mental derangement. The complaint, which earned for George a reputation as the 'mad king', has since been identified [1] as acute intermittent porphyria, one of a number of inherited diseases involving disturbed porphyrin metabolism. In these diseases (the porphyrias) and in similar conditions produced experimentally by treatment of animals with various drugs, a common biochemical defect is thought to be an elevated level of the first and rate-limiting enzyme in the pathway for porphyrin and heme biosynthesis - δ-aminolevulinic acid (ALA) synthetase [2]. The work described in the following pages is concerned with the factors which determine the level of this enzyme in higher cells, particularly in mammalian liver.

SECTION 1. GENERAL SCOPE AND AIMS OF STUDY

The primary aim of work in this thesis was to find an
in vitro system, derived from mammalian liver in which the
level of ALA synthetase could be altered at will by a variety
of experimental stimuli under well controlled conditions and
in which changes in ALA synthetase activity could be measured
directly and with reasonable accuracy. The secondary aim was to
identify the principal variables operating in the regulation of
ALA synthetase in the chosen system, in particular, in relation to
previous findings with mammals *in vivo* and with cultured chick
embryo liver cells. The long term aim of this work was to use
the *in vitro* system to elucidate at a molecular level, the mech-
anisms of control of ALA synthetase in mammalian liver. It is
hoped that an understanding of these mechanisms might lead to
improved methods of treatment for the human hepatic porphyrias as
well as contributing to the understanding of specific gene regula-
tion in higher cells generally.

In Chapter I, existing information on hepatic ALA syn-
thetase will be outlined in the light of these aims. Many aspects
of heme synthesis and the inherited and experimental porphyrias
are covered in recent reviews [2-7], at least two of which [6,7]
devote considerable attention to the role and control of ALA
synthetase in liver, covering findings up to 1967 or 1968. The
introductory material below will not represent a completely
comphrehensive or balanced review but will emphasize recent
developments as well as some aspects not covered elsewhere. Never-
theless, because an attempt has been made to include relevant
aspects from a wide range of studies, the introduction is rather
long. Sections 2B and 3 are less immediately relevant to the
subject of this thesis and may be omitted in reading Chapter I.
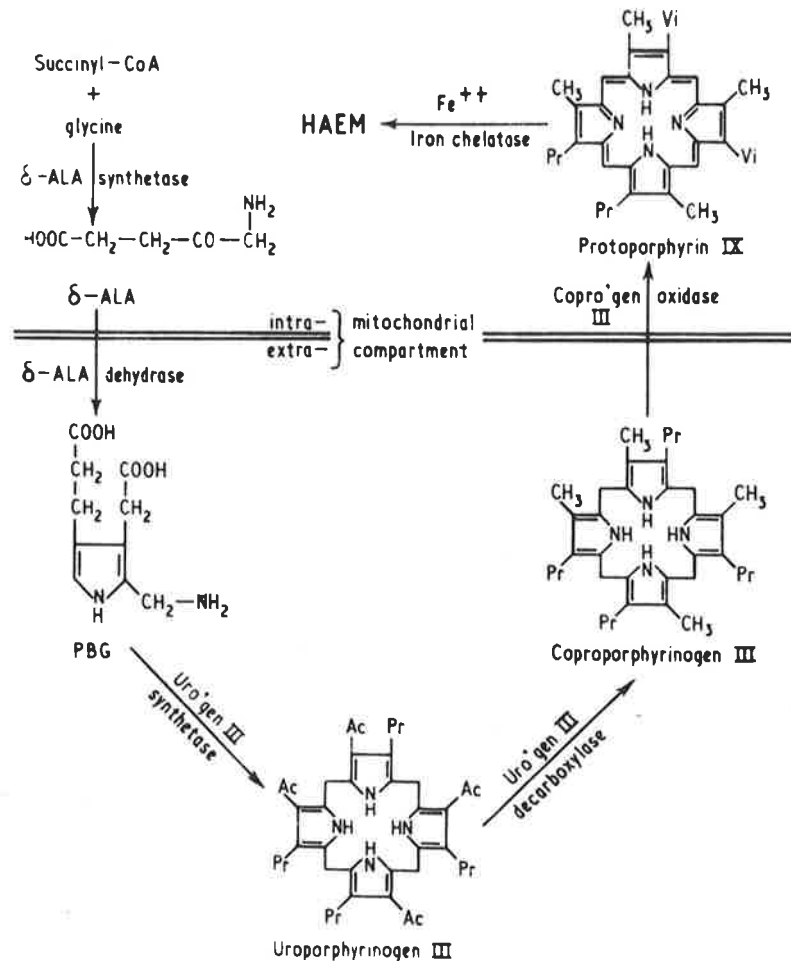
FIG. 1-1. The main steps in the biosynthesis of porphyrins and heme.
Ac: -CH$_2$.COOH; Pr: -CH$_2$.CH$_2$.COOH;
Vi: -CH:CH$_2$.
"Urogen III synthetase" consists of
two different enzymes, a deaminase
and an isomerase. Reproduced from ref.6.

SECTION 2.   THE ROLE OF ALA SYNTHETASE IN LIVER CELL METABOLISM

A.   SIGNIFICANCE OF ALA SYNTHETASE IN CONTROL OF HEME BIOSYNTHESIS

Figure 1-1 shows the heme biosynthetic pathway (for detailed discussion, see Ref.3). The first step, catalyzed by ALA synthetase, commits two metabolites involved in a variety of metabolic processes, to a pathway devoted essentially to formation of heme. Thus ALA synthetase is a logical point of control: that it is indeed the rate limiting step in most of the systems for tetrapyrrole synthesis studied (photosynthetic bacteria [4], yeast [9], erythroid system and livers of birds and mammals [3], rodent Harderian gland [8] and probably in plants [10]) is now well accepted. Even in *spirillum itersonii* [11] and *propionibacteria* [12] where ALA dehydratase is rate limiting, ALA synthetase appears to have an important regulatory role. In bacteria and fungi, ALA dehydratase may also have some significance as a point of control [4,11,12,13,14]: the *Rh. spheroides* enzyme is apparently allosteric and strongly inhibited by heme and protoporphyrin [13]; the *Neurospora* enzyme is repressed by protoporphyrin [14]. In higher cells, ALA dehydratase is inhibited moderately by heme and proto- porphyrin [15,16] but apparently has no regulatory significance except possibly in pathological conditions such as lead poisoning [17]. Thus, in general, the rate of heme and porphyrin synthesis is determined by the availability of ALA. Theoretically, a variable supply of ALA for heme biosynthesis could be achieved by changes in the availability of substrates,

particularly succinyl CoA, or the enzyme cofactor, pyridoxal phosphate, or by changes in the alternative utilization of ALA. The existing evidence [6,7] suggests that these factors are not important. The possibility that movement of intermediates, substrates or cofactors across mitochondrial or other membranes could be relevant to control of the pathway is unexplored. Thus the present evidence indicates that the rate of formation of heme in most systems and certainly in mammalian liver is directly dependent on the level of activity of ALA synthetase.

B. BIOCHEMISTRY OF HEME UTILIZATION AND DEGRADATION

Control of ALA synthetase appears to be primarily devoted to regulating the availability of heme to the cell. At the same time, the level of heme affects the level of activity of ALA synthetase both by feedback inhibition [18,19] and repression [6,7] as discussed in detail in following sections. The biochemistry of heme is thus of central importance in considering the control of ALA synthetase.

Fig.1-2 summarizes current knowledge of synthesis, utilization and degradation of heme in liver. Apart from the interrelation between heme levels and ALA synthetase in determining the rate of heme synthesis, several other points are worthy of note:

(I) Utilization of Heme.

Experiments measuring distribution of newly synthesized
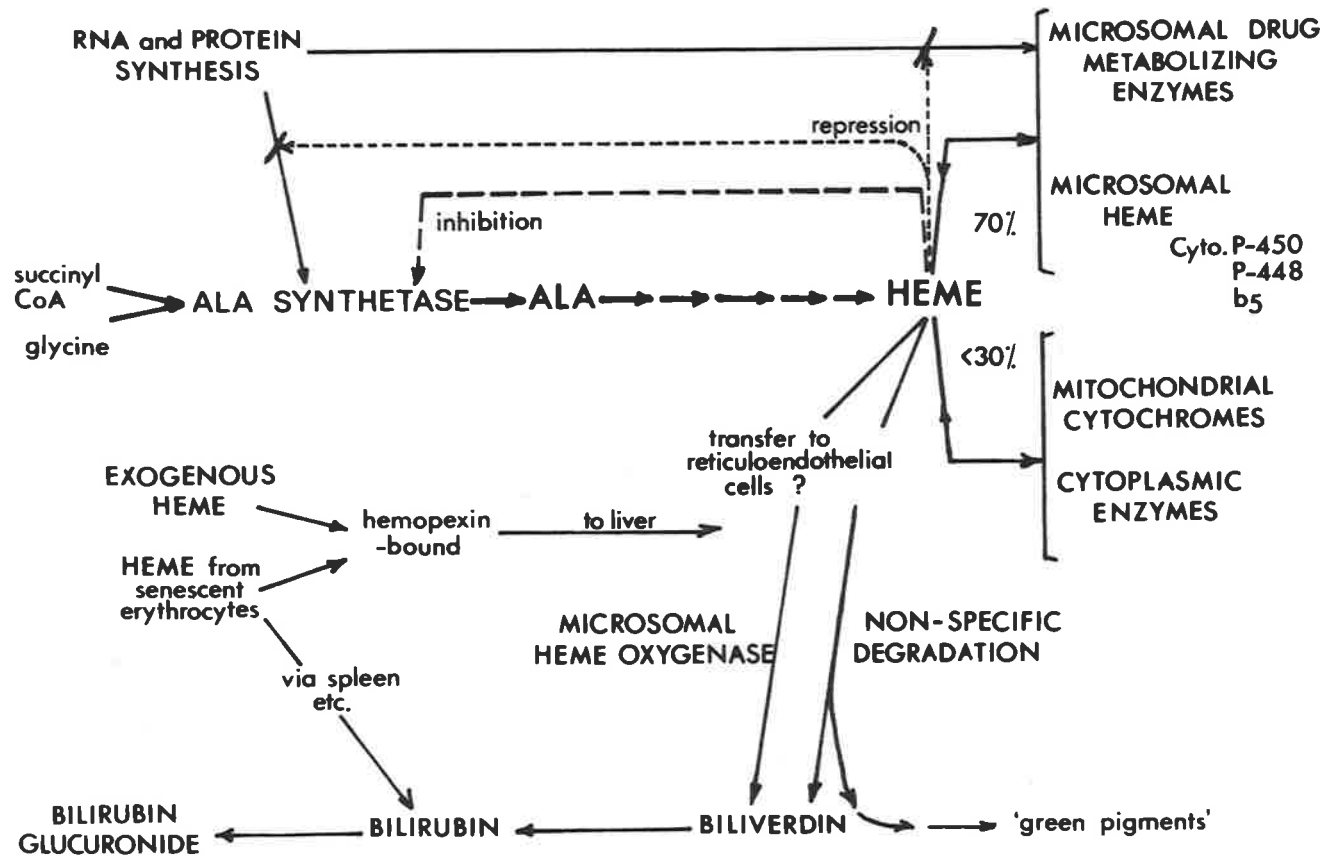
FIG. 1-2. Outline of synthesis, utilization and degradation of heme. See text for full discussion and references.

heme [20] suggest that most heme synthesized in the liver becomes bound in the microsomal fraction, mainly in hemoprotein components of the drug metabolizing system - the two forms of cytochrome P-450 and cytochrome b_5. Consideration of the amounts and rate of turnover of hepatic hemoproteins [21] also implicates P-450 as the major destination of heme, since in normal liver this accounts for roughly 50% of hepatic heme (70-80% in phenobarbital treated liver). It turns over relatively rapidly with an apparent average half life of about 12 hours for the two forms [21]. Of the other haemoproteins, tryptophan pyrrolase turns over rapidly but is present in only minute amounts [21]. Such observations suggest that ALA synthetase regulation might be related at least in part to the cell's cytochrome P-450 requirements. This possibility is discussed further in Section 4B, II.

There are other possibilities for the etiology of ALA synthetase control. For instance, since all the hemoproteins are concenred with redox reactions, control of ALA synthetase might be related in some way to the redox potential of the cell and/or to its energy requirements as appears to be the case for *Rh. spheroides* [4,22,23].

(II) Hemoproteins: Relation between Heme and Apoprotein Synthesis.

While the rate of heme synthesis must be an important factor in determining the activities of various hemoproteins (e.g., ref. 24), the relationship between the level of heme

(or its intracellular distribution) and synthesis of the various apoproteins to which it binds is not entirely clear. Heme stimulates synthesis of cytochrome c in rat liver slices [25], and *in vivo* an increased rate of heme synthesis stimulates synthesis of mitochondrial cytochromes [26]. Some drugs which increase heme synthesis cause an elevation of tryptophan pyrrolase levels [27,28] but depression of catalase levels [29,30]. In the case of cytochrome P-450 the present evidence is difficult to interpret because detection of P-450 depends upon the presence of the heme prosthetic group. There is indirect evidence for the existence of free P-450-apoprotein [31] and for independent turnover of heme and protein moieties of the cytochrome [32]. Nevertheless, P-450 activity is related to the level of heme: increase in P-450 activity requires continuing or augmented heme synthesis since induction of P-450 by drugs is prevented if heme synthesis is prevented by aminotriazole [33] or cobalt ions [34]. Conversely, induction by the same drugs of P-450 and other components of the microsomal drug metabolizing system is repressed by exogenous heme [35].

In short, no general correlation between the rates of heme synthesis and synthesis of various apoproteins has so far emerged.

(III) Degradation of Heme.

The rate of heme degradation is clearly relevant to control of ALA synthetase in view of the effects of heme as

feedback inhibitor and repressor of ALA synthetase. Two path-
ways for heme degradation have recently been described:

In the first system, heme undergoes specific enzymic
conversion to bilirubin which is subsequently glucuronidated
in liver parenchymal cells and excreted in the bile [36].
Conversion to bilirubin involves microsomal heme oxygenase
(of which a form of P-450, distinct from that involved in,
drug and steroid metabolism, is a component) which oxidizes
the α-methene bridge of heme to yield biliverdin [37,38,39].
Biliverdin reductase then reduces biliverdin to bilirubin
[40,41]. This system, which is most active in spleen [38]
may be restricted in liver to the reticuloendothelial cells.
The activity of heme oxygenase is increased by splenectomy,
hemolytic anemia and by methemalbumin administration [39].
Free heme, hemoproteins with loosely bound heme, and hemopexin-
bound heme are substrates for the heme oxygenase [38]. These
observations suggest that a major role of this system may be
degradation of heme synthesized outside the liver. However,
heme synthesized in hepatic parenchymal cells is at least
partly degraded by this system [21,39,42] yielding the
'early labelled' fraction of bile pigment.

Studies with liver homogenates have revealed a
second pathway for heme degradation in which added heme is
converted to a mixture of biliverdin isomers by an oxidation
reaction in which ascorbate is also involved [43]. The reaction
is thought to be non-enzymic although the heme binding site
of various hemoproteins may provide an 'active site' for the

reaction and impart some specificity. The biliverdin formed
is only partially converted to bilirubin because biliverdin
reductase is specific for the α-isomer [43]. Meyer and Marver
[44] have reported that stimulation of lipid peroxidation
(by ADP and ferric ions) results in degradation of heme,
particularly of cytochrome P-450-heme, by a mechanism
apparently similar to that above. Stimulation of lipid
peroxidation is thought to result in disruption of the
structure of microsomal membranes [45]; this might cause
release of P-450 or its heme prosthetic group, and subsequent
non-specific degradation. The fact that degradation of
microsomal heme is biphasic (in rats) may result from the
different susceptibilities of the two forms of cytochrome
P-450 to degradation [46-49]. As discussed below in Section
4B, II, certain drugs apparently increase the rate of
degradation, by the non-specific pathway, of microsomal heme
mainly derived from the major form of P-450 [50-53].

(IV)   Role of Hemopexin in Regulation of Heme Levels in Liver.

The relevance if any, of the serum heme-binding glyco-
protein, hemopexin, to regulation of heme levels in hepatic
parenchymal cells is not clear. Injected heme is rapidly
cleared from plasma; a large excess is initially bound
loosely to albumin and later transferred to hemopexin which
has a high affinity for heme. Hemopexin transfers the heme
primarily to the liver where it is converted to bile pigments
[54-56]. The extent to which heme transported to the liver

by hemopexin contributes to repression of ALA synthetase in hepatic parenchymal cells and whether hemopexin-bound heme is metabolized in both hepatic parenchymal and reticuloendothelial cells (or only in the latter) are unknown at present.

Hemopexin is synthesized in liver; its level in serum is increased by administration of hemin, the drugs AIA, 3-MC[*] and DDC or lead acetate [57].

* 3-MC = 3-methylcholanthrene.

SECTION 3.   LOCALIZATION AND PROPERTIES OF ALA SYNTHETASE
FROM MAMMALIAN LIVER

The enzymology of ALA synthetase will be discussed in detail in a forthcoming thesis from this laboratory [58]. However, several points relevant to the current investigation are mentioned below.

A.   LOCALIZATION OF THE ENZYME

The major fraction of ALA synthetase activity in porphyric liver is localized in the mitochondria: this is consistent with the requirement of the enzyme for succinyl CoA [59,60]. In studies employing a variety of methods for disrupting mitochondria, ALA synthetase is partitioned in parallel with malate dehydrogenase and glutamic dehydrogenase which are distributed between inner membrane and matrix, but generally considered to be matrix enzymes [61,62,63]. Recent work has shown that the distribution of malate dehydrogenase between inner membrane and matrix is altered by treatment with succinate or various cations [64] so that assignment of a single location to ALA synthetase may be somewhat arbitrary.

If ALA synthetase is assayed in the presence of a succinyl CoA-generating system, activity amounting to 30-40% of the total is detected in post-mitochondrial and/or post-microsomal fractions from porphyric liver [18,58,65].

B. POSSIBLE EXISTENCE OF MULTIPLE FORMS OF ALA SYNTHETASE

There is evidence for two types of ALA synthetase (fractions I and II) in *Rh. spheroides* [66,67]; these have similar MW's and kinetic properties but can be separated on DEAE Sephadex [66] and respond differently to changes in environmental conditions. Induction of fraction I is dependent upon a decrease in oxygen tension, while induction of fraction II requires decreased oxygen tension and illumination. Both types are repressed at high oxygen tension [67].

Attempts to purify mammalian liver ALA synthetase have provided clear evidence that the enzyme may exist in various states of aggregation. Thus the rat liver cytoplasmic enzyme, purified 200-fold by Scholnick *et al*. [68] had a MW of 150,000 but tended, in the absence of NaCl, to aggregate with concomitant loss of activity; Hayashi *et al*. found the cytoplasmic enzyme was exluded from Sephadex G-200 and estimated its MW to be 600,000 [69]. Similarly for the mitochondrial enzyme, MW estimates range from 115,000 (ref. 69) up to forms excluded from Sephadex G-200 (ref. 19). The finding in this laboratory that an aggregated, high MW form of the guinea pig liver mitochondrial enzyme (similar to that found by Kaplan in rats [19]) can be dissociated under appropriate conditions, to a soluble form of MW 78,000 [58] could indicate that the various species of different MW are in fact the same enzyme in various states of aggregation with itself or other cellular components. It is not clear whether these forms are artifacts of preparation or have physiological significance.

Considerable interest centres on whether the cytoplasmic and mitochondrial forms of ALA synthetase are isoenzymes. Present evidence suggests that activities in the two cell fractions respond similarly to induction by drugs [68,69,70]. Changes observed are consistent with a model in which a single form of ALA synthetase is synthesized in cytoplasmic polysomes and transferred via a cytoplasmic pool to the mitochondria. The observation that chloramphenicol partly blocks appearance of enzyme in the mitochondrial fraction could indicate that some mitochondrial protein synthesis is required to modify the enzyme or to fit it into the mitochondrial structure [70]. The differences in physical properties between the two forms reported by Hayashi *et al.* [68,69] could be related to differential aggregation while the differences in apparent enzyme half-lives following cycloheximide administration [69] could reflect continuing transfer of recently synthesized enzyme from cytoplasm to mitochondria. The kinetic properties of soluble and mitochondrial enzymes are very similar [18,19,69]. A final decision on whether there are multiple forms of ALA synthetase must await extensive purification or immunological studies.

C. POSSIBLE ROLE OF ENZYME ACTIVATION AND INHIBITION IN CONTROL OF ALA SYNTHETASE

Tuboi and Hayasaka [71] have recently shown that in
Rh. spheroides extracts, the Fraction I ALA synthetase
exists in an active (MW 80,000) and an inactive (MW 100,000)
form. In purified preparations, Fraction I was activated
by incubation with a protein fraction derived from rat liver
mitochondria and it was suggested that activation involved
enzyme modification (possibly cleavage of a peptide) of
Fraction I. In crude extracts, Fraction I could be activated
by a small M.W., heat-stable activator isolated from
Rh. spheroides grown semi-anaerobically [72]. A similar
activation (in crude extracts at 4°C) had previously been
reported by Marriott et al. [23] who also isolated from
oxygenated cells, an inhibitor of the activation. These
observations suggest that activation and inactivation of
ALA synthetase play a role in regulation of bacteriochlorophyll
synthesis by oxygen.

Activation of ALA synthetase has also been reported
in soy bean callus where activity increases 2-3 fold when
newly disrupted tissue is allowed to stand [10].

Several groups have reported the presence of a heat-
stable inhibitor of ALA synthetase in extracts of mammalian
liver [13,73,74,75]. The inhibitor is found in mitochondrial
debris from both normal and DDC treated guinea pig mitochondria
which have been disrupted by extended sonication [73,74].
Neither the nature nor the physiological significance of this

inhibitor are known.

Drugs which *in vivo* cause an increase in enzyme activity have no direct effect on activity in cell extracts and their effects *in vivo* are prevented by inhibitors of protein synthesis [7]. Such studies, however, cannot exclude the possibility that inducers stimulate synthesis of a 'modifier' which increases the activity of existing enzyme as proposed for induction of alkaline phosphatase by hydrocortisone [76].

D. INHIBITION BY HEME

In *Rh. spheroides*, feedback inhibition by heme is a major element of control on the activity of ALA synthetase [77,78]. In crude enzyme preparations from mammalian systems, heme has inhibitory effects on the red cell [79] and liver [18,73,74, 80] enzymes only at concentrations greater than 10^{-4}M, which probably greatly exceed physiological levels. However, in partially purified preparations from red cells [81] and liver [18,19] heme exerts moderate inhibition and with more highly purified preparations heme has marked inhibitory effects: 10^{-5}M heme inhibits the rabbit reticulocyte enzyme 37% (ref. 82) and the guinea pig or rat liver mitochondrial enzyme 30% (ref. 58); 2×10^{-5}M heme inhibits the rat liver cytoplasmic enzyme [68] or mitochondrial enzyme [58] by 50%. Scholnick *et al.* [18] have shown that the inhibition by heme of the partially purified cytoplasmic enzyme is blocked by albumin or by normal rat liver cytosol suggesting

that in crude preparations, added heme is bound by contaminating proteins. However, *in vivo*, heme is formed in the mitochondrion [5] and might exert inhibitory effects on mitochondrial ALA synthetase similar to those in purified systems.

SECTION 4. CONTROL OF THE LEVEL OF HEPATIC
ALA SYNTHETASE

A. STUDIES IN CHICK EMBRYO LIVER TISSUE CULTURE

In 1963, Granick and Urata showed that the normally low
level of ALA synthetase in guinea pig liver could be
dramatically increased by *in vivo* administration of the
collidine derivative DDC [59]. In 1966, Granick found that
AIA similarly induced ALA synthetase in chick embryo liver
[80]. At the same time he described a primary tissue culture
system derived from embryonic chick liver in which AIA, DDC
and a variety of other drugs, steroids and foreign chemicals
caused the excessive formation of porphyrins. The extent of
porphyrin accumulation, assessed by fluorescence under UV
light, was taken as a semi-quantitative measure of the activity
of the rate limiting enzyme in the porphyrin biosynthetic
pathway, ALA synthetase [80]. Studies with this system have
been frequently reviewed(e.g., 6,7,83) and only the principal
findings on control of the level of ALA synthetase in tissue
culture will be given below.

(i) The nature of inducing compounds. The wide range of
compounds which cause porphyrin fluorescence in tissue culture
has been listed by Granick [80] and de Matteis [6]. Structures
of some of these are shown in Fig. 1-3. Although it is
difficult to define common structure-activity relationships
among such a diverse range of chemicals, within a group of

AIA

CH₂CH=CH₂
CH
CH
CH₃
CH₃
C
NH₂   O

DDC

H   CH₃
H₅C₂OOC   COOC₂H₅
CH₃   N   CH₃
H

SULPHONAL

CH₃   SO₂C₂H₅
C
CH₃   SO₂C₂H₅
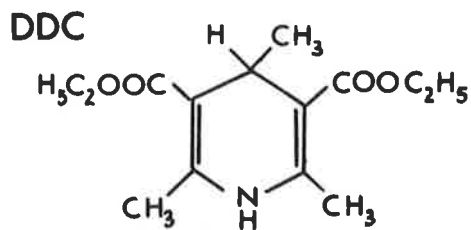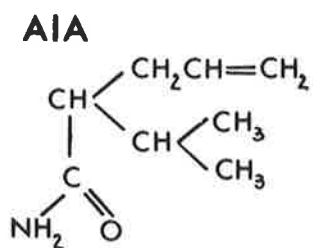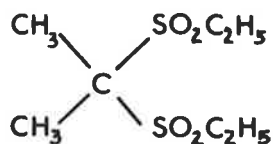
GRISEOFULVIN

PHENOBARBITAL

O
HN   C₂H₅
C₆H₅
O   N   O
H

LINDANE

Cl
Cl   Cl
Cl   Cl
Cl
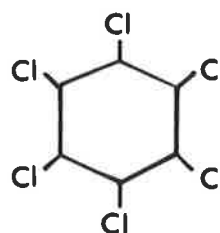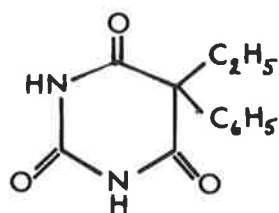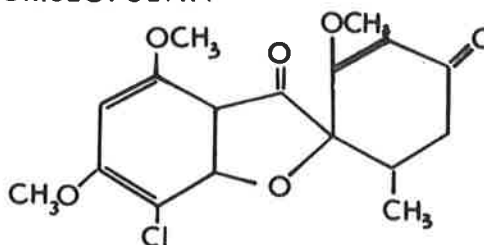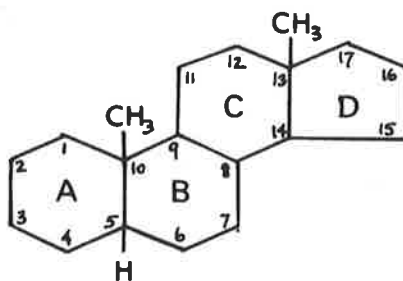
5β-H STEROID NUCLEUS
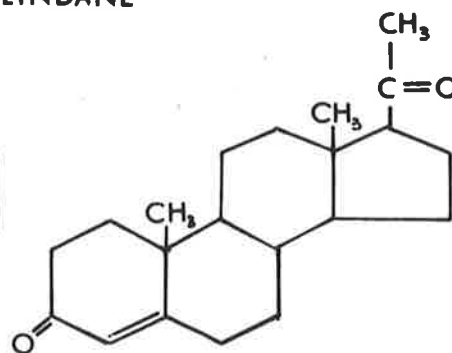
PROGESTERONE

FIG. 1-3. Structures of some compounds which induce ALA synthetase in cultured chick embryo liver cells.

related compounds definite stuctural requirements may be identified: the extensive studies of Marks and co-workers [84] suggest that a common structural feature in inducers of the barbiturate and collidine groups is an ester or amide group sterically hindered from hydrolysis. Granick [85] has suggested that inducing chemicals may all be characterized by a hydrophobic non-planar structure containing a carbonyl group. The most potent inducers of ALA synthetase in tissue culture have proved to be steroids some of which cause fluorescence in concentrations as low as 10^{-8}M. Structural requirements for potent inducing activity in this group are:

(a) a hydrocarbon nucleus of the 5β-androstane or 5β-pregnane type, i.e., with the A:B ring junction angular rather than planar; and (b) alcohol or ketone substituents at C-3, C-17, C-20, and possibly C-11 positions. Ring A-unsaturated steroids such as the hormonal precursors (i.e., sex steroids) of the abovementioned 5β-H metabolites are only weakly active; C-21 hydroxylated steroids such as cortisol and aldosterone are inactive as are steroid glucuronides [86].

(ii) That porphyrin fluorescence induced by drugs or steroids indeed reflects increased levels of ALA synthetase was shown by direct measurement of the enzyme activity in experiments with larger scale cultures [87,88]. Drugs and steroids also induce the enzyme *in vivo* in chick embryo liver [80,89,90]. Following *in vivo* administration of an inducing steroid, enzyme activity, after a one-hour lag, increases to a maximum

at 8 hours then declines to normal by 24 hours. AIA has similar, but more prolonged effects [90].

(iii) Induction of excess porphyrin formation occurs in the liver parenchymal cells [80].

(iv) Induction by drugs [80,87] and steroids [87,91] requres *de novo* protein synthesis and continuing DNA-dependent RNA synthesis, since induction is prevented by inhibitors of protein synthesis such as puromycin and acetoxycycloheximide, and inhibitors of RNA synthesis such as actinomycin D. Induction *in vivo* [90] and in tissue culture [92] is partly prevented by α-amanitin which inhibits the $Mn^{++}/(NH_4)_2SO_4$-stimulated RNA polymerase (or polymerases [93]) in liver nuclei. Steroid inducers have been shown to increase the activity of α-amanitin-sensitive RNA polymerase in isolated nuclei [90,92].

Some groups have suggested that RNA extracted from AIA-induced liver but not that from normal rat liver, will induce ALA synthetase in the chick liver tissue culture system [94,95].

(v) The inducing action of drugs and steroids is considered to be relatively specific since there is no detectable increase in the activity of a number of other enzymes in the heme biosynthetic pathway [59] nor in overall incorporation of labelled precursors into protein or RNA [80,87].

(vi) Inducers do not alter the rate of decay of enzyme activity following addition of cycloheximide [80,87], suggesting

together with items (iv) and (v) above that induction is due
to a relatively specific increase in the rate of synthesis
of ALA synthetase possibly resulting, at least in the case of
induction by steroids, from increased synthesis of some species
of RNA.

(vii) Combinations of sub-optimal concentrations of
different inducers give additive inductions (assessed by
fluorescence) but combinations of optimal concentrations do
not, suggesting a common site of action [91]. However, recently
Sassa and Granick provided evidence that DDC and steroid
inducers act at a transcriptional level while AIA acts at
some post-transcriptional site [87].

(viii) Induction by several drugs and by steroids is blocked
if heme is added to cultures at the same time as inducer [80,
87,88,91,96]. Some other metalloporphyrins also prevent
induction [80,91,97]. The effect of heme on induction by
drugs is not competitive, suggesting that heme may act at a
locus distinct from the site(s) of action of drugs [96].
Findings on the effects of adding heme to preinduced cultures
are difficult to reconcile (but see Section 4B,V): Sassa and
Granick [87] found that in cultures preinduced with AIA for
14 hours, actinomycin D plus heme caused a more rapid decline
in ALA synthetase activity than did actinomycin D alone.
This was interpreted as evidence for a post-transcriptional
site of action for heme. However, Schneck et al. [96] found
that addition of heme alone to cultures which had been
preinduced for 9 hours with AIA had no effect on ALA synthetase

over the next 4 hours even though actinomycin D (alone) caused a significant decrease over the same period.

(ix) UDP glucuronic acid inhibits induction of fluorescence by steroids, presumably by causing increased conversion of steroids to non-inducing glucuronides [91,97]. This is the only observation in the chick system which might explain the glucose repression of ALA synthetase observed in mammalian systems [98]. In chick tissue culture, glucose, glucose phosphates, glucuronic acid, glucagon and insulin all failed to affect induction [80,97]. However, there is one report that glucagon causes a weak and rather variable induction *in vivo* in chick embryo liver [99].

(x) There is a factor in human plasma which induces ALA synthetase in chick tissue culture [100,101,102]. In normal subjects the low MW, ethanol-soluble factor is masked by a protein inhibitor which can be destroyed by heating [102]. Unmasked inducer is found in plasma from patients suffering an attack of acute intermittent porphyria [100]. On the basis of their findings in the chick tissue culture system, Granick and his coworkers [7,80] proposed a model for regulation of the level of hepatic ALA synthetase analogous to the general models proposed by Jacob and Monod [103] for control of enzyme synthesis in bacteria. Granick's model is shown in Fig. 1-4. He proposed that control of the synthesis of ALA synthetase resides in a repressor-operator mechanism which regulates transcription from the structural gene (G_1)
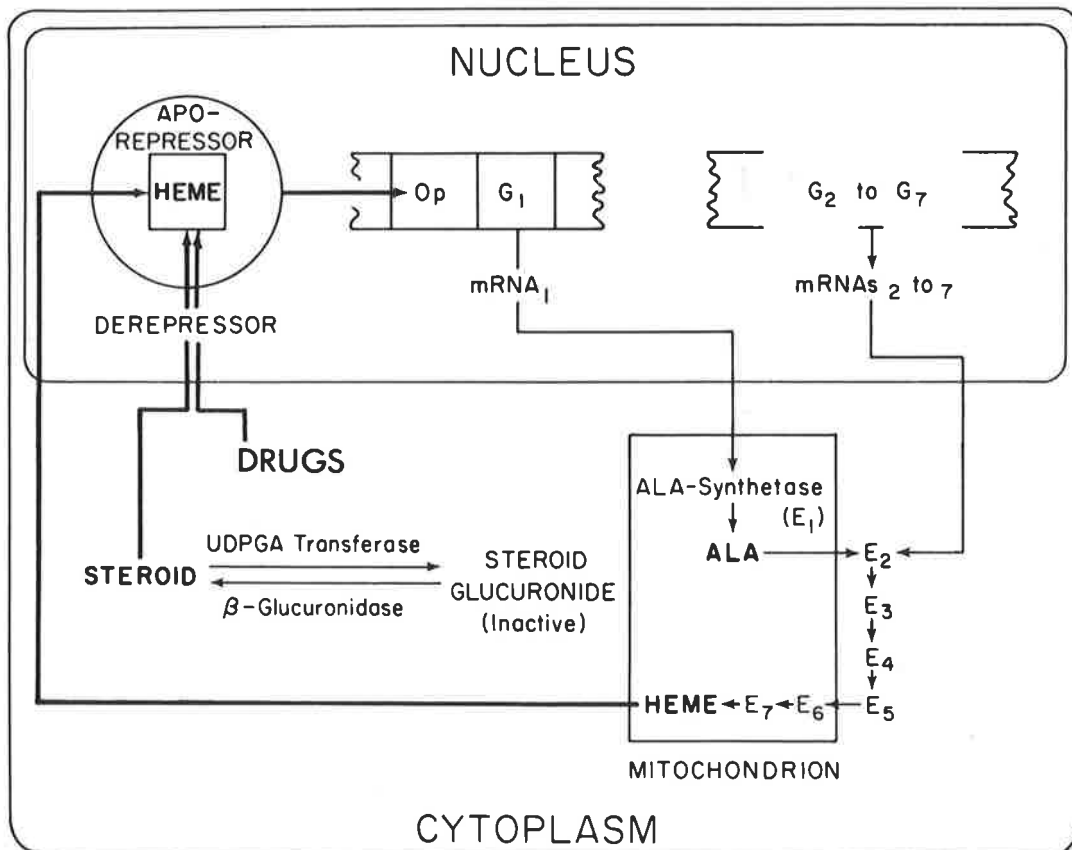
FIG. 1-4. Scheme showing mechanism for
regulation of ALA synthetase proposed by
Granick and coworkers. Reproduced with slight
modification from ref.97.

into mRNA coding for ALA synthetase (E_1). All other enzymes (E_2-E_7) in the heme pathway are present normally, in non-limiting amounts. The active repressor consists of heme bound to an aporepressor protein. The binding of heme to the aporepressor is blocked by inducing drugs and steroids with resulting derepression of G_1 and increased synthesis of ALA synthetase.

Granick further proposed that the genetic lesion in the dominant forms of the human hereditary porphyrias (see next section) might be a mutation in the operator gene which renders it poorly responsive to the repressor [80]. The inducing action of steroids, regarded as the physiological inducers of ALA synthetase and provocative agents in hereditary hepatic porphyria in man, would be modulated by any conditions which altered the activity of UDP glucuronic acid transferase [97].

The essential features of this model compared with others discussed below are that (a) all inducers act essentially at a single site (i.e., on the repressor) and directly on the mechanism regulating synthesis of ALA synthetase; and (b) a bacterial-type mechanism for regulation of enzyme synthesis is assumed. Some recent findings such as evidence for different sites of action for steroids and AIA [87], and the reports by Schneck *et al.* [96] that heme exerts non-competitive repression of induction when administered with inducing drugs but has no effect in preinduced cultures, suggests that the basic model may require at least some

modification. Alternatives to Granick's original proposal
for the nature of the hereditary defect in hereditary porphyria
are discussed in the following section.

B. STUDIES ON ALA SYNTHETASE IN MAMMALIAN CELLS

Many of the features of control of ALA synthetase in
chick embryo liver are also found in mammalian liver. It is
the aim of this section to discuss the extent to which
findings in the tissue culture system apply to mammalian
liver, to outline other findings and proposals for control of
the mammalian liver enzyme and to relate the findings with
experimental systems to the human inherited diseases, the
porphyrias.

(I) ALA Synthetase in the Human Hereditary Porphyrias.

The term 'porphyria' embraces a group of disorders
characterized by greatly increased formation, accumulation
and excretion of porphyrins and/or their precursors. This
section is concerned with forms of porphyria resulting from
inherited metabolic defect(s). The enormous volume of
literature on clinical aspects of the porphyrias has been
comprehensively reviewed [2,5] and will be mentioned only
briefly here. Some properties of the various forms of
hereditary porphyria are summarized in Table 1-1.

A greatly elevated level of ALA synthetase has been found
in all hepatic porphyrias and has been postulated for the
erythropoietic forms. Proposals for the locus of genetic

TABLE 1-1: CLASSIFICATION OF HUMAN HEREDITARY PORPHYRIAS

TYPE OF PORPHYRIA	INHERI- TANCE	ORGAN INVOLVED	TYPE OF SYMPTOMS	PORPHYRINS etc. ACCUM- ULATED or EXCRETED	PPTED BY DRUGS ? (6)	ALA-S INCREASED ?	PROPOSED LESION(S) **
CONGENITAL ERYTHROPOIETIC PORPHYRIA	Recessive No latent cases	Erythro- poietic	Cutaneous	URO,COPRO. Type I>III	No	Postulated	PBG isomerase decreased in relation to PBG deaminase [3,104] with high ALA-s.
ERYTHROPOIETIC PROTOPORPHYRIA	Dominant (Latent cases)	Erythro. & Hepatic (?) [5,105-8]	Mild Photo- sensitivity	PROTO, COPRO III	No	Yes [107]	Fe chelatase deficient, ALA-s increased [107,108]
COPROPORPHYRIA *(109,110)	Dominant (Latent cases)	Hepatic*	Neurological (Rarely Photo- sensitivity)	COPRO III. ALA, PBG in attacks	Barbit- urates etc.	Yes, in attack, not in remission [110]	Increase ALA±§* Possible defect at COPROGEN oxidase [110]
ACUTE INTERMITTENT PORPHYRIA	Dominant (Latent cases)	Hepatic	Neurological	ALA,PBG. Usually greater in attacks	Barbit- urates etc.	Yes in attack & remission [88,111-3]	Increased ALA-s ***[80]. Low PBG deaminase [88,114]. Ab- normal steroid metabolism [83]

TABLE 1-1   (Continued)

TYPE OF PORPHYRIA	INHERI- TANCE	ORGAN INVOLVED	TYPE OF SYMPTOMS	PORPHYRINS etc. ACCUM- ULATED or EXCRETED	PPTED BY DRUGS ?	ALA-S INCREASED ?	PROPOSED ** LESION(S)
PORPHYRIA VARIEGATA	Dominant (Latent cases)	Hepatic	Cutaneous (Neurological in attacks)	COPRO III, PROTO. ALA, PBG in attacks	Barbit- urates etc.	Yes in attack & remission [88,113,115]	Increased ALA-s with possible defect at COPROGEN oxidase and/or Fe chelatase [3]
PORPHYRIA CUTANEA TARDA	? Genetic Predis- position	Hepatic	Cutaneous	URO, COPRO Types I, III	Alcohol, Estrogen Fe	Probable [113,116,117 cf. 88]	Defect in microsomal drug metabolizing enzymes [5]

Notes

　　Information in this table is taken from Reviews [2,5] except where other references are quoted.
　　*There is probably also a distinct (very rare) erythropoietic form of Coproporphyria (see Ref.1).
　　**Watson *et al.* [104] have suggested that differences between erythropoietic and hepatic forms
　　might be explained, at least in part, by greater escape of ALA and PBG from liver cells or more
　　rapid conversion PBG → UROGEN in bone marrow.
　　***ALA dehydratase is also elevated in AIP [42,115] and in coproporphyria, during attacks [110].

lesions involved must account for the common feature of increased ALA-synthetase as well as the differences in the porphyrins or precurosrs excreted or accumulated. The nature of the genetic abnormalities in the porphyrias has been the subject of considerable speculation [2,3,5,6,104,110,115,117-9]. In relation to the hepatic porphyrias as a group, there are two general possibilities:

(a) a common mutation leading to increased ALA-synthetase bringing different latent genetic defects to light [3], or

(b) distinct mutations which lead co-incidentally to ALA-synthetase induction [114,115].

With respect to any one form of porphyria, there would seem to be three general possibilities:

(i) a lesion outside the heme pathway leading to ALA-synthetase induction, among other possible effects;

(ii) a lesion in, or closely related to the heme biosynthetic pathway, resulting in a disturbance of normal controls on ALA-synthetase (e.g., loss of repression by heme);

(iii) a lesion in the genetic control mechanism normally regulating synthesis of ALA-synthetase, possibly of the operator constitutive type.

For the case of AIP, the last possibility (iii) was originally favoured by a number of authors [80,104,111] who based their models on findings on control of enzyme synthesis in bacteria. If the proposal that the primary genetic defect lies in the

control of synthesis of ALA synthetase is to be extended to
other forms of porphyria it is necessary to postulate as in
(a) above, other underlying genetic differences to account
for the different patterns of porphyrin excretion [3].
Marver and his co-workers [88,114] have recently provided
evidence that in AIP, hepatic PBG deaminase activity is only
50% of the level found in normal patients and in patients
with other forms of hepatic porphyria. Thus it might be
suggested that a primary lesion in control of ALA synthetase
together with a latent defect in PBG deaminase could account
for the peculiar excretion pattern in AIP. However, these
authors further showed [114] that in families including AIP
patients, unaffected members had normal PBG deaminase
activity: they concluded that a primary (rather than secondary
latent) defect at PBG deaminase resulted in impaired heme
synthesis leading in turn to derepression of ALA synthetase.
Kaufman and Marver [115] suggested that the primary
abnormalities in the hepatic porphyrias were blocks at
different points in the heme biosynthetic pathway which all
caused reduced repression by heme of ALA synthetase particularly
under conditions of increased demand for heme (e.g., during
induction by drugs or steroids of Cytochrome P-450 and other
components of the microsomal drug metabolizing system).

These proposals are consistent with possibilities
(b) and (ii) above. Kappas and his coworkers [100,101,102]
have recently provided some evidence indirectly supporting
possibility (i): Firstly, there is a small MW, ethanol-
soluble factor in human serum which induces ALA synthetase

when added to cultures of chick embryo liver. The induction
is blocked by puromycin, actinomycin D and heme. In the serum
of normals and AIP patients in remission, the factor is masked
by a protein inhibitor which reversibly binds the inducing
factor. The inhibitor also blocks the action of inducing
drugs and steroids [102]. These findings suggest two
possibilities for the nature of the genetic defect: either
altered metabolism leading to overproduction, under certain
circumstances, of the inducing factor (a steroid?); or a
mutation in the structural or control genes for the protein
inhibitor, giving a protein which is either absent or inactive
during attacks. The most recent work [83] from Kappas' labora-
tory would favour the first of these alternatives. AIP
patients (in remission) have deficient activity of hepatic
steroid-5α-reductase, resulting in decreased metabolism of
steroids to 5α derivatives and preferential conversion to
5β-steroids and/or metabolites with the Δ4,5 double bond intact.
The 5β-steroids are potent inducers of ALA synthetase in chick
embryo liver. It remains to be shown whether the 5α-reductase
deficiency is genetically determined or acquired as the
result of some other primary lesion. A similar 5α-reductase
deficiency is found in the acquired hypothyroid condition,
myxedema. The deficiencies in both myxedema and AIP can be
reversed by thyroid hormone administration, so that an
additional possibility for the genetic lesion in AIP could be
altered metabolism of thyroid hormone [83].

The relation between a possible over-production of

5β-steroids so far found in latent cases of AIP and the excess
of plasma 'inducer' over plasma 'inhibitor' during attacks
(but not in remission) remains to be clearly established.

The sequence of events leading from primary defect
to occurrence of neurological symptoms in the hereditary
porphyrias is unknown.

According to a number of authors, the neurological
symptoms can be explained on the basis of neuropathy in the
somatic and autonomic nervous systems, in which motor, rather
than sensory nerve fibres are principally affected. The
primary cause may be either segmental demyelination of
nerves [120,121] or a 'dying back' of nerve fibres [122-4].
Other authors suggest that the evidence for neuropathy in
porphyria is unconvincing and that a direct effect on nervous
transmission must be involved [125]. Recent reports showing
an effect of PBG on neuromuscular end plate potentials [126]
and of ALA *in vitro* on brain ATPase [125] support such a
direct mechanism. However, *in vivo*, neither direct admini-
stration of ALA [127,128] nor induction of ALA synthetase in
experimental porphyria [128,129] produces the symptoms of an
acute attack. Lead poisoning is the only experimentally
induced disorder of porphyrin metabolism in which such symptoms
are found [17]. It may be that a complex interaction involving
ALA such as proposed by Kosower *et al.* [130,131] is not
reproduced in experimental porphyrias. There is a qualitative
correlation between occurrence of neurological symptoms and
elevated levels of ALA in the hereditary porphyrias (see

Table 1-1). Although in AIP in remission, high levels of ALA are not associated with obvious neurological manifestations, more subtle nervous signs may in fact persist [132,133]. In one study, previously unsuspected porphyria accounted for about 1% of mental case admissions [134]. In hereditary forms of porphyria, changes in diet seem to have parallel effects on ALA levels and the severity of an acute attack (e.g., 117].

A mutation in hereditary porphyria, outside the heme pathway, which leads coincidentally to neurological disturbances and induction of ALA synthetase has also been suggested [118, 119,135].

(II)   Induction of ALA Synthetase by Drugs.

Many aspects of drug-induced disturbances in the heme biosynthetic pathway are covered in a review by de Matteis (6) who lists the wide range of drugs and foreign chemicals which cause excessive formation of porphyrins and their precursors in liver. The excess porphyrins are excreted in the urine and faeces. Because this resembles the excretion of porphyrins and precursors in the human inherited diseases, the porphyrias, the condition induced by drugs is termed 'experimental porphyria' or 'chemical porphyria'. As shown originally by Granick and Urata [59] and subsequently by many workers [6], experimental porphyria is associated with an elevated level of hepatic ALA synthetase.

TABLE 1-2:   EFFECTS OF AIA IN MAMMALIAN SYSTEMS

	Ref. No.

I. Induces hepatic ALA synthetase *in vivo*:

AIA-mediated induction prevented by cycloheximide	98
puromycin, actinomycin D, 5-fluorouracil	98,140
prior adrenalectomy	141,142
administration of heme	143
feeding glucose	60,98
partially prevented by chloramphenicol	70

II.	
Causes excretion of ALA, PBG and URO in urine and accumulation of PBG and porphyrins in liver	6
Increases overall hepatic heme synthesis from glycine	20,144
Does not affect heme synthesis from PBG or alternate utilization of ALA	145
Increases level of ALA dehydratase (prevented by glucose feeding or actinomycin D)	98,140
Increases microsomal heme breakdown	50,51,52
Depresses the level of hepatic catalase	29,30

III.	
Increases liver size (not water uptake)	27,146
Causes hypertrophy of smooth endoplasmic reticulum (prevented by puromycin,	27,146
actinomycin D or adrenalectomy)	27
Increases liver protein synthesis	147
Causes initial decrease, later rise in levels of microsomal cytochromes P-450,	
$b5$	52,148
At later times causes rises in:	
N-demethylase,	148
Glucose-6-phosphatase dehydrogenase,	
NADPH-Cytochrome c reductase	140
Nitroreductase, hexobarbital reductase	147
Sulphobromophthalein-conjugating enzyme	27
and UDP-glucose dehydrogenase	149
Induces tyrosine aminotransferase, tryptophan aminotransferase	150,151
(prevented by adrenalectomy, hypophysectomy, glucose feeding)	151,152
Activates & induces tryptophan pyrrolase	27,28
(prevented partly by adrenalectomy, hypophysectomy or glucose feeding)	152

TABLE 1-2:   (Continued)

	Ref. No.
Induces the serum heme-binding glycoprotein, hemopexin	153
Induces liver cytoplasmic organic anion-binding protein, 'Y'	154

IV. Causes accumulation of Mg^{2+} by liver mito-
chondria   155
fall in liver ATP levels, rise in AMP   156,157
rise in liver NADH:NAD ratio   157
increased excretion of L-ascorbic acid   158
increased cholesterol synthesis in
liver and increased serum levels of
cholesterol   159,160
fatty acid mobilization from adipose tissues →
fatty liver → increased hepatic
lipoprotein synthesis   161
increased phospholipid in SER (decreased
catabolism)   27,146,162

V. Behavioural changes   128
Increased susceptibility to convulsions   130

VI.   Prior treatment with phenobarbital prevents
induction of ALA synthetase by AIA because
AIA is more rapidly metabolized by pheno-
barbital-induced microsomal enzymes to
inactive polar metabolites   163

(a) <u>Effect of drugs in the hereditary hepatic</u> <u>porphyrias</u>. Drugs such as barbiturates, sulphonamides and the fungicide, griseofulvin, can precipitate attacks in the inherited hepatic forms of porphyria (see Table 1-1). However, it should be noted that sensitivity to barbiturates, for instance, is variable and drug administration to porphyriacs even over extended periods may have no adverse effect. The relative sensitivities of different forms of porphyria to barbiturates appears to be in the order variegate >AIP >coproporphyria [6,136]. In general, drugs which tend to precipitate attacks of porphyria in susceptible individuals also cause experimental porphyria in animals [6,137] and induce ALA synthetase.

(b) <u>Effects of drugs on hepatic ALA-synthetase in</u> <u>experimental animals</u>.

(1) <u>Structures of inducing drugs</u>. Structure-activity relationships have not been extensively investigated in mammals but it appears that drugs which induce in chick tissue cutlure also induce *in vivo* in mammalian liver [6] possibly with some variations in relative potency [84].

(2) <u>General site of drug action</u>. Excretion of porphyrins by drug treated animals results primarily from effects of the drug in liver. In bone marrow, AIA has little effect on porphyrin levels [138] or heme synthesis [139] although it reportedly causes a small increase in ALA

synthetase [139] and a marked increase in Fe chelatase [138].
Possible exceptions are griseofulvin and DDC, which in addition
to effects on liver, cause protoporphyrin accumulation in bone
marrow [6].

     (3) <u>Mechanism of increase in ALA synthetase.</u> The
mechanism of induction by AIA administered *in vivo* to rats seems
to be basically similar to the mechanism in chick embryo liver
tissue culture: AIA treatment results in an increase in the
level of ALA synthetase which is blocked by inhibitors of
protein and RNA synthesis, suggesting that *de novo* enzyme
synthesis (requiring DNA-dependent RNA synthesis) is necessary
for induction and that AIA does not activate a pre-existing
inactive form of ALA synthetase [98].

     There is no *proof* that induction results from an
increased rate of synthesis rather than decreased breakdown of
the enzyme although in the presence of puromycin or cycloheximide,
the half life of the enzyme has been found to be about 60-70 minutes
regardless of previous treatment [65,70,98,150] *except*
(i) in cases where soluble and mitochondrial fractions are
measured separately in the early stages of AIA induction [65,70]
when the altered half life of the soluble enzyme (this is
decreased) and of the mitochondrial enzyme (this is increased)
may be explained by transfer of enzyme from cytoplasm to
mitochondria;
(ii) in the presence of chloramphenicol, when the half life of
the mitochondrial enzyme is reported to be 118 minutes [70].

A similar effect has been reported for the bone marrow enzyme [164]. In view of the recent findings of Tomkins' [165] and Kenney's [166] groups on the effects of protein synthesis inhibitors on enzyme turnover, clear evidence on the relative importance of synthesis and degradation requires the use of an antibody to ALA synthetase.

AIA induces ALA synthetase in both cytoplasmic and mitochondrial fractions of the cell [65,70]. As discussed previously (Section 3) it is not clear whether there are distinct forms in the two fractions. It seems likely, however, that the enzyme is synthesized in the cytoplasm and transferred to the mitochondria [70], since cycloheximide completely blocks induction. Since chloramphenicol inhibits induction of the mitochondrial enzyme 50-60% [70], transfer to the mitochondria may require some mitochondrial protein synthesis. This may also apply to the erythroid system [167].

It is frequently stated that ALA synthetase is synthesized on the endoplasmic reticulum. The only evidence for this appears to be a close physical association between rough endoplasmic reticulum and mitochondria in AIA- [27] and progesterone- [168] treated rats. However, this association is also seen in controls.

(4) Specificity of induction by drugs. In contrast to the situation in the tissue culture system, drugs in mammalian liver cause not only induction of ALA synthetase but also a general increase in liver size, proliferation of the endoplasmic

reticulum, increases in a variety of liver proteins and various other changes in liver metabolism. Many of the known effects of AIA are listed in Table 1.2. It remains to be established which of these effects are closely related to induction of ALA synthetase.

(5) <u>Treatments which prevent induction by drugs</u>. Administration of hemin together with inducing drug prevents induction of ALA synthetase [143,144]. In mammals, induction by AIA is partially blocked by prior adrenalectomy [141,142]. Induction by a variety of drugs is prevented by glucose feeding [60,98] (for more detailed discussion, see Sections IV and V below).

In summary then, comparison of findings *in vitro* in chick embryo liver tissue culture with findings *in vivo* for mammalian liver shows

(i) some common features: induction is caused by similar drugs, is rapid and reversible, is dependent on protein and RNA synthesis and is prevented by heme;

(ii) some possible differences: induction by drugs may be less specific in mammalian liver and induction *in vivo* is prevented by adrenalectomy and glucose feeding. In addition, the wide ranging studies on the effect of drugs in mammalian liver have revealed several effects which suggest alternatives to Granick's hypothesis for the mechanism of drug action.

(c) Hypotheses for the mechanism of induction at
ALA synthetase by drugs.

As outlined in section 4A, Granick proposed that
drugs interfere with the ability of heme to bind to an
aporepressor protein. Most alternative proposals which have
emerged from studies with mammalian liver involve less direct,
less specific primary drug effects. It should be mentioned
that there is no definitive evidence that all drugs induce ALA
synthetase by acting at the same site nor even that each drug
acts at only one site, so that different proposals as
discussed below are not necessarily mutually exclusive. These
proposals may be grouped in four general categories:

(1) Induction results from a relatively non-specific stimulation
of RNA and/or protein synthesis.

This general proposal, which apparently could not be
true for the chick tissue culture system, will be discussed in
detail elsewhere [169]. Briefly, the basis for proposing a
non-specific drug effect is the fact that both ALA synthetase
and the mRNA which codes for it have very short half lives
(about 60 - 70 minutes) [170] compared with the corresponding
half lives for other hepatic proteins (mean half life about 3
days [171]) and their respective mRNAs. A drug effect which
for instance, reduced all mRNA breakdown would result in the
relatively greater accumulation of mRNAs turning over most
rapidly. This might lead in turn to relatively more frequent
translation of these messengers and the apparent induction of

of enzymes such as ALA synthetase. Similar arguments could apply to a generalized increase in mRNA synthesis, a generalized increase in protein synthesis or decreased protein breakdown. These general concepts have been discussed by Schimke [172,173]. This type of model clearly requires a vast amount of data for proof although demonstration of some change in synthesis or degradation of RNA or protein at times early enough to account for induction of ALA synthetase would provide a basis for further work.

Recent work in this laboratory has shown that AIA administration *in vivo* to rats causes a fall in the level of ribonuclease activity in microsomes and in cytoplasmic ribonuclease when the ribonuclease inhibitor is blocked with PCMB [169]. A similar phenomenon has been observed in pheno-barbital-treated animals [174-6]. While these effects might provide a mechanism for decreasing mRNA breakdown, our studies suggest that induction of ALA synthetase precedes any significant change in ribonuclease activity [169]. Thus while an effect of AIA on ribonuclease could account for some of the broad effects of AIA on protein synthesis listed in Table 1-2, it apparently does not account for induction of ALA synthetase. Furthermore, corticosterone also depresses ribonuclease [177,178] but does not induce ALA synthetase [80]. These findings of course do not exclude other possibilities for induction by some non-specific mechanism.

(2) <u>Drugs alter the metabolism or distribution of some</u>
<u>endogenous compound(s) resulting in accumulation of primary</u>
<u>inducer(s) of ALA synthetase.</u>

The tissue culture studies (Section 4A) suggest that
drugs act directly on liver cells. Nevertheless, it is
conceivable that drugs alter the metabolism or distribution of
some compounds present in the fetal calf serum added to
cultures [80], or even of a compound present in the cells
themselves. Published results do not indicate whether the fetal
calf serum contains 'inducer' and protein 'inhibitor' analogous
to those found in human serum [100].

The only obvious candidates for the role of primary
inducers are steroids. Active steroids (5β-H steroids?) might
accumulate as the result of changes in steroid metabolism or
distribution caused by drugs. Drugs and steroids are metabolized
by the same cytochrome P-450-containing enzyme system in liver
microsomes [179-184] and thus compete for the same binding site
on P-450. By competitive inhibition of steroid hydroxylation,
drugs might enhance metabolism of steroids by pathways leading
to active inducers. AIA and DDC for instance reportedly [50]
bind strongly to P-450 ($K_s = 10^{-6}M$). The fact that ester or
amide groups are sterically hindered from hydrolysis in these
and a number of other potent inducers [83], may make them
effective inhibitors. However, SKF-525A which strongly
inhibits drug and steroid metabolism [180] induces ALA
synthetase only weakly and blocks induction by phenobarbital
[144]. The major objection to this model is that apart from the

observation that adrenalectomy largely prevents *in vivo* induction by AIA [141,142], there is little direct evidence at present that steroids have a vital or obligatory role in induction of ALA synthetase in mammalian liver (see also Section 4B,III).

(3) <u>Drugs cause changes in energy metabolism which result in induction of ALA synthetase.</u>

This general heading covers two related hypotheses which differ on the exact nature of the 'primary effect' which leads to induction:

(i) Labbe and his coworkers propose that impairment of mitochondrial electron trnasport is the primary effect of drugs. The mechanism by which this might lead to induction is unknown, although Labbe suggests that this might involve an increased NADH:NAD ratio [185,186].

(ii) Gajdos and coworkers propose that drugs cause depression of intracellular levels of ATP resulting in induction by some unknown mechanism [157].

For both hypotheses, it might be postulated that induction of ALA synthetase depends on the effects of inducers on the mitochondrial respiratory chain. Many drugs and steroids are known to interact with the respiratory chain on the O_2 side of the flavoprotein, NADH dehydrogenase [187,188].

NADH →

Dehydrogenase
(FMN)

ATP

AMYTAL, STEROIDS

$CoQ \to b \to c_1 \to c \to a \to a_3 \to O_2$

Dehydrogenase
(FAD)

Succinate

STEROIDS
at high conc.
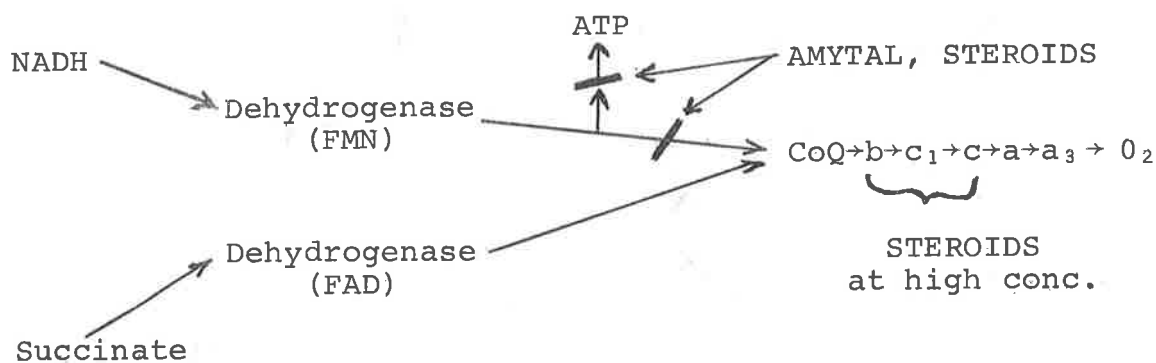
Drugs (of which the barbiturate, amytal, is often taken as
prototype) and some steroids bind not to the dehydrogenase
itself, but possibly to a lipid component of the membrane [188]
and inhibit both electron transport and coupled energy transfer:
inhibition is partly relieved by uncouplers [189,190]. Some
drugs e.g., thiopental also uncouple energy transfer and
activate ATPase [189,191]. Except at higher concentrations
of steroids when electron transport between cytochromes b and c
is inhibited, succinate oxidation is relatively unaffected [192].

Labbe's group has clearly established that many
porphyria-inducing drugs do inhibit NADH dehydrogenase [186,
193,194] and that within this group inhibition does correlate
reasonably well with inducing activity [186]. As might be
expected a number of these drugs have been shown to depress
ATP levels [157]. Changes both in NADH:NAD ratio and in ATP
level precede induction of ALA synthetase [157]. Either
hypothesis is supported by the reports that malonate which
inhibits succinic dehydrogenase, increases excretion of
porphyrins and their precursors [157,195] and that ethanol
which raises the NADH:NAD ratio and depresses ATP, induces

ALA synthetase [157,196].

In *Rh. spheroides* there is now good evidence that changes in energy metabolism affect the activity of ALA synthetase: both oxidation:reduction potential [22,23] and ATP levels [197] have been implicated. However, in mammalian liver, despite correlations between induction by drugs and their effects on energy metabolism, there is no direct evidence linking the two actions. Furthermore, there are a number of cases where correlations between the two effects break down [6,186,198]. Thus, at present the relevance of inhibition of electron transport or depression of ATP levels to induction of ALA synthetase is uncertain.

(4) Induction by drugs results from, or is modulated by changes in the availability of heme to act as 'repressor' of ALA synthetase.

The various hypotheses covered by this general heading all assume that a primary control on the level of ALA synthetase is exerted in some way through the level or distribution of heme in liver. Inducers could decrease the level of heme by decreasing its synthesis, increasing its utilization or increasing its degradation:

(i) At least one potent inducer causes *decreased heme synthesis*: DDC administered *in vivo* greatly depresses Fe chelatase activity [33,199] and significantly reduces the rate of heme synthesis [199]. It seems, however, that few inducers have a similar action. For instance, AIA has no effect on liver Fe

chelatase [138] and increases overall heme synthesis [20,144];
phenobarbital induces Fe chelatase [33,200] and increases over-
all heme synthesis [20,33]. In fact, inhibition of heme
synthesis alone may not be sufficient to cause induction of
ALA synthetase since aminotriazole, which inhibits heme synthesis
40-50% *in vivo*, neither induces itself nor enhances induction
by other drugs [33,201]. Another inhibitor of heme synthesis,
lead, causes the symptoms of porphyria in animals but there is
little compelling evidence from *in vivo* studies that it marked-
ly induced ALA synthetase [138,150 cf. 202, see also Section
4B,VII).

(ii) Increased heme utilization may account for induction of
ALA synthetase *in vivo* by tryptophan and some of its
analogues which increase heme binding to apoenzyme tryptophan
pyrrolase [203]. As discussed in Section 2, the major
destination for liver heme is the microsomal fraction, where
heme is bound as the prosthetic group of cytochromes P-450
and b_5 [20]. Apart from their quantitative importance as
hemoproteins in normal liver, the following observations suggest
that the level of ALA synthetase may be related in some way
to heme utilization in P-450 and to a lesser extent, b_5.

(a) A variety of drugs induce P-450 along with other
components of the microsomal drug metabolizing system [204-6].
An increase in P-450,which also turns over relatively
rapidly [20], is not possible if heme synthesis is restricted
by aminotriazole inhibition to near normal levels [33]. It
seems therefore, that induction of the drug metabolizing enzymes

requires induction of ALA synthetase.

(b) As might be expected from (a), drugs which induce the detoxifying system generally induce ALA synthetase (compare lists of ALA synthetase inducers in refs. 6,207 with list given by Conney, ref. 205). In the absence of direct comparative studies, it is difficult to assess how good a correlation exists between inductions by drugs in the two systems.

(c) Heme which has generally been regarded as a relatively specific feedback repressor of ALA synthetase also blocks induction by a variety of drugs, of components of the microsomal drug metabolising system: heme represses not only the hemoproteins but also the flavoprotein NADPH cytochrome c reductase and prevents the general increases in microsomal protein and phospholipid [20,35].

(d) Inductions by drugs of ALA synthetase and the detoxifying system have some features in common: both inductions are prevented by inhibitors of RNA and protein synthesis [6,205]; inductions of both by phenobarbital are blocked by SKF-525A [20]; inductions of both by AIA are prevented by adrenalectomy [27,141] although adrenalectomy does not prevent induction of the drug metabolising enzymes by phenobarbitone [208-211]; inductions of ALA synthetase and P-450 [20,212] but not other components of the detoxifying system [212] are prevented by glucose feeding.

These observations establish that inductions of ALA synthetase and the microsomal drug metabolising enzymes (or at

least cytochrome P-450) are related. On existing evidence several hypotheses on the nature of this relationship might be considered. Drugs might primarily induce the cytochrome P-450 (or the detoxifying system in general) with the resulting depletion of free heme leading to derepression of ALA synthetase. This seems unlikely, since following drug administration, induction of ALA synthetase precedes by several hours any marked increase in P-450 levels [33,52,148]; interpretation of time course data, however, is complicated by the difference in the half lives of ALA synthetase and P-450. The converse proposal - that primary induction of ALA synthetase and the resulting increase in heme synthesis cause induction of P-450- also seems unlikely in view of the finding that exogenous heme represses rather than induces P-450 [20].

Several authors (e.g., refs.135 ,80] have implied that drugs might induce both systems and that the ability of a drug to induce one system would greatly enhance its apparent ability to induce the other, since a parallel induction supplies the heme necessary for increases in P-450 activity and minimizes by utilization, accumulation of the repressor, heme. Such a proposal is consistent with the known facts but leaves open the question of how drugs cause induction, in particular whether they act through a common mechanism or independent mechanisms to induce ALA synthetase and component(s) of the microsomal detoxifying system. Considerable information is available on the mechanism of induction of the drug metabolising enzyme system by both phenobarbital-type inducers and polycyclic

hydrocarbons. (See following refs: reviews 205, 206, 209; role of ALA synthetase 20, 33; diet and induction 20, 211-213; role of steroids 208-211; additive inductions of distinct forms of P-450 214-7; increased synthesis v. decreased degradation 218-220; tissue culture studies on induction 217, 221-4; site of synthesis 225; effects of phenobarbital on RNA metabolism 174-6, 226-9.)

(iii) <u>Increased heme catabolism</u> is the third process which might cause or modulate induction of ALA synthetase by reducing the availability of heme to act as repressor. The mechanisms for heme breakdown in liver were described in Section 2. Two potent inducers, AIA and DDC have been shown to cause loss of cytochrome P-450 from microsomes [50-53,148,230]. The P-450-heme is degraded to unidentified 'greenpigments' [51,53] presumably biliverdin. The action of AIA apparently requires binding to P-450 and possibly metabolism to some active derivative since AIA-enhanced heme degradation is blocked by SKF 525A [51,53]. The effect of AIA is possibly confined to increasing the catabolism of heme from the major form of cytochrome P-450 in microsomes [52, see also Section 2] and it remains to be shown whether this in any way changes the availability of heme to act as a repressor of ALA synthetase.

(III) <u>The Role of Steroids</u>

    (a) <u>Hydrocortisone.</u>

        In 1966, Marver *et al.* [141] reported that prior

adrenalectomy partly prevented induction of ALA synthetase by AIA *in vivo*. This effect of adrenalectomy could be reversed by administration of hydrocortisone. Neither adrenalectomy nor injection of hydrocortisone alone changed the basal level of the enzyme. The authors concluded that hydrocortisone had a permissive role in induction of ALA synthetase. Kikuchi's group [142] suggested that hydrocortisone potentiated the action of drugs, since in normal intact rats the steroid increased the extent of induction by suboptimal doses of AIA and if given an hour before the AIA, reduced the lag period preceding the increase in ALA synthetase activity. In rats treated with AIA plus hydrocortisone, a peak in ALA synthetase activity at 2-3 hours is followed by a 'refractory period' in which further doses of AIA have no effect.

These observations, together with the facts that adrenalectomy prevents AIA-induced proliferation of the smooth endoplasmic reticulum [27] and increases a number of liver proteins [151,152] are consistent with a permissive role for hydrocortisone which is known to have general stimulatory effects on hepatic RNA and protein synthesis as well as causing relatively specific induction of some enzymes (see ref. 231 for a review). In the chick embryo liver tissue culture system there is apparently no requirement for hydrocortisone (but see Section 4B, II, p.38); neither does hydrocortisone cause induction [80].

(b) <u>Sex steroids and their derivatives</u>.

As already discussed in Section 4A, the sex steroids (androgens, estrogens and progestins) are weak inducers and a number of their derivatives, notably those with a 5β-H structure, potent inducers of ALA synthetase in chick embryo liver. With a few exceptions discussed below there is little direct evidence that sex steroids or their metabolites are potent inducers of ALA synthetase in mammalian liver [89]. There is, however, considerable indirect evidence implicating sex steroids in the control of ALA synthetase:

(1) <u>Effects in the hereditary hepatic porphyrias</u>.

(i) Symptoms of AIP and porphyria variegata rarely appear before puberty [2,3,5,232];

(ii) The recorded occurrence of AIP is at least $1.^1/_2$ times greater in women than in men and female AIP patients have more frequent attacks. Porphyria variegata occurs equally in men and women, but women suffer neuological symptoms more often, and cutaneous symptoms less often than men. The different occurrence of cutaneous symptoms could reflect vocational differences [2,5,120,233].

(iii) In a small proportion of female porphyriacs, attacks are associated with the luteal phase of the menstrual cycle, i.e., just prior to menstruation [2,5,120,233]. Both estrogen and progesterone levels are elevated in the luteal phase although peak estrogen levels occur in the ovulatory phase [234,235]. In such cases there seems to be little correlation,

however, between progress of the menstrual cycle and levels of ALA and PBG in urine [233]. In those cases of porphyria where symptoms are related to the menstrual cycle, treatment with contraceptive steroids reduces the occurrence of symptoms possibly by eliminating (by feedback on the pituitary) peaks in estrogen and/or progesterone production in the ovaries (e.g., ref. 236). This contrasts with the adverse effects of steroids noted in some other porphyric patients (see (v) below).

(iv) In a few women porphyriacs, attacks are associated with pregnancy, usually occurring either in the first trimester or just after delivery [2,5,120,233]. At these times, estrogen and progesterone levels are high, though not maximal [235].

(v) Treatment of porphyric patients with estrogens [233, 237,238], progesterone [239] or contraceptive mixtures [238] may in some cases (cf. item (iii) above) precipitate attacks and/or increase excretion of porphyrins and their precursors.

(vi) Goldberg *et al.* [240] found significant increases in excretion of conjugates of a number of 17-keto steroids (dehydroepiandosterone, etiocholanolone and epiandrosterone) by patients with AIP both during attacks and in remission. A similar finding was reported for coproporphyria patients during attacks, though not in remission [110].

(vii) Kappas' group [83] investigated metabolism of testosterone and 11-hydroxy Δ^4-androstenedione in AIP patients

in remission, and found evidence of impaired steroid-5α-reductase activity.

Items (i) to (v) show that in genetically susceptible individuals, estrogens and progesterone in some situations increase the excretion of porphyrins and their precursors and/or increase the occurrence of symptoms, while items (vi) and (vii) suggest that in some forms of porphyria at least, steroid metabolism may be abnormal. Despite the suggestive finding that plasma from AIP patients causes an induction (blocked by UDP glucuronic acid) in chick tissue culture [100,101], it remains to be shown whether sex steroids or some abnormal metabolite(s) actually induce ALA synthetase: the observations (i) to (v) could conceivably be explained for instance, by inhibition by steroids at some later point in the heme biosynthetic pathway.

There are several reports of extended estrogen administration causing porphyria cutanea tarda [116,233,241-3], an acquired form of porphyria for which there may be some genetic predisposition [207, see also Table 1-1]. In one such case, ALA synthetase was significantly elevated [116]. However, in many of the cases described, liver damage may have been involved (e.g., ref. 233].

(2) Effects of steroids in normal humans and experimental animals.

Treatment of normal humans with contraceptive steroid mixtures [244] increased excretion of ALA and PBG but extended

treatment with estrogen alone [245] had no effect. This, together with studies with contraceptive components in chick embryo liver [246] suggests that progestins or their metabolites, but not estrogens have ALA synthetase-inducing activity in normal humans (cf. effects in porphyric patients described above). This conclusion is supported by studies with rats: estrogen causes an initial fall followed by oscillations in hepatic ALA synthetase [247] whereas progesterone in high doses has been shown to induce the enzyme about 10-fold in 16 hours [163] or 3-fold over 5 days [168]. Goldberg *et al.* [240] have also shown that daily administration of the 17-keto steroid, dehydro-epiandrosterone or its sulphate caused a maximum 2-3 fold induction after 3 days.

A number of groups have shown that the 5β-H steroids known to be potent inducers of ALA synthetase in chick embryo liver [89] also stimulate heme and globin synthesis in mammalian bone marrow [248-251]. However, attempts to demonstrate induction by these steroids in mammalian liver have been unsuccessful [89] perhaps because of some difficulty in getting the relatively polar steroids to accumulate in liver in adequate concentrations.

(3) Mechanism of steroid action

From the discussion above, there is direct evidence for induction of ALA synthetase in normal animals by progesterone and dehydroepiandrosterone. There is little further information from mammalian studies on the mechanism

by which these steroids induce.   Extrapolation from the chick
embryo liver studies would suggest that the effects of
steroids are moderately specific with no general increase
in RNA or protein synthesis [87,90] but causing increases
in ALA synthetase and cytochrome P-450 and mixed-function
oxidase activity [83]:   in rat liver, progesterone causes
marked proliferation of the endoplasmic reticulum and induction
of P-450 [168].   The tissue culture studies also suggest that
induction by steroids depends on *de novo* protein synthesis and
stimulation of (α-amanitin-sensitive) synthesis or accumulation
of some RNA species [90,91,92].   An effect on the transcription
or accumulation of the mRNA for ALA synthetase would be
consistent with current proposals for the mechanism of
action of steroids in other mammalian systems (e.g., see
ref. 252).

While the role of 5β-H steroids in mammalian liver
might be regarded as doubtful, it seems unlikely that
estrogens themselves induce ALA synthetase.   They may,
however, exert indirect effects, e.g., on the levels of other
inducers.   Estrogens are known at least in rats, to decrease
metabolism of some steroids and drugs in the microsomal drug
metabolising system (e.g., 180,184,253-5) and to alter
metabolism of steroids by other pathways [255-7].

(IV)   Repression by Glucose.

As mentioned in earlier sections, feeding high
carbohydrate (or high protein, but not lipid [258]) diets

to patients with AIP [259,260] or porphyria variegata [117]
reduces the clinical symptoms and decreases the excretion of
porphyrins and their precursors: dietary restriction has
the opposite effects [117,259]. Glucose feeding also
prevents induction of ALA synthetase by a variety of drugs
in vivo [20,60,98] as well as induction by drugs of
tyrosine aminotransferase, tryptophan aminotransferase,
tryptophan pyrrolase [152] and cytochrome P-450 [20].

Little is known about the mechanism of glucose
repression of ALA synthetase. From other studies [261] it
is known that glucose does not decrease overall protein
synthesis. Feeding glucose to a rat in which hepatic ALA
synthetase has been preinduced with AIA results in a rapid
decay in enzyme activity. At later times after AIA
administration (14-16 hours) the initial rate of decay after
glucose feeding is more rapid than the rate of decay following
actinomycin D administration [98] suggesting that glucose
might cause repression at a translational level. As
mentioned in Section 4A, neither glucose, glucagon nor
insulin have any effect in chick embryo liver tissue culture
[80] although there is one report of weak induction by
glucagon, but not cyclic AMP, in chick embryo liver *in vivo*
[99]. In rats, glucagon failed either to induce ALA
synthetase or to augment induction by AIA [98].

The following proposals have been made for the
mechanism of glucose repression of ALA synthetase:

(i) that high glucose levels might hasten the removal by liver cells of active inducers either by increased glucuronidation of steroids [91] or by providing NADPH for detoxification reactions in the microsomal drug metabolising system [20];

(ii) that glucose reverses the effects of drugs such as AIA [262] and of ethanol [263] on ATP and NADH levels and thereby prevents induction.

A direct involvement of glucose in metabolic control, as proposed in (i) and (ii) has been reported in some other mammalian systems [264-7]. Glucose represses a number of hepatic enzymes, many of them involved in amino acid catabolism and gluconeogenesis [261,268-271]. In a number of these systems, the 'glucose effect' involves changes in the levels of insulin and glucagon and complex interaction with other hormones, particularly glucocorticoids (e.g., 261, 271-3). The resultant changes in enzyme activity are probably mediated at least in part through changes in hepatic levels of cyclic AMP (e.g., 273-8). Recent work has shown that catabolite repression in bacteria involves control of enzyme synthesis by cyclic AMP [278,279].

In the light of findings in other systems, it is tempting to suggest that cyclic AMP may be involved in glucose repression of ALA synthetase, despite the apparent lack of effect of either insulin or glucagon mentioned above. Stimulation of bone marrow ALA synthetase by low concentrations

of cyclic AMP has recently been reported [280].

(V) Mechanism of Repression by Heme.

As mentioned in earlier sections, heme administered
at the same time as inducer prevents (i) induction of porphyrin
fluorescence by moderate (but not high) concentrations of
AIA [80] and by steroids [91] in chick embryo liver tissue
culture, (ii) induction of ALA synthetase by AIA in tissue
culture [87] and (iii) in rat liver *in vivo*, induction by
AIA of ALA synthetase measured either in homogenates [20] or
in separate mitochondrial [143,281] and cytoplasmic [281]
fractions. Sassa and Granick [87] found that in the tissue
culture system, heme neither inhibited general protein
synthesis nor increased the rate of breakdown of ALA
synthetase in the presence of acetoxycycloheximide. Heme
caused a more rapid fall in ALA synthetase activity in AIA-
preinduced cultures than did actinomycin D, suggesting that
heme has a relatively specific effect at some posttranscript-
ional site which results in decreased activity of ALA
synthetase. These observations are consistent with the
basic premise in most models for control of ALA synthetase,
namely that heme decreases the rate of synthesis of ALA
synthetase. However, as mentioned in Section 4A, the recent
finding by Schneck *et al*. [96] that heme had no apparent
effect on porphyrin synthesis when added to chick embryo
liver cultures preinduced with AIA, has raised some doubt
that this premise is always valid. Similarly, in rat liver,

it has been reported [281] that w███e heme administered 2-10 hours after AIA does not decrease total induction by AIA, but alters only the relative proportions of enzyme in mitochondrial and cytoplasmic fractions of the cell by preventing appearance of newly synthesized enzyme in the mitochondria. A redistribution phenomenon of this type may have been the post-transcriptional effect of heme observed by Sassa and Granick [87] who apparently measured ALA synthetase activity in isolated mitochondria.

A lack of effect by heme in preinduced liver cells is not consistent with the simple model of Granick [7,80] and suggests that regulation of ALA synthetase may be more complex than previously suggested.

(VI) Lead Poisoning: the Effects of Lead on Heme Synthesis.

(a) Significance of studies with lead.

The effects of lead on the heme biosynthetic pathway are of interest, particularly within the context of the present study, for three reasons:

(1) The clinical features of lead poisoning are remarkably similar to those of acute intermittent porphyria. This fact has been discussed at length by Goldberg and his co-workers [17,282] and by Chisolm [283] and will be mentioned only briefly here. In both diseases the most common symptoms are neurological: abdominal pain, constipation, vomiting, paralysis and psychological disturbances. Both diseases are

characterized biochemically by excessive excretion in urine
of ALA, URO and COPRO, although the high levels of PBG
excreted in AIP are generally not found in lead poisoning
[17,282,283]. As with AIP, the extent to which the symptoms
of lead poisoning are a result of disturbed porphyrin metabolism
remains to be determined (see ref.17 for discussion). Neverthe-
less, the facts that the diseases have very similar symptoms
and that both involve disturbed porphyrin metabolism, suggest
that lead poisoning might provide a useful experimental
model for the hereditary AIP.

(2) As an inhibitor of heme synthesis (see below), lead may be
a useful tool for testing the importance of feedback controls
by heme and the relevance of these controls to AIP.

(3) Lead poisoning is of considerable importance in its own
right. There is growing concern over atmospheric pollution
by lead from car exhausts. In a recent study, 2.6% of
particulate matter in air near a busy highway was lead [284].
Lead poisoning is still a major pediatric problem in some
cities [285]. Workers exposed to lead in scrap metal
burning, battery manufacturing and ship breaking industries
for instance, may have elevated levels of lead in blood
even in situations where extensive safety precautions are in
force [286].

(b) <u>Effects of lead on heme and porphyrin synthesis</u>
    <u>in erythroid cells.</u>

The major site of action of lead in mild lead
poisoning is probably the bone marrow, since a small dose of
lead disappears from soft tissues over several days but
remains in a tightly bound form in bone marrow [287]. Most
studies to date have concentrated on the erythroid system.
In more severe or prolonged exposure the highest levels of
lead are found in liver, kidney, blood and bone marrow [282,288]
and heme synthesis in all those tissues may be affected.

Studies on circulating red cells [289] or bone marrow
cells [288] from lead poisoned subjects have led to the
proposal that lead reduces heme synthesis from ALA by
depressing the activity of the sulphydryl enzymes, ALA
dehydratase and Fe chelatase [17,283,288]. These effects
could explain the reduced hemoglobin, excretion of ALA and
COPRO and accumulation in erythroid cells of PROTO
observed in lead poisoned patients [17,282]. In bone marrow
cells from lead poisoned rabbits, the activity of ALA
synthetase was found to be normal [288].

Direct inhibition of ALA dehydratase [290] and Fe
chelatase [288,291] by lead has been confirmed with partially
purified preparations of these enzymes. However, as Haeger-
Aronson has pointed out [292] several metal ions, more
potent as -SH inhibitors than lead, do not produce the
symptoms of lead poisoning. Furthermore, the suggestion
that inhibition of ALA dehydratase and Fe chelatase might

account for the biochemical abnormalities in lead poisoning
is only partially supported by studies in which lead is added
to normal intact red cells or lysates. These studies show
that

(i) lead strongly inhibits overall heme synthesis from acetate
or glycine in intact rabbit red cells [293], intact chick
red cells [294] and in avian red cell lysates [291,295].
Lead concentrations $>10^{-4}$M give 80% or greater inhibition;
10^{-5}M Pb inhibits 50-60% (refs. 291,293-5).

(ii) the major effect on the heme biosynthetic pathway in red
cells *in vitro*, particularly at lower concentrations
(10^{-5}M) is on the formation of ALA from a glycine precursor
rather than on subsequent utilization of ALA [291,294,295].

(iii) at high concentrations (10^{-4}M Pb) the conversions
ALA→PROTO [291,295], PBG→PROTO [291] and PROTO→HEME
[291,296] are also inhibited.

From these observations it might be concluded that higher
concentrations of lead inhibit Fe chelatase and ALA dehydratase
and/or PBG deaminase and/or UROGEN decarboxylase,all of which
are sulphydryl enzymes [3]: but the major action of lead is to
reduce synthesis of ALA, (although *in vitro* lead inhibits ALA
synthetase itself only at relatively high concentrations [297]).
Since it is difficult to explain the excessive excretion and
accumulation of porphyrins of *in vivo* lead poisoning on this

basis, it may be that *in vitro* studies on the circulating red
cell population (in which only a small fraction of cells
actively synthesize heme [3]) do not reproduce the conditions
of lead poisoning *in vivo*.

In lead poisoning *in vivo*, there are various changes
in the erythroid system whose relation to altered heme and
porphyrin synthesis is as yet unclear. Anemia due to lead
poisoning [292,293] probably results from two effects -
decreased half-life of circulating erythrocytes [298] and
decreased production of erythroid cells in bone marrow [299].
The latter effect is probably linked with impaired red cell
maturation which leads to the appearance of basophilic stippled
red cells in bone marrow [17,282,283]. Sano [300] has
suggested that retention of mitochondria by these cells which
fail to undergo normal maturation to erythrocytes, may account
at least in part for excessive excretion of ALA and porphyrins.

In short, while it seems clear that lead does
inhibit some sulphydryl enzymes of the heme biosynthetic pathway,
it may be that its effects on the erythroid cell pathway are more
complex than this simple inhibition.

(c) Effects of lead on heme synthesis in liver.

The only comparative study to date [288] suggests
that *in vivo*, lead has very similar effects in bone marrow and
liver. In livers from lead poisoned rabbits [288,301], rats
[150], and mice [202], ALA dehydratase activity is depressed
about 50%. ALA dehydratase is also inhibited by lead *in vitro*

[302]. Lead at levels comparable to those in lead poisoned tissues [288] inhibits hepatic Fe chelatase *in vitro* [34,288].

The effect of lead on ALA synthetase is of some interest since the similarity of lead poisoning to AIP, the excessive excretion of porphyrins and the fact that lead probably impairs synthesis of heme, the putative co-repressor of ALA synthesis, all provide some basis for suggesting that lead might cause induction of ALA synthetase. Administration of lead to rabbits over several months [288] or to rats over 5 days [150] did not increase the level of hepatic ALA synthetase. However, in a recent abstract, Morse *et al.* [202] reported that a single intravenous dose of lead acetate produced a 3-fold increase in ALA synthetase in mouse liver. Since this rise was preceded by lowered heme synthesis and prevented by cycloheximide, they suggested that lead induced by relieving heme repression of the synthesis of ALA synthetase.

SECTION 5. REVIEW OF LITERATURE IN RELATION TO EXPERIMENTAL AIMS

The long term aim of work in this laboratory is to elucidate at a molecular level the nature of controls on the activity of ALA synthetase in mammalian liver and to apply these findings to the human hepatic porphyrias. The present investigation is concerned particularly with control on the level (i.e., amount) of ALA synthetase in liver cells.

The chosen experimental approach to this problem, as outlined in Section 1, involved development of an *in vitro* system derived from mammalian liver, in which the level of ALA synthetase can be altered at will by various known stimuli, in which complex *in vivo* interactions are avoided and possible variables directly controlled and in which relatively complex experimental manipulations are possible.

The review of the literature above suggests several reasons for such an approach. While the basic elements of control of ALA synthetase in mammalian liver (induction by drugs and possibly some steroids, repression by heme and by glucose *in vivo*, and possible permissive effects of steroids) are known, the mechanism of these effects is only partly understood. Existing evidence suggests control involves changes in the rate of synthesis of ALA synthetase but definitive evidence on the relative importance of enzyme breakdown must await studies with a specific antibody to ALA synthetase. A number of hypotheses on the mechanism of action of inducers and

repressors have been discussed above. Differentiation between
proposed mechanisms will require firstly identification of those
compounds which act directly on the liver followed by detailed
investigation of their effects under well-defined conditions.

In vivo studies, particularly with porphyric patients,
have provided suggestive evidence for the involvement of
steroids in control but direct evidence for such involvement
is lacking. A more definitive investigation of the role of
steroids should be possible *in vitro*. Investigation of the
'glucose effect' is also difficult *in vivo* because of the
complex endocrine interactions present in whole animals.

The chick embryo tissue culture system employed by
Granick and his coworkers has already proved the worth of
an *in vitro* system; however, possible dangers in extrapolating
results with chick cells in tissue culture to mammalian liver
are suggested by apparent differences between the two systems
(e.g., effects of 5β-H steroids, effects of inducers on RNA
and protein synthesis). Thus for the present studies it was
desirable that the chosen *in vitro* system should be derived in
particular, from mammalian liver.

These considerations, then, provide the rationale
for experiments described in the following chapters.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

A. CHEMICALS AND MEDIA

(a) Compounds used in ALA synthetase assays.

ATP (disodium salt Type II, 98% pure), CoA (90% pure), glutathione (reduced form) and pyridoxal-5-phosphate were Sigma products. Solutions of these compounds were adjusted to pH 7.4 with NaOH and stored at -15°C. ^{14}[C]-1,4,-succinic acid was obtained from the Radiochemical Centre, Amersham. Solutions of ALA-HCl (Sigma, 95% pure) were prepared freshly when required in 10^{-3}M HCl.

Ehrlich reagent was prepared freshly as required as a 2% (w/v) solution p-dimethylaminobenzaldehyde in glacial acetic acid containing 16% (v/v) of 70% perchloric acid and 0.3% (w/v) of HgCl$_3$.

Scintillation fluid was a solution of 0.3% (w/v) 2,5 diphenyloxazole and 0.03% (w/v) 1,4-bis 2-(4-methyl-5-phenyloxazolyl) benzene in toluene.

(b) Compounds tested as modifiers of ALA synthetase activity.

AIA, a gift from Hoffmann-LaRoche, Basle, Switzerland, was dissolved in saline (20 mg/ml) before use in *in vivo* or perfusion experiments or in culture medium before addition to cell suspensions or cultures. DDC, purchased from Eastman

Kodak, was recrystallized twice from ethanol before use; for tissue culture studies, DDC was dissolved directly in the culture medium. The steroids progesterone, pregnanolone, etiocholanolone and 17β-estradiol were Sigma products; hydrocortisone was obtained from Calbiochem. For *in vivo* experiments, steroids were dissolved in maize oil (15 mg/ml); for perfusion experiments, steroids were dissolved directly in the Ringer solution before perfusions were commenced. Dibutyryl cyclic AMP (monosodium salt, Sigma) was dissolved in a small volume of saline. Hemin (ferric protoporphyrin - Bovine Type I) was obtained from Sigma; solutions were prepared freshly when required as described by Burnham and Lascelles [77]. L-tryptophan was a Mann Assay product. Neutral insulin (NOVO, ACT RAPID) was obtained from Evans Medical Aust., and glucagon from Eli Lilly, Indianopolis. Solutions of actinomycin D (Merck, Sharpe and Dohme Research Lab.) and cycloheximide (Sigma) in saline were stored at -15°C.

(c) Compounds and media used in cell suspension and tissue culture experiments.

Tissue culture medium was prepared as described by Granick [80] except that as noted for some experiments, 5% fetal calf serum was used and Myocostatin was omitted in most experiments. The components, Eagles Basal Medium (as a solid preparation), glutamine and fetal calf serum were all purchased from Commonwealth Serum Laboratories (CSL), Melbourne.

Hanks medium (Ca, Mg free) and Earle's salt-glucose solution were prepared [303] with phenol red as indicator.

Dialysed albumin. Bovine serum albumin Fraction V, purchased from Sigma was dissolved in Krebs Ringer-Bicarbonate, dialysed against the Ringer solution for 12 hours and stored at -15°C.

Respiration Medium had the following final composition: K_2HPO_4 (16.4mM), KH_2PO_4 (3.6mM), KCl (90mM), $MgCl_2$ (5.0mM), NaCl (25mM), Na lactate (25mM).

Suspension medium was Krebs Ringer-Bicarbonate containing 1% dialysed albumin and 0.5% glucose.

All media were sterilized by millipore filtration.

(d) Compounds and media used in perfusion experiments.

Nembutal (pentobarbitone sodium 60 mg/ml) for injection was obtained from Abbott Laboratories, Sydney, Australia, and heparin for injection from CSL. Casein hydrolysate (acid-hydrolysed) was purchased from British Drug Houses Ltd. and stored dessicated. In some experiments where indicated, a synthetic amino acid mixture designed for use with Eagles Minimal Essential Medium (as 100x concentrate, CSL) was employed as amino acid source.

Phosphate-Bicarbonate Ringer was prepared by mixing 0.154M NaCl (94 volumes), 0.154M KCl (4 vol.), 0.11M $CaCl_2$ (3 vol.), 0.154M KH_2PO_4 (1 vol.), 0.154M $MgSO_4$ (1 vol.) and Solution X (13 vol.). Solution X was prepared by diluting

80 ml 0.125M Na_2HPO_4 + 2 ml 1M HCl to 100 ml. The mixture was stored at 4°C. Before use, 13 volumes of 0.154M $NaHCO_3$, saturated with CO_2, were added to give the complete Ringer.

Standard perfusate. Casein hydrolysate (0.6 g) and glucose (0.5 g) were dissolved in 55 ml phosphate-bicarbonate Ringer. The pH of this solution was adjusted to 7.3 by addition of HCl or NaOH if required. To this solution, 38 ± 1 ml freshly prepared rat blood were added. Test compounds, except in the case of steroids, were dissolved in 2 ml saline and added to the perfusate immediately after perfusion was commenced: the final volume was thus 95 ml. The circulating perfusate was gassed before and during perfusions with carbogen (CO_2 + O_2, 5 : 95).

(e) Standard (PEST) buffer for liver cell preparations.

PEST buffer contained pyridoxal-5-phosphate, 10^{-4}M; EDTA, 10^{-4}M; sucrose, 0.25M; and Tris-HCl (pH 7.4), 5 x 10^{-3}M. This was stored at 4°C.

B. ENZYMES

Succinyl CoA synthetase was prepared from *E. coli* (Crooke's strain) by a method based on that of Hildebrand and Spector [304]. Cells were grown and sonic extraction, streptomycin sulphate precipiation and ammonium sulphate fractionation carried out as described [304]. The pellet from the ammonium sulphate precipitation was resuspended in 0.05M Tris-HCl, 0.05M KCl, pH 7.2 and dialysed against this buffer for 12 hours.

The dialysed enzyme was stored overnight at -15°C then clari-
fied by centrifugation for 10 minutes at 15,000 rpm. The
preparation was stored in small aliquots at -15°C. This
procedure gave an enzyme preparation with specific activity
approximately 14 μmoles succinhydroxamic acid/mg protein/
30 min. when assayed by the method of Kaufman [305].

Collagenase (Type I), hyaluronidase and recrystallized
lyophilized trypsin were purchased from Sigma. Trypsin (1: 250)
and pangestin were Difco products.

C. ANIMALS

Guinea pigs, obtained from the Institute of Medical and
Veterinary Science, Adelaide, South Australia, were males
weighing 400-500 g.

Chick embryos were kindly provided by Dr. B. Moore,
Institute of Medical and Veterinary Science. Eggs were
maintained in a humidified cabinet at 37°C.

Rats (Wistar, albino) were obtained, unless otherwise
noted, from the Central Animal House, Waite Agricultural
Research Institute, Adelaide. Both male and female
rats weighing between 150 and 250 g (in most cases 180-220 g)
were used in *in vivo* experiments and to provide livers for
perfusion. Depending on their availability, larger rats
(up to 300 g) were used as blood donors for perfusion
experiments. Unless otherwise stated, all rats were fasted
for 42 hours before use.

In experiments involving *in vivo* induction of ALA synthetase by AIA, the AIA in saline was administered by subcutaneous injection. The dosage used in all cases was 350 mg/kg.

D. ANALYTICAL METHODS

(a) Estimation of ALA synthetase activity.

The colorimetric assay was based on the method of Granick [80] and essentially as described by Irving and Elliott [73]. The 1 ml incubation mixture contained, in μmoles: glycine, 50; potassium succinate, 50; Tris-HCl (pH 7.4), 50; $MgCl_2$, 20; CoA, 0.125; pyridoxal-5-phosphate, 1; ATP, 5; glutathione, 2; EDTA, 10; 0.01 ml succinyl CoA synthetase preparation (activity equivalent to 2 μmoles succinyl CoA/hour as measured by the method of Kaufman [305]); liver preparation and water to final volume. The EDTA, added to inhibit ALA dehydratase present in liver cytosol was omitted in assays of mitochondria. The volumes of liver preparations included in the incubation mixture were mitochondria, 0.2 ml; post-mitochondrial supernatant, 0.4 ml; cell debris, 0.4 ml; homogenate, 0.4 ml. Incubation times were 20 min. for homogenates and 60 min. for other cell fractions unless otherwise stated. Incubations were carried out in tubes at 37°C in a shaking water bath and stopped by addition of 2.0 ml of 3.75% TCA. Colorimetric estimation of enzymically-formed ALA was as described by Granick [80], except that the formula for calculation of ALA formed per assay was adjusted for the

1 ml incubation volume in place of the 2 ml used by Granick:

$$\text{m}\mu\text{moles ALA/assay} = 8.7 \quad (21.7Z - W)$$

where W and Z are optical densities at 552 mμ before and after
ether extraction respectively.

All assays were carried out in duplicate and
corrected for a blank to which TCA was added at zero incubation
time. In some experiments an enzyme blank was also included
but this was invariably lower than the zero time blank.

The radiochemical assay was as described by Irving
and Elliott [73]. Assays were carried out in duplicate and
corrected for an enzyme blank. The product in the radiochemical
assay was identified by thin layer chromatography on Kieselgel
G7731 (Merck) as described by Irving and Elliott [73].

(b) Estimation of protein.

Protein in liver cell preparations was estimated
by the Biuret procedure [306].

(c) Measurement of amino acid incorporation.

Amino acid incorporation by cell suspensions and
cultures was measured by incubating cells with labelled amino
acids as indicated. Incubations was terminated by adding 1
volume ice-cold 5% TCA - 1% casamino acids. The precipitate
was extracted with 3 vols. cold 5% TCA - 1% casamino acids,
then digested with 1 vol. 1M NaOH. Protein was precipitated
with cold 10% TCA and the precipitate, retained on GFC glass

fibre filter paper, washed with cold 5% TCA and then ether.
The filter paper with precipitate was dried and ^{14}C content
estimated by scintillation counting.

(d) Measurement of respiration rates.

Respiration rates were measured by standard manometric
techniques in a Warburg apparatus. Isolated cells or liver
slices equivalent to approximately 20 mg protein were
equilibrated for 15 min. in a Warburg flask in respiration
medium minus the substrate, lactate. At zero time, lactate
was added from the side arm and subsequent O_2 consumption
measured. The centre well contained 0.2 ml KOH to trap CO_2.

E. PREPARATION OF LIVER HOMOGENATES AND CELL FRACTIONS

Liver samples were immediately cooled and washed in
ice-cold PEST buffer; after 10 min., samples were weighed then
homogenized using a Potter-Elvejhem homogenizer (clearance
0.003 inches) in 9 volumes PEST buffer. In most experiments
as indicated, the unfractionated homogenate was rapidly frozen
using a dry ice-ethanol bath and stored at -15°C. Where cell
fractions were required, cell debris, mitochondria and post-
mitochondrial supernatant were prepared from homogenates by
differential centrifugation [307]. Mitochondria were
resuspended in PEST buffer using a Potter-Elvehjem homogenizer
so that 1 ml of mitochondrial suspension was equivalent to
0.5 g wet weight of liver. Cell debris was resuspended in
PEST buffer to give a preparation equivalent to 1.0 g wet

weight of liver/ml. Cell fractions were rapidly frozen and
stored at -15°.

F. CULTURE OF CHICK EMBRYO LIVER CELLS

Two different methods were used in tissue culture
experiments. One procedure was essentially as described by
Granick [80] except that cells in some experiments were grown
in 1 ml culture medium in Leighton tubes and in later experi-
ments in 10 ml culture medium in 100 ml milk dilution bottles,
rather than on cover slips.

A second, modified procedure was used in the majority
of tissue culture experiments reported below. Five or six
livers were removed from 15 or 16 day old chick embryos and
placed in sterile Earle's salt-glucose solution. The livers
were washed once then cut into tiny pieces. The Earle's
solution was drawn off and the liver fragments transferred to
a 50 ml screw cap erlenmeyer flask containing 15 ml dissociating
solution (Earle's solution containing 3.0 mg/ml recrystallized
lyophilized trypsin) adjusted to pH 7.3 with a carbogen gas
phase. The liver fragments were incubated at 37°C for 30 min.
in a gently rotating water bath, after which the fragments,
still essentially intact were transferred to the culture
medium (with 5% fetal calf serum) and dissociated by drawing
them in and out of a wide bore pasteur pipette. From the
resulting cell suspension, cells were isolated by centrifugation
at 100 g for 3 min., and resuspended in 12 ml of culture medium.
Cell numbers were assessed using a hemocytometer, then innoculum

added to culture vessels to give a cell concentration of
approximately 10^5 cells per 1 ml of culture medium (5% fetal
calf serum). In early experiments cells were grown in 1 ml
of culture medium in Leighton tubes and later in 10 ml of
medium in milk dilution bottles.

Thereafter, in all experiments, cultures with pH
adjusted to 7.3 - 7.4, were incubated with a carbogen gas
phase for 24 hours at 37°C. The medium was then replaced with
fresh medium containing inducing compounds as indicated. After
a further period of incubation, the culture medium (containing
detached cells) was discarded and growing cells removed from
the glass by shaking gently with Earle's solution containing
1% Difco (1:250) trypsin. Cells were collected by centrifuging
at 1000 g for 5 min. In some cases, cell smears were micro-
scopically examined for porphyrin fluorescence under U.V.
light. Where indicated, cells were rapidly frozen, stored
at -15°C and later thawed and assayed for ALA synthetase
activity.

G. PREPARATION OF CELL SUSPENSIONS FROM MAMMALIAN LIVER

Guinea pig livers provided starting material for all
experiments on preparation of cell suspensions.

Mechanical preparation of liver cell suspension was
based on methods of Anderson [308] and Branster and Morton
[309]. Guinea pigs were anaesthetized with ether; the
abdominal and thoracic cavities were opened and the vena cava
cut; the liver was perfused under pressure with 0.25M sucrose

buffered with 0.01M Tris-HCl pH 7.4 until the liver was blanched and extended. In experiments reported, the perfusing medium was at 4°C; in some experiments medium at 37°C was used. The liver was then removed, cut into large pieces and dispersed with about 20-30 strokes of the pestle in a loose fitting Potter-Elvejhem homogenizer.

Separation in the presence of citrate was as above except that 0.027M sodium citrate was added to the perfusing medium.

Separation with trypsin. Liver was excised and cut into small pieces which were incubated with 0.25% Difco (1:250) trypsin in Ca, Mg free Hank's solution for 2 hours at 37°C. Remaining cell masses were dispersed by drawing the preparation in and out of a wide bore pasteur pipette.

Separation with tetraphenylboron was as described by Rappaport and Howze [310].

Separation with collagenase and hyaluronidase was as described by Howard and Pesch [311].

Separation with collagenase alone was by the method of Rodbell [312].

The yields of cells obtained by the procedures above were estimated by hemocytometer counts and viability by addition of 0.2% trypan blue or toluidine blue which stain cells with broken plasma membranes. For measurement of respiration, cells were collected by centrifugation at 500 g for a few minutes and resuspended in respiration medium.

H. PERFUSION OF RAT LIVER *IN SITU*

Collection of fresh rat blood. Rats were anaesthetized with ether or nembutal as indicated; heparin (0.1 ml = 100 units) was injected into the saphenous vein and the abdominal cavity opened by midline incision. Blood was collected by inserting a syringe into the vena cava or by drawing blood from the abdominal cavity as it drained from a cut vena cava. Blood was stored for use the same day in heparinized vials and filtered through nylon mesh before addition to the phosphate-bicarbonate Ringer. The complete standard perfusate was circulated for 30 min. before commencement of liver perfusion to allow equilibration with carbogen gas phase and to bring the perfusate to 37°C.

Perfusion apparatus was almost exactly as described by Hems *et al.* [313]. Hydrostatic pressure was generally 20-25 cm.

Operative technique was as described by Hems *et al.* [313] except that ether was used as anaesthetic in some experiments as indicated and the bile duct was not cannulated. When connection to the perfusion apparatus and removal of a zero-time liver sample was complete, the liver was covered to minimize cooling due to evaporation.

Sampling of perfused liver. In experiments in which mitochondrial ALA synthetase was assayed, whole liver lobes were removed as described by Hager and Kenney [314]. In later experiments in which ALA synthetase was assayed in unfractionated homogenates, small liver samples were taken as described by Seglen and Jervell [315].

CHAPTER III

EXPERIMENTS ON ASSAY OF ALA SYNTHETASE IN

LIVER PREPARATIONS

CHAPTER III

EXPERIMENTS ON ASSAY OF ALA SYNTHETASE IN LIVER PREPARATIONS

SECTION 1. RESULTS

Several methods for the assay of ALA synthetase have been described and used extensively but each of the published procedures has been designed for use with a particular enzyme preparation: it was therefore desirable to check that assay methods chosen were suitable for the present investigations. In this chapter some preliminary studies on the preparation of liver samples and on use of assay methods (developed for other tissues) with rat liver preparations are described. These experiments were designed to establish standardized procedures for use in later studies most of which involve rat liver samples.

Previous work in this laboratory [73,74] was directed towards finding a method of assay for ALA synthetase which was sensitive, specific and reproducible: a radiochemical assay method with these characteristics was developed [73] and optimal incubation conditions for use with the more convenient colorimetric assay of Granick [80] were also established. The assay methods used in the present study were based on these findings.
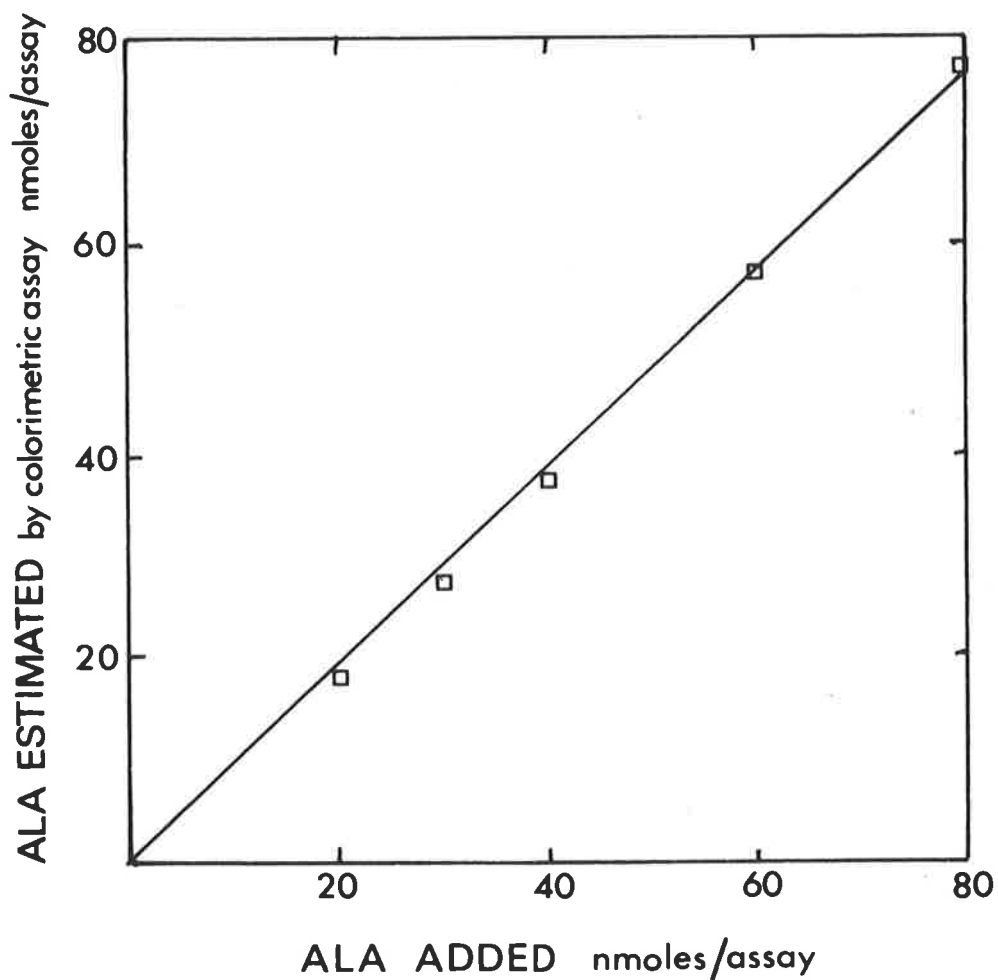
FIG. 3-1.   Standardization of the colorimetric assay using chemically prepared ALA.   See text for experimental procedure.

———————   amount standard ALA added (95% pure)

□   amount ALA calculated from optical densities using formula in Materials and Methods.

A. STANDARDIZATION OF THE COLORIMETRIC ASSAY PROCEDURE.

In the majority of experiments described in later chapters, where relatively large tissue samples were available, the more rapid and convenient colorimetric assay (see Materials and Methods) was used to assess changes in the level of ALA synthetase. The estimation of ALA formed in enzyme assays depends upon conversion of ALA to ALA pyrrole and the subsequent reaction of the pyrrole with Ehrlich's Reagent to give a red colour. A correction is made for colour due to aminoacetone by an ether extraction step in which most of the interfering aminoacetone pyrrole is removed. The amount of ALA formed in the original incubation mixture is then calculated, using the formula given in Materials and Methods. The validity of this calculation was checked by applying the colorimetric procedure to samples containing known amounts of chemically prepared ALA. As shown in Fig. 3-1, the amounts of ALA calculated from optical densities obtained by the colorimetric procedure corresponded closely to the actual amounts of ALA.

Further experiments using the colorimetric method for estimation of ALA were carried out to establish standard incubation conditions for assay of various rat liver cell fractions. Because ALA synthetase activity is low in normal liver, these experiments were done, in most cases, with preparations from rat livers in which ALA synthetase had been preinduced *in vivo* with AIA. The considerable variety of incubation conditions used by different workers for assay of ALA synthetase, in general fall into two categories which
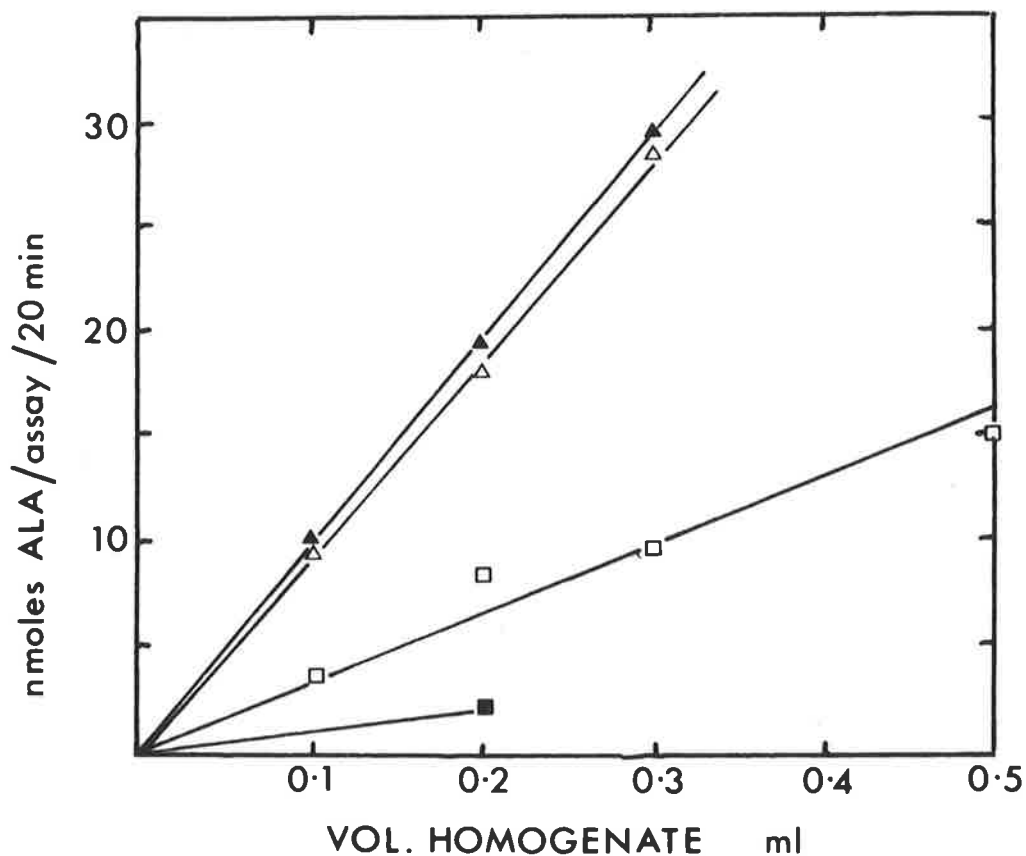
FIG. 3-2. Comparison of results obtained for estimation of ALA synthetase in homogenates using different incubation conditions for assays. A homogenate was prepared from the liver of a rat killed 28 hr after AIA administration.

Δ————————Δ   incubation conditions as in Materials and Methods; fresh homogenate

▲————————▲   incubation conditions as in Materials and Methods; frozen and thawed homogenate

□————————□   incubation conditions of Marver *et al*. [316]; fresh homogenate

■————————■   incubation conditions of Marver *et al*. [316]; frozen and thawed homogenate

differ in the means employed for generation of the substrate,
succinyl CoA. In procedures used by Irving and Elliott [73,74]
and others [18,65] a bacterial succinyl CoA-generating system
is added while some groups [e.g., 70,314] have used assays
for fresh liver preparations which depend on endogenous (mito-
chondrial) generation of succinyl CoA. The most commonly used
procedure in the second category is that of Marver *et al.* [314];
results obtained by preparing and assaying homogenates exactly
as described by these authors, are compared in Fig. 3-2 with
results obtained by the procedures listed in Materials and
Methods and based on those of Irving and Elliott [73,74].
The effect of freezing and thawing samples before assay is also
shown. In our hands, the method of Marver *et al.* [314] gave
significantly lower results and in further work the incubation
conditions of Irving and Elliott were used. This had the added
advantage as shown in Fig. 3-2, of allowing assay of frozen and
thawed samples since succinyl CoA generation was not dependent
on the presence of intact mitochondria.

Figs. 3-3 to 3-8 show the results of experiments
designed to establish the times of incubation and volumes of liver
preparations for which the colorimetric assay gives a linear
response. Variation of product formed with time of incubation,
shown in Fig. 3-3 (homogenates), Fig. 3-5 (mitochondria) and
Fig. 3-7 (post-mitochondrial supernatant), was similar for all
fractions tested, increasing in a linear fashion for 20-30 minutes
but thereafter at a decreasing rate. This deviation from linearity
at later times probably reflects the relative instability of ALA
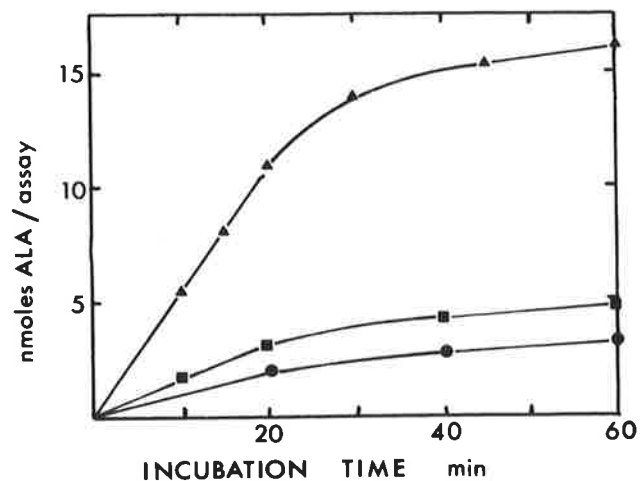
FIG. 3-3.   Estimation of ALA synthetase activity
in rat liver homogenates by the colorimetric assay.
Variation of ALA fomred with incubation time.   Assay
procedure was described in Materials and Methods.
Homogenates tested were prepared from rats pre-
treated as indicated.

●————●	untreated
■————■	preinduced for 2 hr *in vivo* with progesterone, 200 mg/kg
▲————▲	preinduced for 16 hr *in vivo* with AIA, 350 mg/kg.
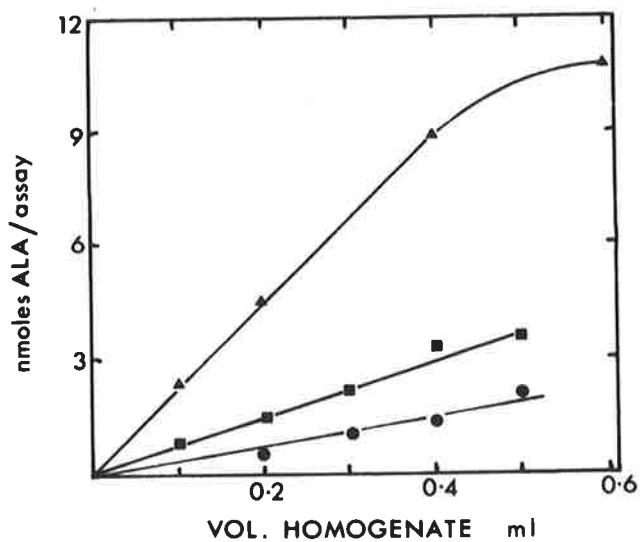
FIG. 3-4.   Estimation of ALA synthetase activity in
rat liver homogenates by the colorimetric assay:
variation of ALA formed with volume of homogenate.
Procedure was as in Fig. 3-3.

●————●	untreated
■————■	preinduced with progesterone
▲————▲	preinduced with AIA.
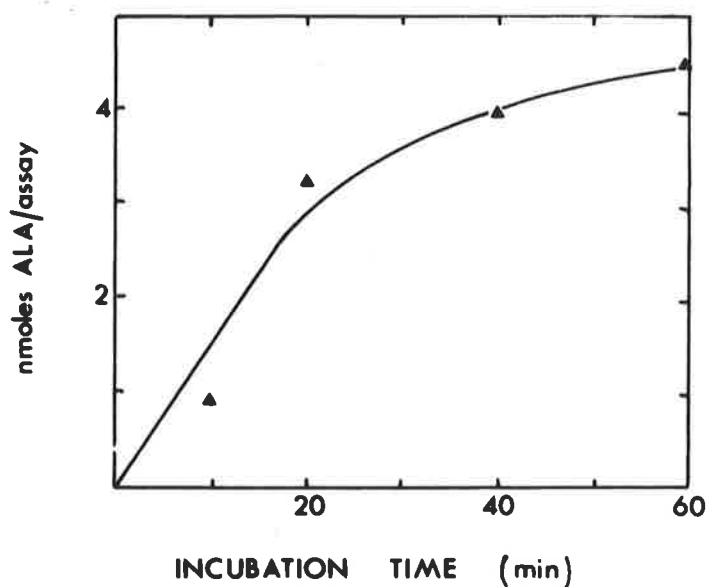
FIG. 3-7. Estimation of ALA synthetase activity
in rat liver post-mitochondrial supernatants:
variation of ALA formed with incubation time.
Post-mitochondrial supernatant was prepared from
liver of rat killed 6 hr after AIA administration
and assayed as described in Materials and Methods.
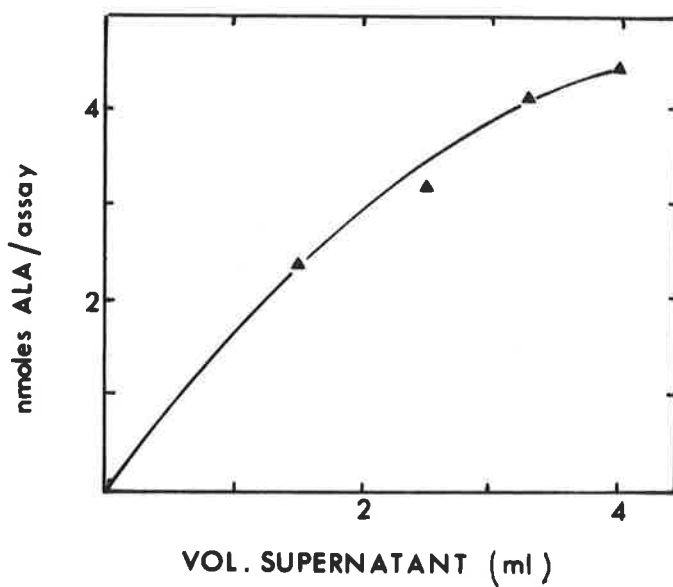
FIG. 3-8. Estimation of ALA synthetase activity
in rat liver post mitochonrial supernatants:
variation of ALA formed with volume of supernatant.
Procedure was as in Fig. 3-7. Incubation times of
60 min. were used.

synthetase since preincubation of homogenates for 20 minutes
at 37°C was found to cause a significant loss of activity in
subsequent assays. To test the possibility that linearity of
assays might vary according to the nature of pretreatment, the
variation of ALA formation with incubation time was also
measured with homogenates from progesterone-induced and
untreated livers (Fig. 3-3). The behaviour of these preparations
paralleled that observed with liver homogenates from AIA-treated
rats.

Fig. 3-4 shows that with a 20 minute incubation time,
ALA formed was proportional to the amount of unfractionated
homogenate up to 0.4 ml (corresponding to 40 mg wet weight of
liver or 10 mg of protein per 1 ml assay) of 10% homogenates
prepared from AIA-treated, progesterone-treated or untreated
livers. As shown in Fig. 3-6, the assay, using either 20 or
60 minute incubations, was linear for volumes of mitochondrial
suspension up to 0.2 ml (corresponding to approximately 0.1 g
wet weight of liveror 5 mg of protein). In the case of
post-mitochondrial supernatants, activity was generally quite
low and to measure activity reproducibly, it was necessary to
assay volumes of 0.4 - 0.5 ml (40 - 50 mg wet weight of liver
or 5 - 7 mg of protein); at this tissue concentration, ALA
formation was not proportional to the amount of supernatant
added (Fig. 3-8). Thus in assays of post-mitochondrial super-
natants, ALA synthetase activity was underestimated in relation
to other cell fractions.

TABLE 3-1: EFFECT OF STORAGE OF LIVER HOMOGENATES ON ALA

SYNTHETASE ACTIVITY

TREATMENT	% ACTIVITY
Frozen once and immediately thawed	[100]
Fresh (not frozen)	86
Frozen, stored one day, thawed	95
Frozen, stored two days, thawed	92
Frozen, stored one week, thawed	90
Twice frozen and thawed	92

A liver homogenate was prepared from a rat 26 hours after AIA administration. Activity was assayed in aliquots after storage as shown and compared to activity in samples which had been rapidly frozen and thawed immediately. All values are averages of determinations made in triplicate.

B. EFFECTS OF DIFFERENT PROCEDURES FOR PREPARATION AND STORAGE OF LIVER SAMPLES.

Previous work in this laboratory [317] established that in guinea pig liver fractions prepared in PEST buffer (see Materials and Methods), ALA synthetase was relatively stable if

fresh samples were rapidly frozen and stored at -15°C. Table
3-1 shows the effects of freezing and thawing and of various
periods of storage at -15°C on enzyme activity in rat liver
homogenates. Freezing and thawing samples once, slightly
increased activity in this experiment (cf. Fig. 3-2). A second
freeze-thaw, or storage at -15°C for periods of up to a week
resulted in only slight loss of activity.

In the course of some experiments with liver from
untreated rats it was noticed that where liver samples were
allowed to stand at 0-4°C in PEST buffer for some time before
homogenization, the enzyme activity detected in subsequent
assays was apparently higher than where liver samples were
homogenized immediately. To check this observation a series
of experiments was carried out in which a single liver was
removed from a starved (but otherwise untreated) rat, briefly
cooled in iced buffer then rapidly cut into slices. A part of
the liver (combining slices from different lobes) was immediately
homogenized and frozen using a dry ice-ethanol bath. The
remaining liver was stored in PEST buffer at 0-4°C; after
various times portions were removed, homogenized and frozen.
Enzyme activity and protein were later assayed. The means of
results from a number of such experiments, expressed as change
in activity, are shown in Fig. 3-9. Standing resulted in a small
but significant rise in activity over the first hour followed
by a fall in the second hour to approximately the original
level. The maximum activity corresponded to an increase over
zero-time levels of 50 - 100% in different experiments. When
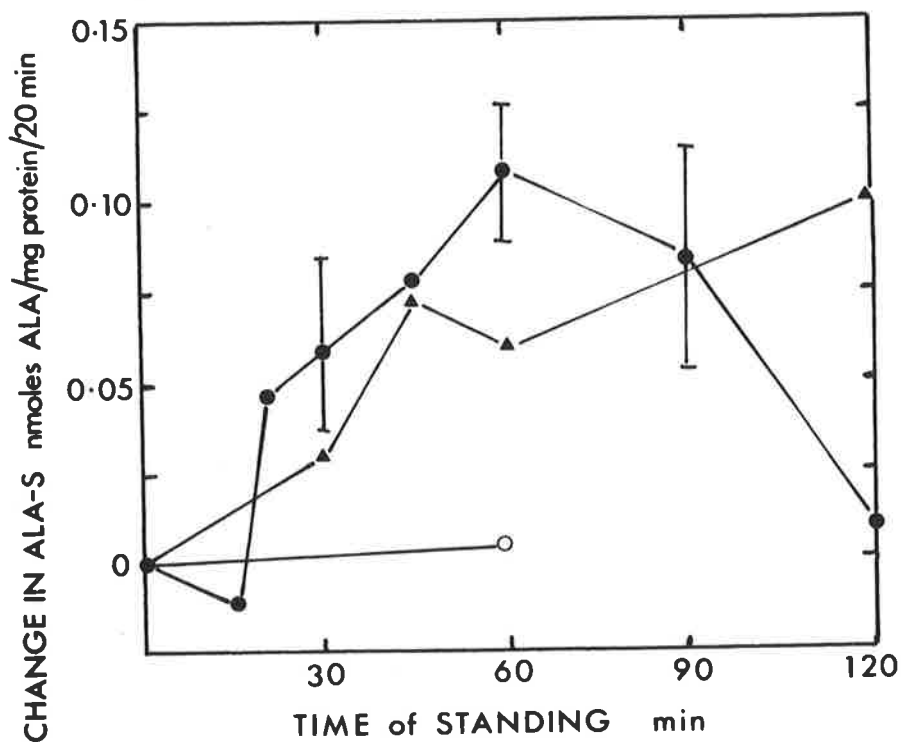
FIG. 3-9. Effect of standing at 4°C on total
ALA synthetase activity in liver slices. See text
for experimental procedure. Activity in homogenates
was assayed by the colorimetric method. The numbers
in brackets in the legend indicate the number of
experiments contributing to each curve. Standard
deviations of means indicated by the vertical
bars were calculated where four experiments
contributed to the mean.

●━━━━━●   liver from fasted but otherwise
                untreated rats [4]

▲━━━━━▲   liver from rats preinduced for
                4 hr with AIA *in vivo* [2]

○━━━━━○   liver from untreated rats,
                homogenized before period
                of standing [ ].

the same experimental procedure was followed using rats
pretreated with AIA, a small rise over the preinduced level of
activity was observed upon standing (Fig. 3-9); this was
similar in magnitude to the increase in untreated liver but
represented only a small percentage increase (15%) over the
preinduced level of 0.7 mµmoles ALA/mg protein/20 min.
The change in activity was not observed when samples of
untreated liver were homogenized before, rather than after
the period of standing at 0-4°C (Fig. 3-9).

SECTION 2. DISCUSSION

The experiments described in this chapter were to provide information necessary to define standard procedures for preparing, storing and assaying rat liver samples in an investigation of the regulation of ALA synthetase. The results together with previous work in this laboratory [73,74, 317] suggest that the assay conditions developed by Irving and Elliott are appropriate for assay of ALA synthetase in homogenates or mitochondrial suspensions prepared from rat liver. The incubation times and tissue concentrations for which assays of these fractions are linear were also established. In assays of post-mitochondrial supernatants, the variation of product formed with volume of enzyme was found to be non-linear in the range where activity was sufficient to measure reproducibly. Assays of this fraction thus provide only a relative measure of activity: the results suggest that that the true activity would be underestimated. Poor linearity may result from the presence of ALA dehydratase [3] in the supernatant fraction even though EDTA (10 mM) was added to assay mixtures to inhibit this reaction.

The results in Table 3-1 establish that homogenates can be rapidly frozen and stored at -15°C without major losses in activity. However, an unexpected source of variation in preparation of liver samples was revealed in experiments where slices of fresh liver were allowed to stand in buffer at 0-4°C before being homogenized (Fig. 3-9). Because standing led to

increases in activity over the level detectable in freshly

excised liver it was essential to homogenize samples soon after

excision so that activity in later assays reflected only

experimental manipulations (*in vivo* or in perfusion) before

sampling, without additional variations due to the method of

preparation. In studies reported in later chapters, liver

samples were routinely allowed to stand at 4°C for 10 minutes,

then homogenized and rapidly frozen. Little increase in activity

occurs over this short period of standing (Fig. 3-9).

The observed increases in activity with standing have

not so far been investigated in detail although the phenomenon

may well reflect a physiologically important process. An

effect which doubles the basal activity of the rate limiting

enzyme could provide fine control on the rate of heme biosynthe-

sis. The results to date suggest that the increase in activity

occurs in intact tissue but not after homogenization. In this

respect it differs from 'spontaneous activations' of ALA

synthetase observed when homogenates of semi-anaerobically grown

Rh. sperhoides were allowed to stand at 4°C [23] or when extracts

of cultured soybean callus were stored at 4°C [10]. This

difference might be more apparent than real: for instance,

an 'activation' in homogenates of rat liver might be counter-

acted by increased degradation of ALA synthetase. Discussion

of possible mechanisms for the observed activity-increase must

be purely speculative at present. It might be expected that

an increase occurring at 4°C would represent an 'activation'

rather than 'induction'. An activation of ALA synthetase in

mammalian liver could be analogous to activation of the enzyme
in *Rh. spheroides* (see Chapter 1, p.13; also 23,71) or
perhaps involve the heat-stable inhibitor in extracts of
mammalian liver reported by a number of groups [13,73,71]. On
the other hand, some effect such as release of preformed
ALA synthetase from polysomes might be involved. The numerous
possibilities remain to be investigated.

CHAPTER IV

STUDIES ON CONTROL OF ALA SYNTHETASE IN ISOLATED CELLS:

PRELIMINARY EXPERIMENTS

CHAPTER IV

STUDIES ON CONTROL OF ALA SYNTHETASE IN ISOLATED CELLS:
PRELIMINARY EXPERIMENTS

As discussed in Chapter I, a review of the existing literature on control of the level of ALA synthetase in liver cells suggested that for further investigations of control at a molecular level it was desirable to establish appropriate *in vitro* system(s) in which the level of ALA synthetase could be both measured directly and changed by various experimental perturbations. Furthermore it seemed desirable that such *in vitro* system(s) should be derived from mammalian liver since a major reason for undertaking any study of regulation of ALA synthetase was the possible relevance of the findings to the human hereditary hepatic porphyrias.

Previous work in this laboratory [73,74] established a radiochemical method for the assay of ALA synthetase more sensitive and specific than any previously described. Since with this assay it was possible to estimate ALA synthetase activity in quite small samples of tissue it became feasible to directly measure the enzyme level in small cell cultures or suspensions.

A. PRELIMINARY EXPERIMENTS WITH CHICK EMBRYO LIVER TISSUE CULTURE: RESULTS AND DISCUSSION.

Because of the lack of any previous experience with cell suspensions or cultures in this laboratory, it seemed desirable to establish the necessary techniques with a system which had been studied extensively by other workers. The primary tissue culture system derived from embryonic chick liver developed by Granick [80] and subsequently used in studies on control of ALA synthetase by a number of groups [7,87,88,90-92,96,102] appeared to be ideal for such preliminary studies. Furthermore at the time when this work was begun, changes in the level of ALA synthetase in tissue culture had not been directly measured but had been inferred from porphyrin formation as judged by fluorescence or estimation of extracted porphyrins [e.g., 80,86]. It seemed that direct measurement of enzyme activity by the radiochemical assay might provide more precise information on control of ALA synthetase.

This phase of the present studies was not successful despite prolonged efforts. The results obtained are therefore reported only briefly below.

Initial attempts to establish viable cultures using the methods of Granick [80] were unsuccessful. On the advice of local tissue culture experts some modifications were made in the methods for preparing cultures - these involved mainly less severe conditions for dissociating the embryonic liver and resuspension of cells to remove trypsin before placing the dissociated cells into culture medium. The culture medium

used was the same as that of Granick [80] except that a lower
concentration (5%) of fetal calf serum was used.

Cells prepared by this modified method (see Materials
and Methods for details) attached to the glass of Leighton
tubes and grew for about 3 days with filmy processes at the
edges of colonies, typical of healthy cultures. After 2-3
days some cells began to round up and detach from the glass.
Incubation of one-day-old cultures for a further 12-24 hours
with ALA or AIA occasionally resulted in appearance of weak
red fluorescnece when cultures were examined under U.V. light*.
For direct radiochemical assay of ALA synthetase in small
1 ml cultures, it was necessary to use [^{14}C] succinate with
very high specific activity. To provide a greater amount of
tissue for assay, attempts were made to grow cultures on a
larger scale. After considerable difficulty mainly involving
maintenance of correct pH, larger scale cultures were success-
fully established in 100 ml 'milk dilution bottles'. An
initial innoculum of 10^6 cells was grown in 10 ml of culture
medium. Cell counts indicated the cells divided 2-3 times over
48 hours, yielding 10-15 mg cells for enzyme assays. Cultures
incubated with ALA or AIA fluoresced weakly or not at all

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*It is possible that the apparently poor fluorescence by
 these cultures reflected an inadequate optical set-up since
 in some later experiments where a modified optical system
 was used, bright red fluorescence *was* seen after incubation
 of cultures with ALA. This possibility, however, does not
 alter the validity of direct assays of ALA synthetase activity,
 which were the main object of the work.

under U.V. light.

Table 4-1 shows that larger scale cultures prepared by the modified procedure actively incorporated labelled amino acids into TCA-insoluble material. Amino acid uptake was prevented by cycloheximide suggesting that uptake reflected active protein synthesis.

Table 4-2 shows results of experiments in which one-day-old cultures were incubated for a further period (8-22 hours) with AIA or other drugs. ALA synthetase activity was measured using the radiochemical assay. The results represent relative activities expressed in arbitrary units (counts per minute, minus background, in a small aliquot of final methanol extract). The results show that AIA at concentrations about 100 μg/ml caused a 3-4 fold increase in ALA synthetase activity over 22 hours. This increase was prevented by a high concentration of cycloheximide. The induction by AIA was considerably less than the 10-40 fold induction which might have been expected from the previous semiquantitative experiments of Granick [80] and from recently published studies with the chick tissue culture system in which ALA synthetase was directly assayed [87,88,90]. Other drugs also failed to cause the dramatic inductions reported elsewhere [6,80].

Fig. 4-1 shows the effect on the level of ALA synthetase, of incubating one-day-old cultures with 100 μg AIA/ml for varying times. The shape of the curve is similar to that reported by Incefy and Kappas [90] but the extent of induction as noted above, is considerably less.

TABLE 4-1:   AMINO ACID UPTAKE BY CULTURED CHICK EMBRYO

LIVER CELLS

Additions	^{14}[C] amino acids in acid-insoluble material : cpm/mg protein		
	0 min.	40 min.	90 min.
None	696	24,971	58,778
Cycloheximide, 20 μg/ml	1069	2,741	2,421

Chick embryo liver cells were prepared and grown for 45 hours by the procedure given in Materials and Methods. The culture medium was replaced with Krebs phosphate-ringer plus ^{14}[C] algal hydrolysate and cultures incubated at 37°C. After times shown, ^{14}[C] amino acid incorporation to acid-insoluble material was estimated as described in Materials and Methods.

90.

TABLE 4-2: EFFECT OF DRUGS ON ALA SYNTHETASE ACTIVITY IN CULTURED CHICK EMBRYO LIVER CELLS.

Additions, µg/ml	ALA synthetase activity cpm/0.1 ml methanol extract
I. None	47
AIA, 50	126
AIA, 100	146
AIA, 150	91
AIA, 100 + cycloheximide, 250	4
II. None	34
AIA, 100	135
III. None	24
AIA, 100	129
Sedormid, 200	147
DDC (saturated solution)	27

Three batches (I, II and III) of chick embryo liver cells were prepared and grown for 24 hours as described in Materials and Methods; additions as shown were made and cultures grown for a further 22 hours. ALA synthetase activity was estimated by radiochemical assay as described in Materials and Methods. Each result is the average of activities measured in 3 cultures.
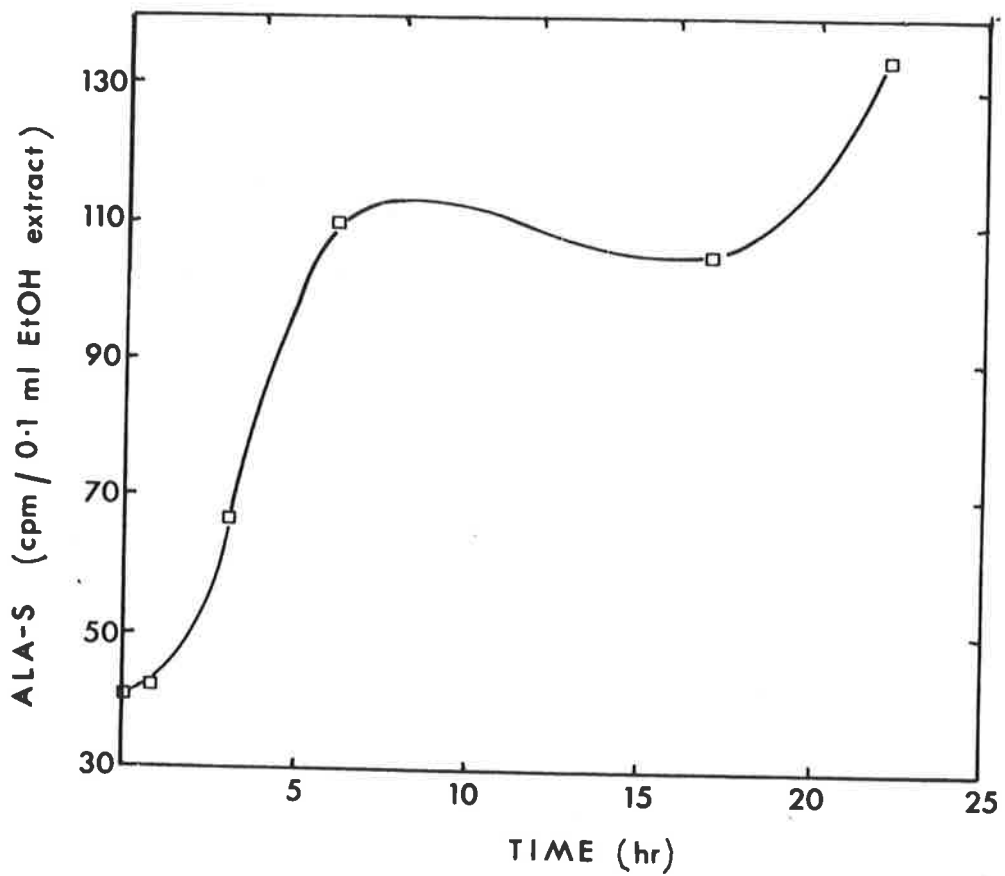
FIG. 4-1. Effect of AIA on total ALA synthetase activity in cultured chick embryo liver cells. Procedure was as in Table 4-2, except that cells were isolated and frozen after varying periods as indicated.

☐————————☐   + AIA, 100 µg/ml

In further experiments similar to those above, it was found that variation in the age of chick embryos used to provide liver cells, in the concentration of trypsin or time of incubation used in the liver disaggregation step or in concentrations of the culture medium components, fetal calf serum or glutamine, all failed to cause any marked improvement in the extent of induction by AIA. Since the tissue culture system was being used primarily as a convenient pilot system for gaining experience rather than for a major investigation *per se*, it was decided not to proceed further with work on it.

The failure to obtain more than about 3-fold induction in culture chick liver cells was not regarded as necessarily significant in relation to the mechanism of ALA synthetase regulation since various trivial explanations of this failure were possible. Schneck *et al*. [96] noted that the extent of induction by drugs in cultured chick liver cells varied over an 8-fold range with different batches of serum used in the culture medium; Granick [318] noted a similar phenomenon. It is thus possible that batches of fetal calf serum used in the present experiments lacked components necessary for induction or contained inhibitory factors. However, the only conclusion to be drawn from this work is that achievement of successful growth of cells is not a sufficient prerequisite for major induction of ALA synthetase.

B. EXPERIMENTS WITH CELL SUSPENSIONS PREPARED FROM ADULT GUINEA PIG LIVER: RESULTS AND DISCUSSION.

In parallel with the chick tissue culture studies, an attempt was made to establish an experimental system involving cells derived from adult mammalian liver. It was desirable that such cells should exhibit properties similar to those of cells in intact liver and also that it should be possible to obtain a uniform preparation of cells large enough so that several different experiments could be conducted with a single preparation.

Other workers have found that normal adult mammalian liver cells do not grow in tissue culture. However, liver slices or isolated liver cells can be maintained in a viable state for a few hours. Because of its short half life, changes in the level of ALA synthetase occur rapidly: it was therefore possible that control of ALA synthetase might be studied in cell suspensions or liver slices maintained in culture medium for a few hours.

(I) Comparison of Methods for Preparing Cell Suspensions.

Several methods for preparing cell suspensions from intact mammalian tissues have been described [e.g., 308-312]. Before proceeding with studies on ALA synthetase it was desirable to establish which method of preparation gave isolated cells most closely resembling cells in the intact tissue. Cell suspensions prepared using different disaggregation procedures were compared to determine the
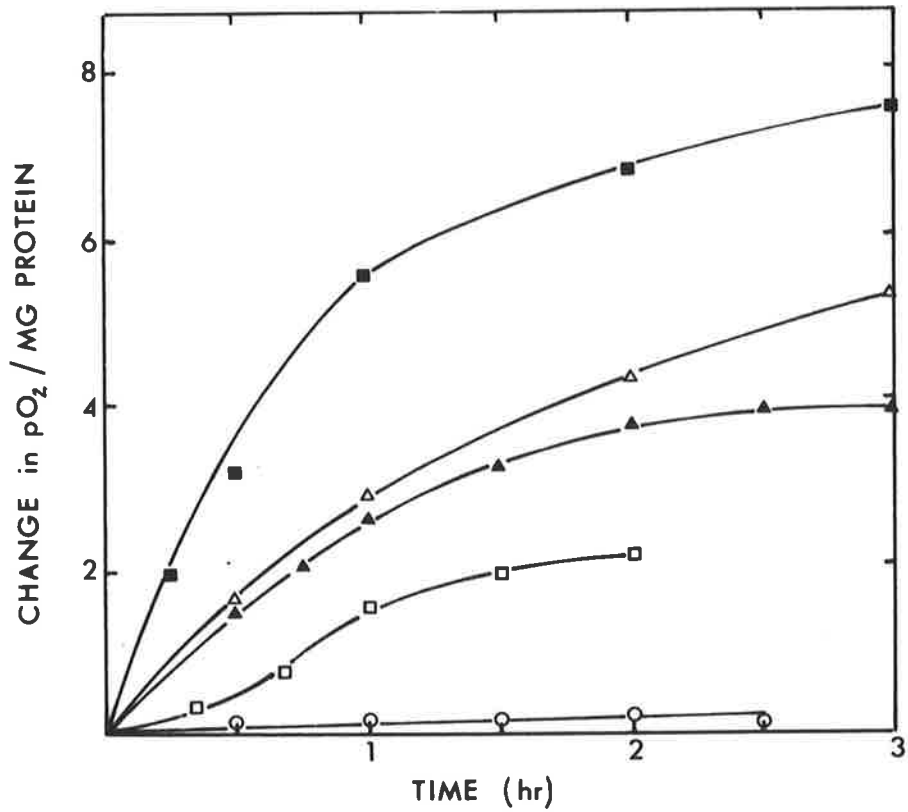
FIG. 4-2.   Respiration by isolated guinea pig
liver cells prepared by different methods compared
to respiration by liver slices.   Respiration rates
were determined as described in Materials and
Methods.   Results are expressed in arbitrary units
per mg cell protein.

■———■	liver slices
△———△	cell suspension, prepared with collagenase alone
▲———▲	cell suspension, prepared with collagenase plus hyaluronidase
□———□	cell suspension prepared with citrate
O———O	cell suspensions prepared mechanically, with trypsin or with tetraphenylboron.

yields of isolated cells, the viability of cells as indicated by staining with toluidine blue or trypan blue and the metabolic activity as indicated by respiration rate, measured manometrically. Oxygen uptake of various cell preparations compared with that of liver slices is shown in Fig. 4-2. The various methods for preparing cell suspensions are given in detail in Materials and Methods. For manometry experiments, cells were maintained in a buffered salt solution with lactate present as a substrate.

Cells prepared using mechanical separation, incubation with Difco trypsin at 37°C or incubation with tetraphenylboron at 4°, 25° or 37°C all failed to exclude toluidine blue indicating damage to cell membranes and in all cases respiration was negligible. When livers were perfused with a cold Ca^{2+} and Mg^{2+} free Hanks solution containing 27mM citrate and then disaggregated by mild mechanical means, there was a good yield of isolated cells but the majority of these failed to exclude toluidine blue or trypan blue. With these cells significant oxygen uptake was observed but was only about 30% of the rate in liver slices.

Recently, Howard and Pesch [311] described a method for disaggregation of rat liver using a crude preparation of collagenase, together with hyaluronidase. Incubation of guinea pig liver with either collagenase alone or with collagenase plus hyaluronidase produced relatively low yields of isolated cells (about 20% of original tissue) but about 95% of these cells excluded toluidine blue or trypan

blue and their respiration rate was about 50-60% of the rate in liver slices. Direct comparison of respiration in suspensions prepared with collagenase with and without hyaluronidase, suggested that particularly after longer periods of cell maintenance, oxygen utilization was slightly greater in cells prepared with collagenase alone.

The suitability of cell suspensions prepared with collagenase for studies on enzyme induction was further tested by assessing the ability of cells to incorporate amino acids. For this and later experiments, cell suspensions were incubated in Krebs phosphate Ringer to which glucose and 1% albumin were added. Respiration in this improved medium was somewhat greater than in the buffered salt solution used in most manometry experiments. In cell suspensions in the new medium, ^{14}[C]valine incorporation into TCA-precipitable material was found to be essentially linear over 3 hours as shown in Fig. 4-3; amino acid incorporation was prevented by cycloheximide.

The findings on the relative viability and respiration rates of cell suspensions prepared by different methods were in general, consistent with those of other workers. Electron microscope studies [319] have revealed broken plasma membranes in cells prepared by mechanical means, with or without citrate and several groups have reported leakage of enzymes from such cells [e.g., 320-2]. Loss of cytoplasmic factors probably account for impaired protein-synthetic capacity and low respiration rates of cells prepared mechanically [309,324,325] or with the complexing agents,
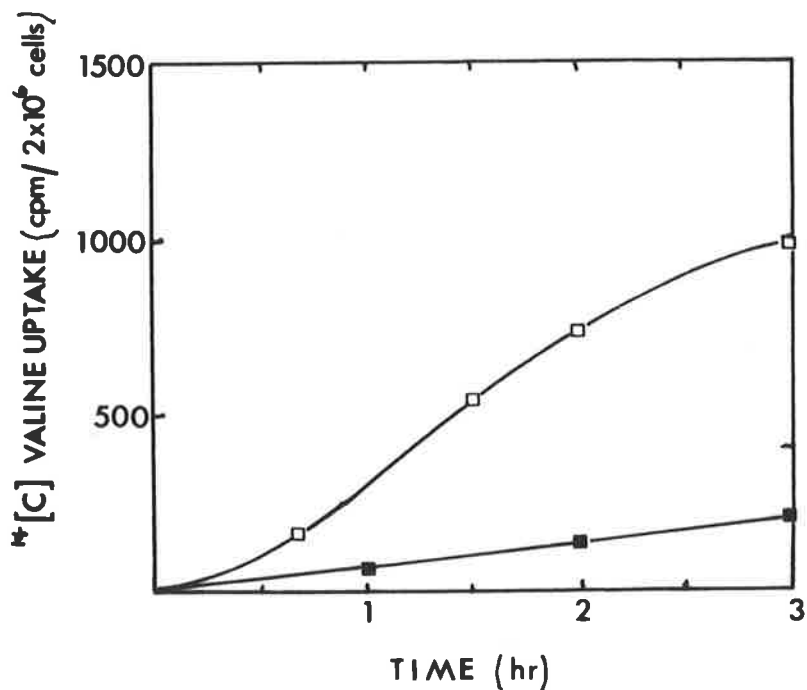
FIG. 4-3. Amino acid incorporation by guinea pig liver cell suspensions prepared using collagenase. One ml of suspension medium containing 2×10^6 cells plus 1 μC ^{14}[C] valine was incubated with gentle shaking at 37°C for the times indicated. Amino acid incorporation was estimated as described in Materials and Methods. Each point is the average of three experiments.

□————□ cells alone

■————■ cells + cycloheximide, 10^{-4}M.

citrate or tetraphenylboron [326]. By contrast, cells prepared with collagenase and hyaluronidase have recently been shown by others to exclude trypan blue [311], to respire at a greater rate than cells prepared by other methods [311, 324] and to incorporate amino acids linearly for more than three hours [324]. Thus on the basis of the experiments above and the findings of others it was concluded that cell suspensions prepared enzymically either with collagenase and hyaluronidase or with collagenase alone were most suitable for further studies.

(II) Effect of ALA on the Level of ALA Synthetase in Liver Cell Suspensions.

Table 4-3 shows the effect on the level of ALA synthetase of incubating liver cell suspensions for 2 hours with increasing concentrations of AIA. Hydrocortisone was also added because of its reported permissive and/or stimulatory effects on induction by AIA *in vivo* [141,142]. Concentrations of AIA about 100-200 µg/ml caused a 5-fold induction of ALA synthetase (assayed by the radiochemical method) over the 2 hour incubation period.

Fig. 4-4 shows results from ~~three~~ two experiments in which cell suspensions were incubated at 37°C with AIA (100 µg/ml and hydrocortisone (10 µg/ml) for varying periods. AIA caused a rapid increase in enzyme activity over the first two hours of incubation but thereafter the level of activity fell. Maximal activity after two hours corresponded to a 4-5 fold

TABLE 4-3:   EFFECT OF INCREASING CONCENTRATIONS OF AIA ON

THE LEVEL OF ALA SYNTHETASE IN LIVER CELL

SUSPENSIONS.

Concentration of AIA μg/ml	ALA Synthetase Activity cpm/0.1 ml methanol extract
0	43
20	80
50	91
100	212
200	219
300	142

A cell suspension was prepared with collagenase [312]

and 2×10^6 cells in 1 ml of suspension medium containing

20 μg/ml hydrocortisone and AIA as shown, were incubated for

2 hours at 37°C. Suspensions were chilled and cells, isolated

by centrifugation, were rapidly frozen. Cells were later

thawed and assayed using the radiochemical method.

increase over the basal level. Little increase in activity

occurred when suspensions were incubated in the absence of AIA.

These preliminary results suggested that the cell

suspension system was potentially useful for studies on control
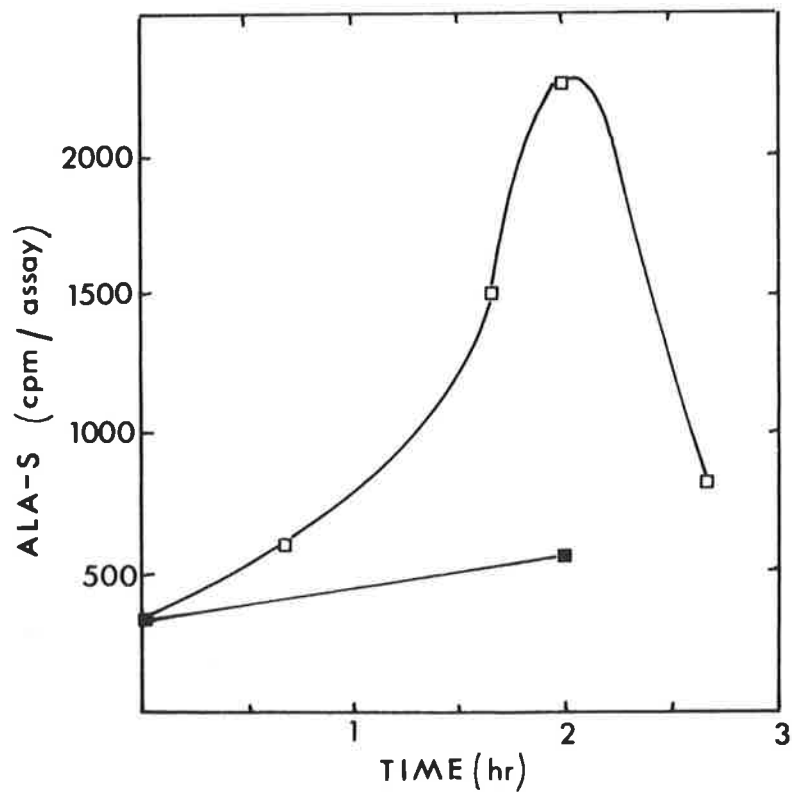
FIG. 4-4. Effect of AIA plus hydrocortisone on ALA
synthetase activity in guinea pig liver cell suspen-
sions prepared with collagenase. Procedure was as
in Table 4-3 except that cells were frozen after
varying periods as indicated. Each point is the
average of two experiments.

■————■ no additions

□————□ + AIA, 100 µg/ml + hydro-
cortisone,10µg/ml.

of ALA synthetase in mammalian liver. The main disadvantage of the existing system was the relatively low yield of cells (in our hands) in the disaggregation step. The poor yield made it necessary to work with small cell numbers and hence with either high specific activity ^{14}[C]succinate or low counts in the final steps of the radiochemical assay. Another apparent disadvantage was the fact that induction by AIA did not continue after two hours incubation as would be expected from *in vivo* studies (e.g., see Chapter V, Section A).

Further attempts to exploit the liver cell suspension system were deferred in favour of studies with perfused rat liver as described in the following chapter.

CHAPTER V

DEVELOPMENT OF A RAT LIVER PERFUSION SYSTEM

FOR STUDIES ON THE REGULATION OF ALA

SYNTHETASE - RESULTS

CHAPTER V

DEVELOPMENT OF A RAT LIVER PERFUSION SYSTEM

FOR STUDIES ON THE REGULATION OF ALA

SYNTHETASE - RESULTS

A. PRELIMINARY STUDIES ON INDUCTION *IN VIVO* BY AIA OF ALA
SYNTHETASE IN RAT LIVER

The primary objective of work described in this Chapter was
the development of a liver perfusion system in which control of
ALA synthetase could be studied in a mammalian liver at the
level of the isolated organ. A preliminary investigation of the
effects of AIA *in vivo* was carried out despite the fact that
induction by this compound has been studied in considerable detail
by other workers [6,53,65,70,98,150]: previous reports show marked
strain differences, within a given species, in the response of
hepatic ALA synthetase to induction by drugs [327]. This response
may also be dependent on age [328-9], nutritional status [24,
98,258] previous exposure to drugs [163] and possibly the sex of
experimental animals [163,247]. It therefore seemed desirable to
establish for the rats used in this study appropriate conditions
both for maximal *in vivo* induction as a basis for selecting an
appropriate strain of rats and their pretreatment for perfusion
studies and *in vivo* figures for comparison with findings in
perfused liver.

In all *in vivo* experiments with AIA, rats were
injected subcutaneously with 350 mg AIA/kg body weight.
This dose was shown to be optimal by Marver *et al.* [98] and
Hayashi *et al.* [65]. Table 5-1 compares induction of
mitochondrial ALA synthetase in three strains of rats

TABLE 5-1:   INDUCTION OF MITOCHONDRIAL ALA SYNTHETASE BY

AIA *in vivo* IN RATS FROM DIFFERENT COLONIES.

Rat species and source	No. of experiments	Mitochondrial ALA synthetase activity nmoles/g liver/hr
Wistar (albino), Waite Inst.	13	148 $\pm$ 10
Wistar (hooded), Waite Inst.	2	45 $\pm$ 15
Wistar (albino) IMVS	6	69 $\pm$ 20

Starved rats (males, 180-220 g) were injected s.c. with AIA.
After 13 hours, rats were killed; livers were removed and
mitochondria prepared and assayed by the colorimetric
procedure as described in Materials and Methods. Basal
enzyme levels were similar in all rats tested.

available for study. The large differences in extent of
induction were not investigated in detail: they may reflect
genetical differences or possibly, despite uniform fasting
before use, differences in nutritional status (e.g., in
vitamin E levels [24]) or different rates of metabolism of
AIA resulting from previous exposure to insecticides [162].
Albino Wistar rats from Waite Institute Central Animal House
were used in further studies unless otherwise stated.

Fig. 5-1 summarizes data from several experiments
on induction of the mitochondrial enzyme in rats of varying
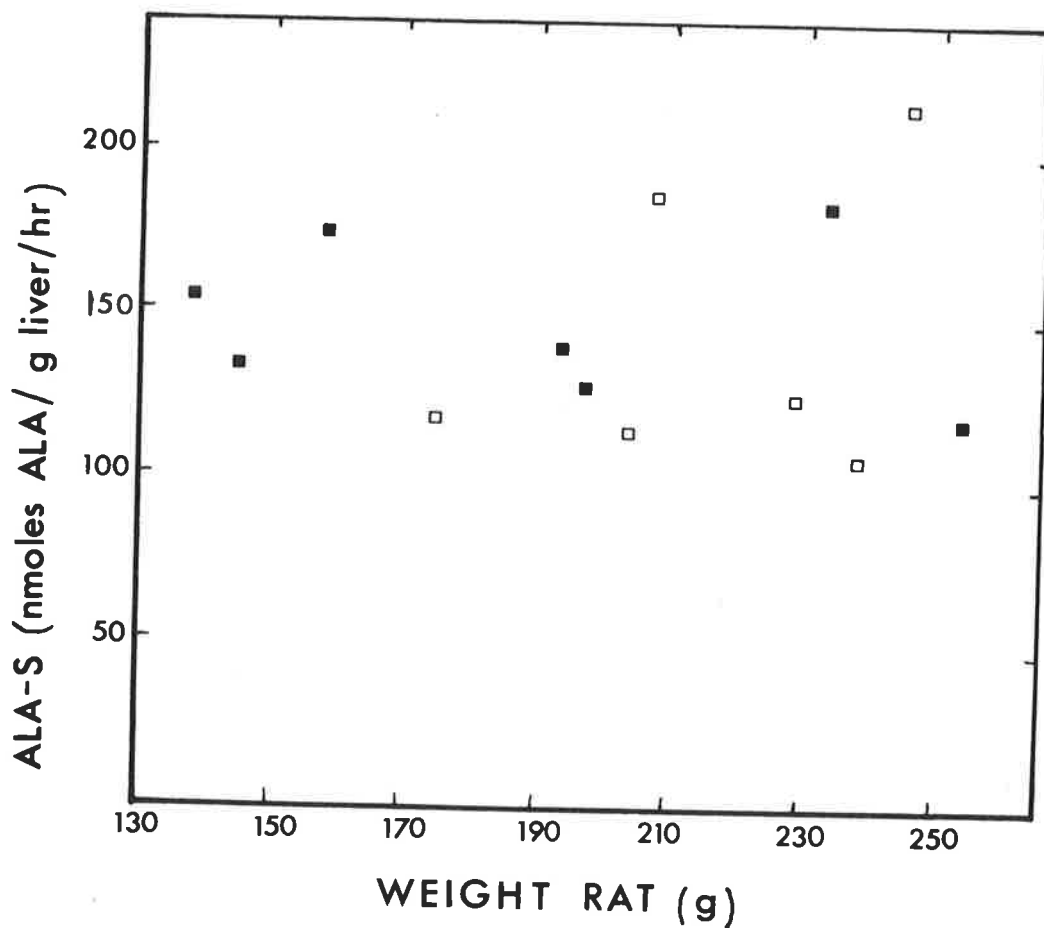
FIG. 5-1.   Effect of weight and sex of
rats on *in vivo* induction of mitochon-
drial ALA synthetase by AIA.   Procedure
was as in Table 5-1.   The overall mean
activity for all 14 rats was 148 $\pm$
10 nmoles ALA/g liver/hr.   Mean activity
for 8 males (■) was 150 $\pm$ 9 and for
6 females (□), 143 $\pm$ 19.

size and sex.   From this limited data it appears that for
rats of weight 200 ± 60 g, induction is independent of weight
(age) and sex.

The results in Table 5-2 (a) and (b) show that fasting
rats before use increases both the basal level of activity
(a) and the extent of induction by AIA (b). The results are
consistent with previous findings [6,98,327].

There is some apparent disagreement in the literature
on the time course of induction by AIA *in vivo*, although
direct comparison of published time courses is difficult
because of differences in dosing and in the cell fractions
assayed [e.g., 65,70,98]. The change in ALA synthetase
after *in vivo* administration of AIA was therefore followed
by assaying both homogenates and isolated mitochondria in an
attempt to establish the time course of change of total
activity and also the relative change in the mitochondrial
fraction.

The pooled results from a number of experiments
conducted on a single batch of rats, are shown for homo-
genates in Fig. 5-2(a) and for isolated mitochondria in
Fig. 5-2(b).   The two curves are not directly comparable
quantitatively because different assay times were used. Total
activity as measured in homogenates increased in approximately
linear fashion for about 11 hours after AIA administration.
Over the same period, changes in mitochondrial activity
followed a different course - an initial rapid rise over
three hours then a lag before a secondary rise.   The

TABLE 5-2(a): EFFECT OF FASTING ON THE BASAL LEVEL OF

ALA SYNTHETASE IN RAT LIVER.

Period of fasting	No. of experiments	Total ALA synthetase activity nmoles ALA/20 min.	
		per g liver	per mg protein
0	7	18 + 3	0.085 + 0.014
20	12	24 + 3	0.099 + 0.010
42	28	38 + 3	0.168 + 0.013

(b): EFFECT OF FASTING ON INDUCTION OF ALA

SYNTHETASE *in vivo* IN RAT LIVER BY AIA.

0	3	64	0.278
20	3	203	0.977
42	3	244	1.048

Rats fasted for the periods shown were killed immediately
(a) or injected s.c. with AIA and killed 4 hours later (b).
Livers were removed and homogenates prepared and assayed by
the colorimetric procedure as described in Materials and
Methods.

FIG. 5.2   Time course of induction of ALA synthetase
            by AIA *in vivo*.

        Fasted rats were injected s.c. with AIA,
350 mg/kg and killed after the times shown. Preparation
and assay (by the colorimetric method) of liver
samples were as described in Materials and Methods.
In (a) unfractionated homogenates were assayed;
in (b), isolated mitochondria.

Each point represents the average of results from
at least three experiments. In this, and all other
experiments, standard deviations of means shown
by the vertical bars, were calculated where four
or more experiments contributed to the mean.

(a)

ALA-S (nmoles ALA/g liver/20 min) ——— ●

ALA-S (nmoles ALA/mg protein/20 min) ○

TIME AFTER AIA ADMINISTRATION (hr)

(b)

ALA-S (nmoles ALA/g liver/hr) ——— ■

ALA-S (nmoles ALA/mg protein/hr) □

TIME AFTER AIA ADMINISTRATION (hr)
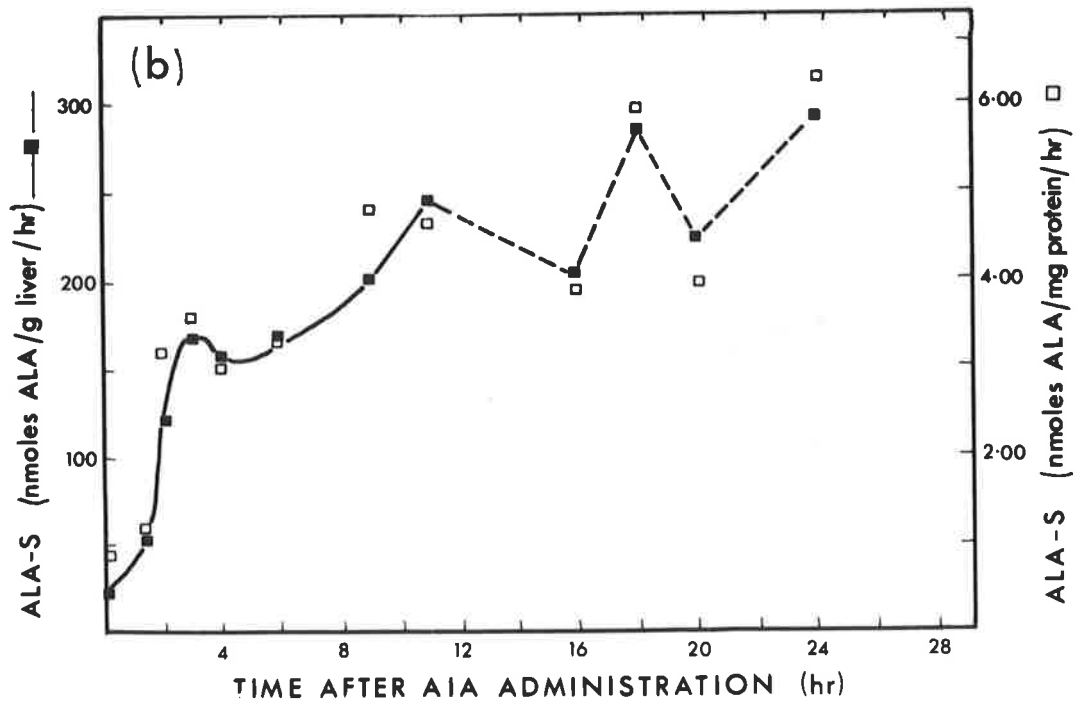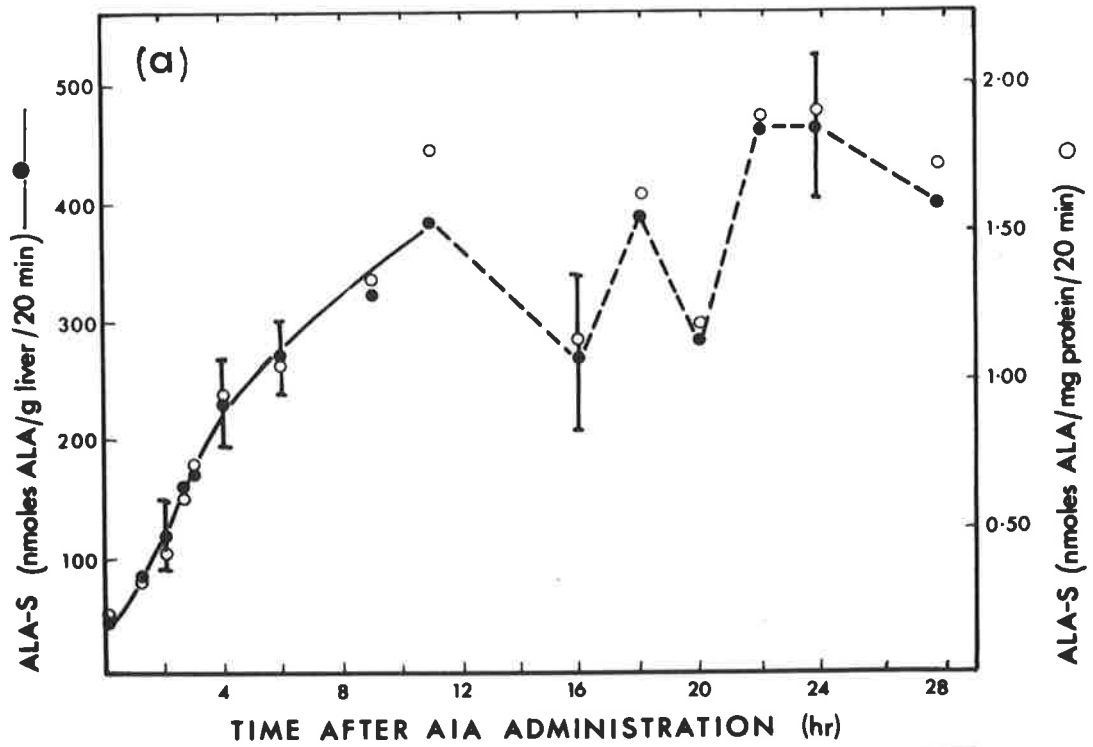
changes in total activity are similar to those reported by
Marver *et al.* [98] while the variation in mitochondrial
activity is reasonably consistent with the findings of
Hayashi *et al.* [65] and Beattie and Stuchell [70].

At times greater than 11 hours after AIA administra-
tion, ALA synthetase activity varied considerably from
rat to rat for a similar period of induction (large
standard deviations of means in Fig.5-2(a)) and even more
markedly with respect to time of induction (irregular shape
of curve). Marver *et al.* [98] also noted considerable
variation of time course in different experiments. While
variation might result from several factors, it is possible
that the rise and fall in activity reflect a real phenomenon,
the timing of which varies slightly from rat to rat resulting
in the large standard deviations observed. For instance the
level of ALA synthetase may undergo oscillation in AIA-treated
rats as previously observed for rats treated with heme [330]
or estrogen [247]: detection of oscillation would depend on the
choice and frequency of time intervals in time course experiments.
The curve in Fig.5-2(a) resembles the time course of induction
of ALA synthetase by benzpyrene [33].

The relation between mitochondrial and total activity
during *in vivo* induction by AIA was examined directly in a
series of experiments in which both homogenates and mitochondria
prepared from the same liver were assayed. The change in
mitochondrial activity, expressed as a percentage of the total
is shown in Fig. 5-3. The given percentages represent relative

rather than true values since no allowances were made for incomplete recoveries of mitochondria during their preparation. A standard preparative procedure was, however, used throughout. The results show that the proportion of activity located in the mitochondria increased somewhat during the first 2-3 hours after AIA administration but thereafter a distribution of activity similar to that in untreated animals was restored.

B. STUDIES ON THE LEVEL OF MITOCHONDRIAL ALA SYNTHETASE IN PERFUSED RAT LIVER.

(I) Development of the Basic Perfusion System.

Liver perfusion offered, potentially, one means of studying changes in the level of hepatic ALA synthetase meeting most of the requirements for an *in vitro* system set out in Chapter I. As a prelude to any detailed investigations, it was necessary to establish that the level of ALA synthetase could be increased in perfused liver, as in whole animals. Thus in initial experiments the effects on enzyme level, of perfusion with a known inducer were investigated. AIA was chosen as inducer since it was known to act both *in vivo* in rats and *in vitro* on cultured chick liver cells. The concentration of AIA used (0.55 mg/ml) was relatively high compared to that employed by Granick [80] in tissue culture studies (30-300 µg/ml). Hydrocortisone (80 µg/ml) was also included in the perfusate because of evidence that it exerted a permissive and/or stimulatory effect on induction by AIA

in vivo [141,142]. Choice of other components of the perfus-
ate (40% rat blood, phosphate-bicarbonate Ringer and casein
hydrolysate) was based on perfusates used by other workers in
studies of enzyme induction in liver [e.g., 314,315], except
that glucose, was omitted from initial experiments since
feeding of this to rats represses induction of ALA synthetase
by AIA [98]. The perfusion apparatus and procedure were as
described by Hems *et al.* [313].

On the basis of experiments described above, albino
Wistar rats from the Waite Institute colony, about 200 g
weight and fasted for 42 hours wereused as liver donors and
all perfusions were started at approximately 11 a.m. These
factors were designed to minimize genetic, nutritional and
diurnal variations. Because of limited animal supply, it was
necessary to use rats of both sexes but as shown above, sex
did not affect induction *in vivo* by AIA.

The effect of perfusion on the level of ALA
synthetase was assessed by clamping off whole lobes of liver
after various times, preparing mitochondrial suspensions
and assaying these using the colorimetric and/or radiochemical
assays. The results are shown in Fig. 5-4(a). Over 2-3 hours
there was a significant increase in mitochondrial ALA synthetase
activity whether assessed by colorimetric or radiochemical
assay. There was reasonable agreement between the two assay
methods although as previously noted by Irving and Elliott
[73] the more specific radiochemical assay gave lower results
particularly in untreated liver. As an additional safeguard,

FIG. 5-4(a).   Effect on the level of mitochondrial
ALA synthetase of perfusion with standard perfusate minus
glucose, plus AIA (550 μg/ml) and hydrocortisone (80 μg/ml).
Perfusion procedure and preparation of mitochondria were
as described in Materials and Methods. Mitochondria
were assayed by the procedures indicated. Curves in this
and all perfusion experiments represent pooled results
from the number of experiments indicated in brackets in
the legend. Each point is the average of results from
at least two experiments. Standard deviations of means,
indicated by vertical bars, were calculated where four
or more experiments contributed to the mean.

■————■   assayed by colorimetric method [10]
▲————▲   assayed by radiochemical method [6]

   (b)   Identification of product from radio-
chemical assay. The radioactive product was identified
by comparison with authentic ALA pyrrole by thin layer
chromatography as described in Materials and Methods.

(a)

ALA-S (nmoles ALA/g liver/hr)

TIME (hr)

(b)

cpm

DISTANCE FROM ORIGIN (ins)
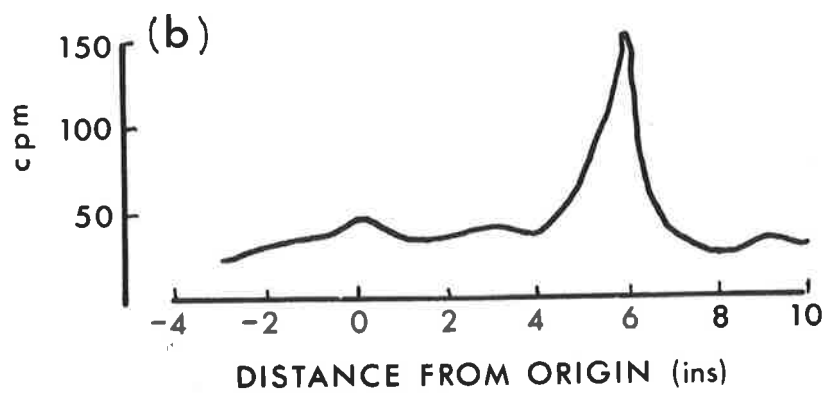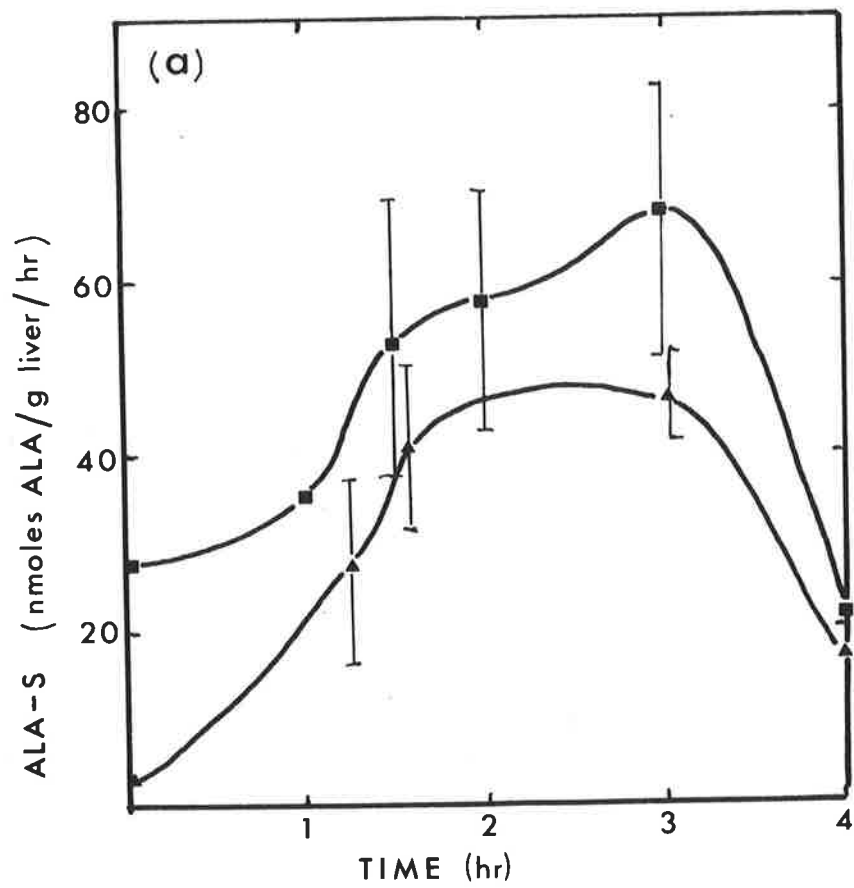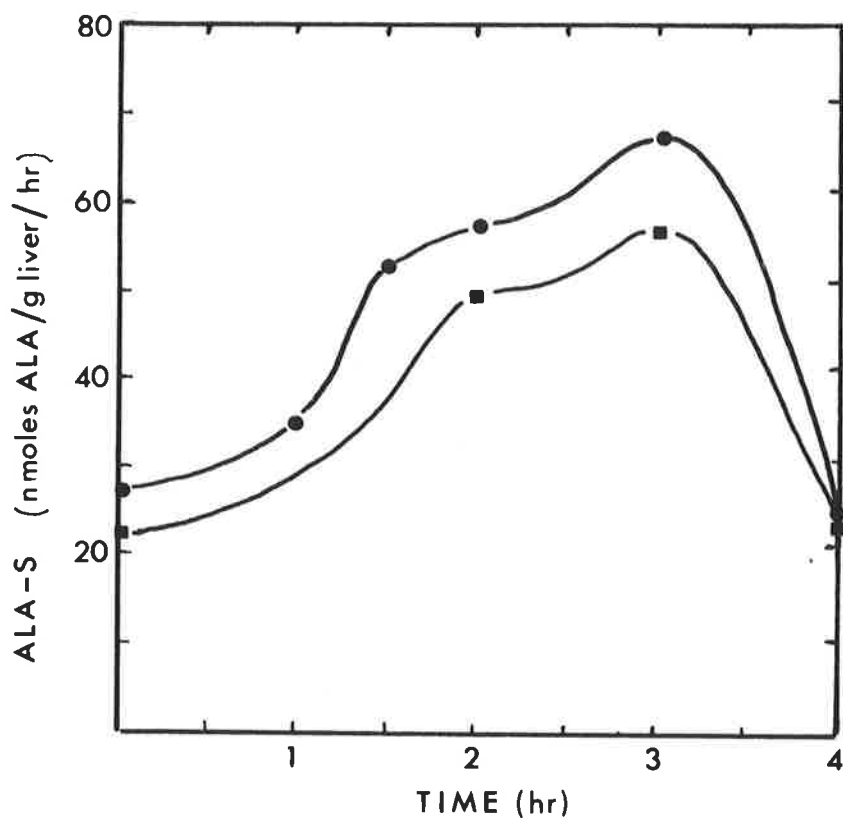
FIG. 5-5. Effect of rate of flow of perfusate on induction of mitochondrial ALA synthetase in perfused liver. Procedure was as in Fig. 5-4(a). Mitochondria were assayed by the colorimetric method.

●———● normal flow rate [10] (15-20 ml/min)

■———■ slow flow rate [3] (5-10 ml/min)

the product in the radiochemical assay was identified as ALA pyrrole by thin layer chromatography: the radioactive pyrrole cochromatographed with pyrrole prepared from authentic ALA as shown in Fig.5-4(b).

In all further experiments, the colorimetric assay was used to measure ALA synthetase activity.

Since it has been suggested [157,186] that control of ALA synthetase may be related in some way to the oxidation: reduction potential of the cell, it seemed that it might be possible to enhance induction by limiting the supply of oxygen to the liver. If this were the case, rate of flow of perfusate through the liver could affect the level of ALA synthetase. Fig.5-5 shows the result of reducing the flow rate so that the perfused liver was partially anaerobic as judged by the colour of perfusate leaving the liver; the reduction in flow rate had no significant effect.

As shown by the large standard deviations of means in Fig. 5-4(a), the initial perfusion conditions gave rather variable results. An attempt was made to improve the reproducibility of induction by supplementing the original perfusate with a substrate to supply the energy requirements of the liver. Addition of lactate (5 mg/ml) resulted in increased and more reproducible induction of the enzyme as shown in Fig. 5-6. In view of this result, the effects on induction of adding different substrates were compared (Fig. 5-7). Addition of glucose or lactate but not butyrate resulted in induction greater than in the absence of any added substrate.
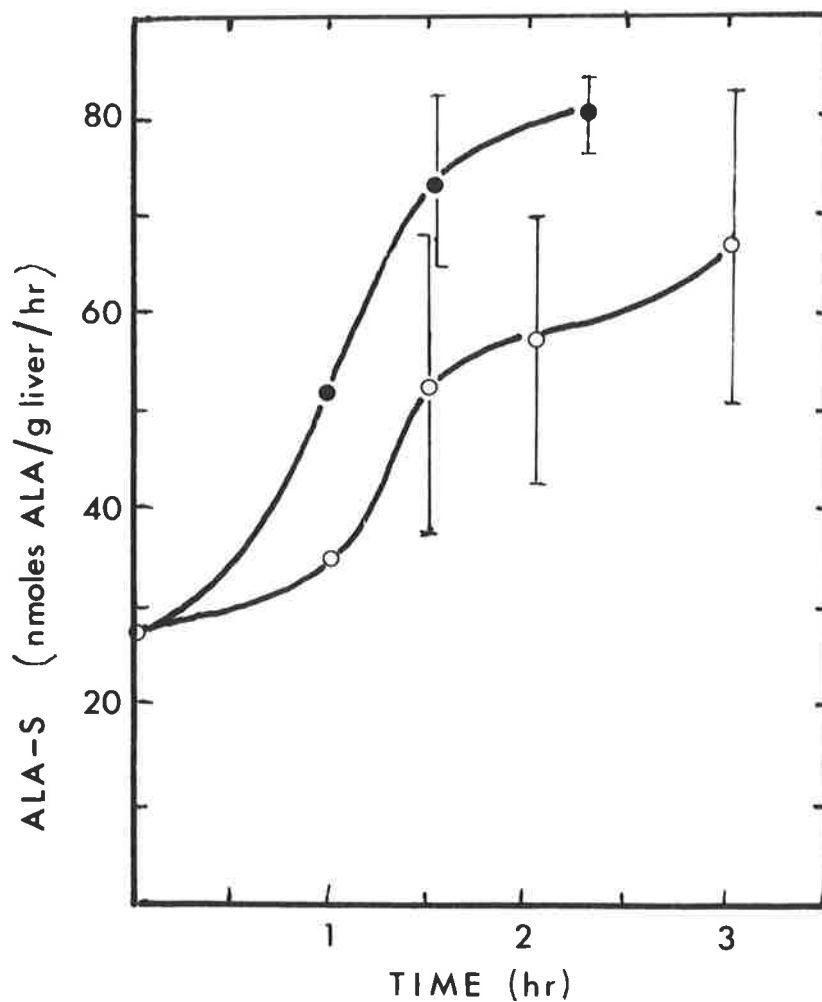
FIG. 5-6.   Effect of added sodium
lactate on induction of mitochondrial
ALA synthetase in perfused liver.
Procedure was as in Fig. 5-5,
with normal flow rate and
additions as indicated.

o———o   none [10]

●———●   + lactate,
         5 mg/ml [7].
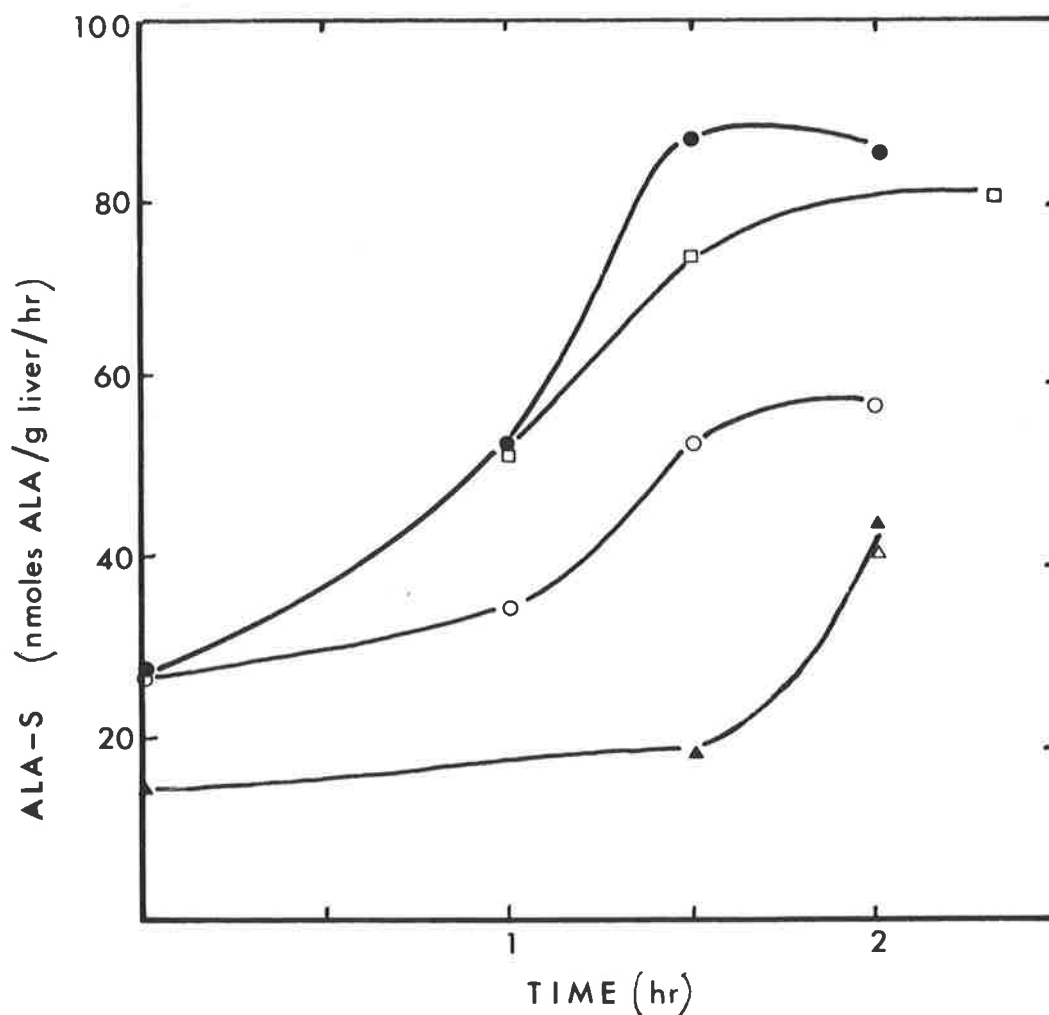
FIG. 5-7. Effect of added substrates on
induction of mitochondrial ALA synthetase
in perfused liver. Procedure was as in
Fig. 5-6 with additions as indicated.

O———o   none [10]

□———□   + sodium lactate, 5/mg/ml
             [7]

△———△   + sodium butyrate, 10mg/ml
             [2]

▲———▲   + sodium butyrate, 2.5 mg/
             ml [4]

●———●   + glucose, 5.5 mg/ml [2]
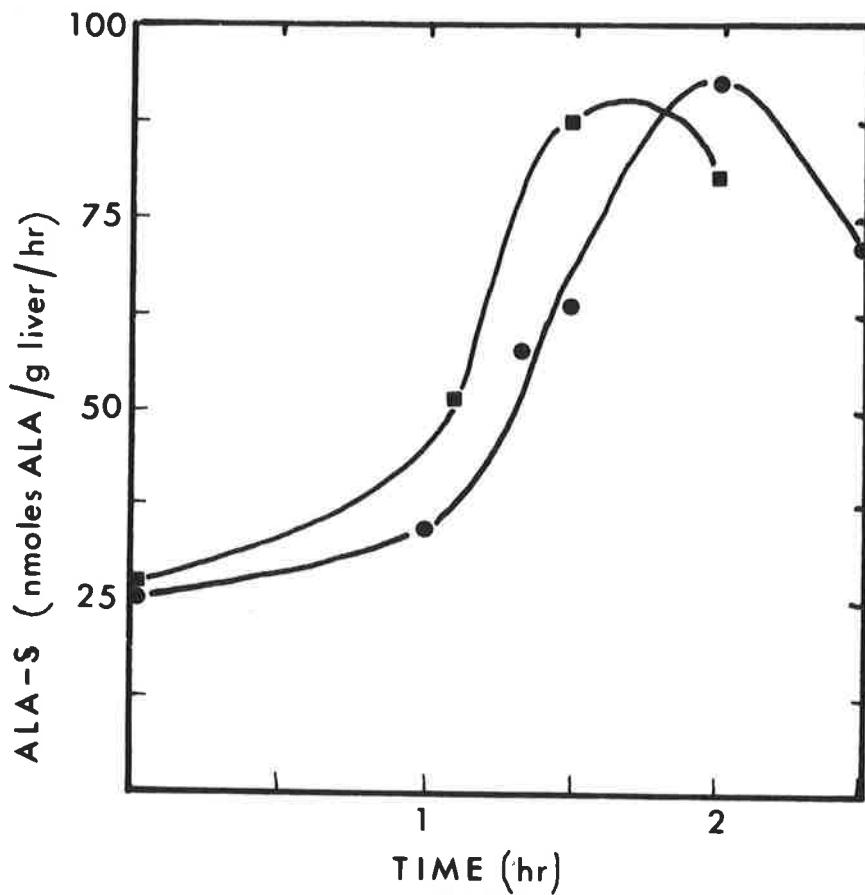
FIG. 5-8. Effect of hydrocortisone on induction of mitochondrial ALA syntetase by AIA in perfused liver. Procedure was as in Fig. 5-4(a), except that standard perfusate with additions as shown was used. Mitochondria were assayed by the colorimetric procedure.

●————● + AIA, 550 mg/ml [6]

■————■ + AIA + hydrocortisone, 80 µg/ml [2]

In all further experiments, glucose was included as a component of the standard perfusate.

In the perfusions described above, hydrocortisone was included in the perfusate since *in vivo* experiments had suggested its presence might be a prerequisite for observing induction by AIA [141,142]. As shown in Fig. 5-8, omission of hydrocortisone from the perfusate had only a small (not necessarily significant) effect on the time course and apparently no effect on the overall extent of induction. While it is not possible to draw conclusions on the role of glucocorticoids in regulation of ALA synthetase from these preliminary experiments, the results suggested that addition of hydrocortisone to the perfusate was unnecessary: it was omitted in further experiments.

The preliminary experiments described above provided an *in vitro* mammalian system in which a significant and fairly reproducible increase in the activity of mitochondrial ALA synthetase occurred. This basic system was now used to investigate the effects of some of the agents implicated in ALA synthetase regulation by *in vivo* studies, on the enzyme level in perfused liver.

(II) Use of the Perfusion System for Preliminary Studies on Aspects of Control of ALA Synthetase.

(a) The effects of AIA.

Figs. 5-9(a) and (b) show changes in the level of mitochondrial ALA synthetase resulting from perfusion with
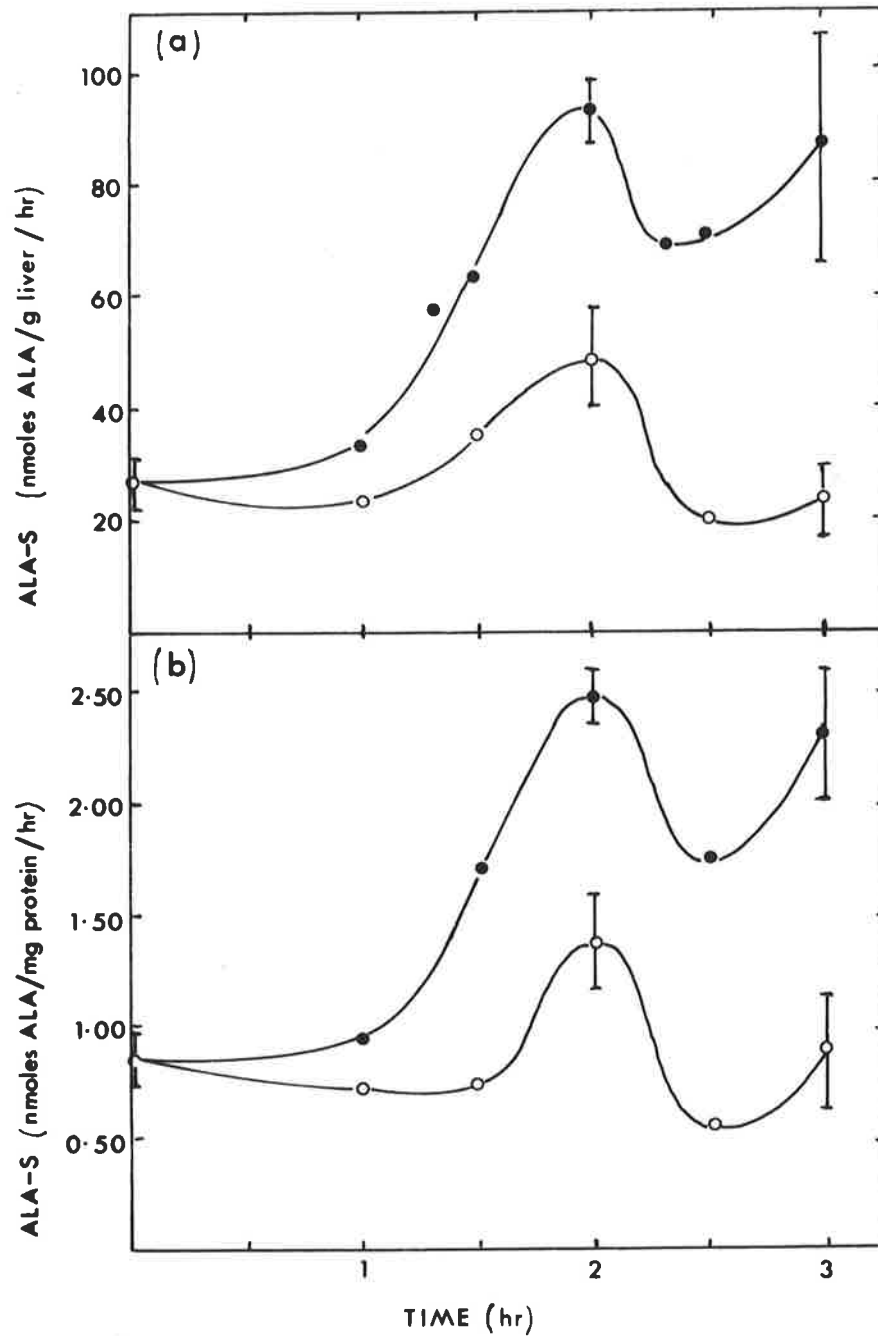
FIG. 5-9(a) and (b). Effect of AIA on
the level of mitochondrial ALA synthet-
ase in perfused liver. Procedure was
as in Fig. 5-8. Results are expressed
as activity per g wet liver (a) and
per mg mitochondrial protein (b).

o————o   no addition [8]

●————●   + AIA, 550 µg/ml [6]

and without AIA. The results are expressed as activity per g
of wet liver (a) and activity per mg of mitochondrial protein
(b). Perfusion without AIA resulted in a small increase in
activity; perfusion with AIA caused a significant and
reproducible 3-4 fold increase in the level of mitochondrial
ALA synthetase. After a 1-hour lag phase, enzyme activity
increased to a maximum after 2 hours; a second rise in activity
between $2.^1/2$ and 3 hours raised the possibility that induction
occurred in more than a single phase or involved at least
two separate processes. These possibilities are discussed
further below.

(b) <u>Perfusion of livers preinduced *in vivo* with AIA.</u>
Fig. 5-10 summarizes the results of experiments
which were carried out with the original object of establishing
whether induction once initiated *in vivo* by AIA, could
be continued in livers perfused without the inducer. Rats
were injected with optimal doses of AIA and after varying
periods of induction *in vivo*, their livers perfused and
changes in mitochondrial ALA synthetase measured. In Fig. 5-10,
the solid line indicates the time course of induction *in vivo*
by AIA (similar, but somewhat higher results were obtained
in the experiment shown in Fig. 5-2(b)). The dashed lines
indicate the overall increases (though not the actual time
course) in mitochondrial ALA synthetase over a two-hour
perfusion commenced at the times indicated by arrows. As
shown in Fig. 5-9, perfusion of an untreated liver (i.e.,
zero preinduction time) results in a small increase without
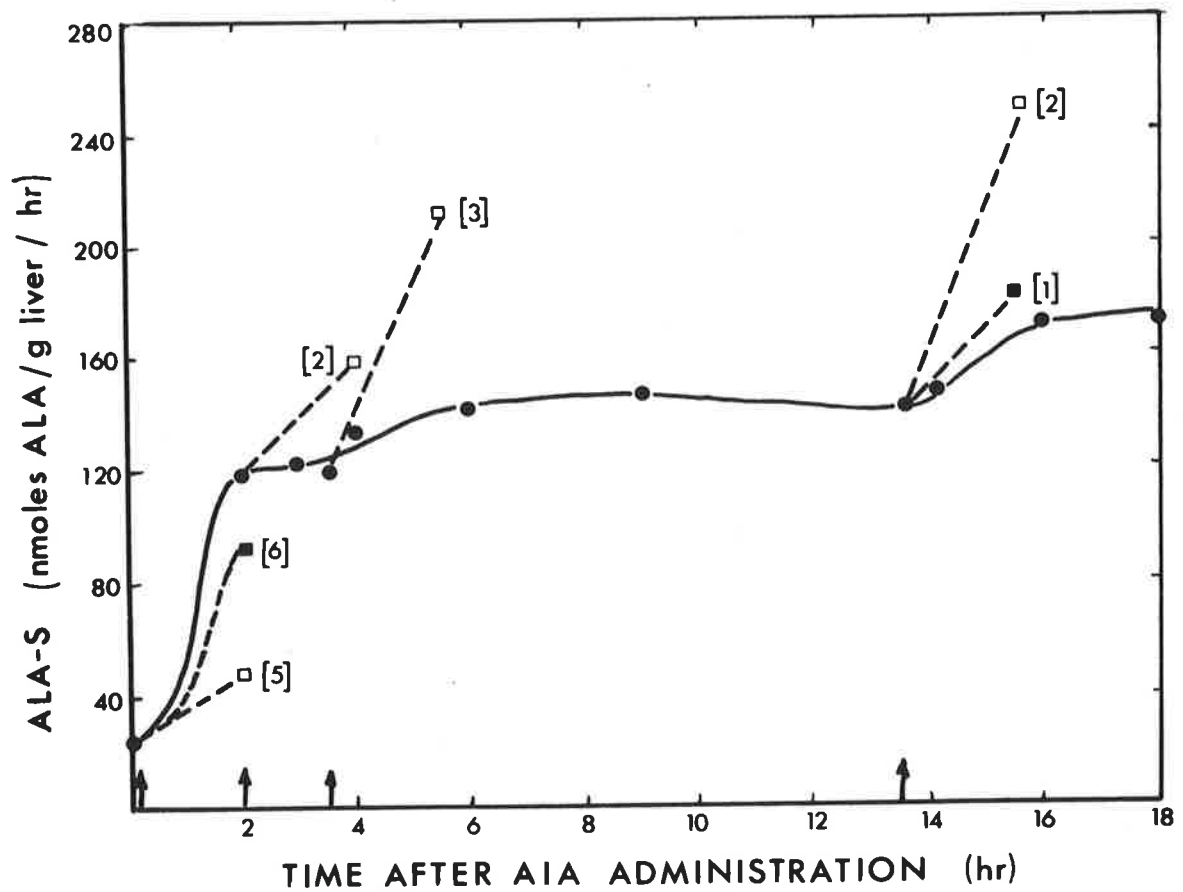
FIG. 5-10. Effect on the level of mitochondrial
ALA synthetase of perfusion of livers preinduced
in vivo with AIA. See text for experimental details.

●————●   level of activity due to preinduced
                         in vivo by AIA (each point is the
                         average of two or more determinations).

--------   increase in activity over 2 hour
                         perfusion (no. experiments shown
                         in brackets).

-------□   without AIA, i.e., control perfusion
-------■   with AIA, 550 ug/ml.
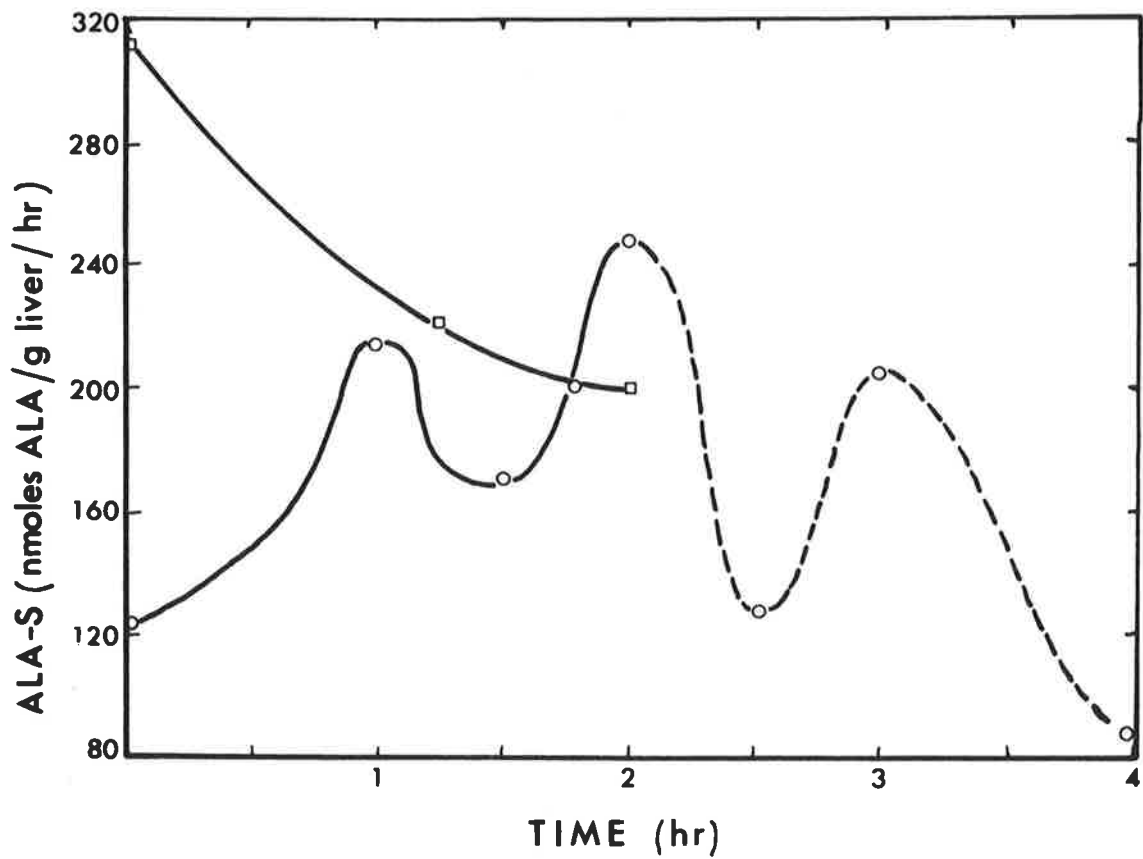
FIG. 5-11. Effect on the level of mitochondrial
ALA synthetase of control perfusion (no added
inducer) of livers preinduced *in vivo* with AIA
for 13.[1]/2 hours. Procedure was as in Fig.5-10.

o————o   perfusion of livers in which the
          preinduced level of activity was
          'normal' [8]

□————□   perfusion of livers in which the
          preinduced level of activity was
          high [3].

AIA and a larger increase when AIA is present. However, after a period of preinduction *in vivo*, this situation was surprisingly reversed. Where the perfusate contained no added AIA, perfusion resulted in large increases in enzyme activity. The increases were much greater than those which apparently occurred over a comparable time interval *in vivo*.

Also in the limited number of experiments conducted, the extent of the further induction during perfusion appeared to increase with increasing periods of preinduction by AIA. In one experiment in which a liver, preinduced for $13.^1/2$ hours, was perfused with AIA, the drug appeared to inhibit rather than augment further induction during a 2 hour perfusion.

Fig. 5-11 shows the actual time course of change in mitochondrial ALA synthetase activity which occurred when livers, preinduced for $13.^1/2$ hours, were perfused. The curve represents pooled results from several separate perfusions; each individual point represents the mean of 2 or 3 results, except for values in the dashed section of the curve which were measured in one experiment only and remain to be confirmed. The level of enzyme activity increased rapidly during the first hour of perfusion then apparently oscillated, reaching maximal activity (approximately double the preinduced level) after 2 hours. While these changes were observed in most experiments, in 3 cases, the level of activity after $13.^1/2$ hours preinduction was unusually high. In these cases subsequent perfusion resulted in a fall in enzyme activity to an intermediate level. A possible explana-

tion for the oscillation of enzyme activity observed was
(as suggested for previous cases where oscillation of ALA
synthetase activity was noted [247,330]) that oscillations
might reflect periodic application and release of repression
by the feedback repressor, heme. In the cases where, for
unknown reasons, the preinduced level of ALA synthetase (and
therefore of heme?) was high, greater feedback repression might
account for the observed fall in activity.

The general observation that perfusion without addition
of inducers can cause large increases in mitochondrial ALA
synthetase activity in particular circumstances was unexpected.
One possible explanation of this phenomenon was that washing
out remaining AIA or one of its metabolites relieved some
general toxic or perhaps specific block to full ALA synthetase
induction. But the results also raised the possibility that
perfusion of a liver altered the effect of some other element
of control on the enzyme level, so that further induction was
possible. In view of this, it seemed desirable to investigate
in a preliminary way some of the variables operating in control
of ALA synthetase in perfused liver before embarking on
detailed investigations of the mechanism of action of AIA or
other inducers.

(c) Studies related to repression of ALA synthetase
   in vivo by glucose.

From *in vivo* experiments, it is known that glucose
has a repressive effect on the induction of ALA synthetase
by drugs [60,98]. Experiments described above (Fig.5-7)
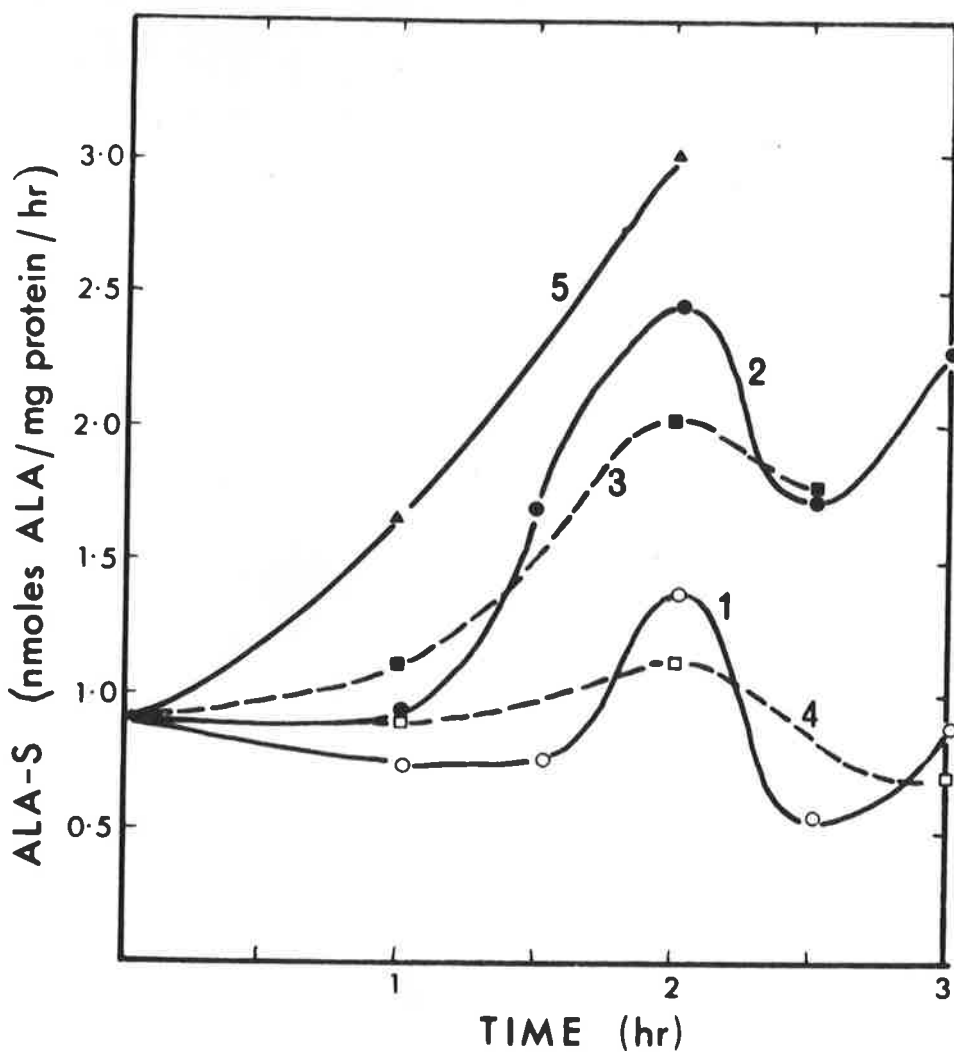
FIG. 5-12. Effects on the level of mitochondrial ALA synthetase in perfused liver of possible mediators of *in vivo* glucose repression. Procedure was as in Fig. 5-8 with variations as indicated.

○————○ Curve 1, no addition [7]
●————● Curve 2, + AIA, 550 µg/ml [5]
■– – –■ Curve 3, + AIA, with liver and blood from fed rats [2]

See text for further details.

suggested that this effect of glucose is not a direct one on the liver. It therefore seemed that the 'glucose effect' observed *in vivo* might be mediated for example, by pancreatic hormones which in turn might affect hepatic levels of cyclic AMP. Such indirect effects are thought to be involved in glucose repression of other liver enzymes [e.g., 272]. The results of experiments designed to test these possibilities are shown in Fig. 5-12. When livers from fed rats were perfused with perfusate containing blood from fed rats, induction by AIA (curve 3) was slightly less than when blood and livers were taken from 42 hour-starved rats (curve 2); the difference may not be significant. This observation suggested that glucose repression which operates in fed rats *in vivo* (Table 5-2) was at least partly relieved in perfused liver. In more direct tests for the possible mechanism of the glucose effect, it was found that a high level of insulin almost completely prevented induction by AIA (curve 4) and that dibutyryl cyclic AMP caused a significant enhancement of induction by AIA (curve 5).

These results together with those in Fig. 5-7 provided preliminary evidence that the *in vivo* 'glucose effect' is an indirect one, possibly involving pancreatic hormones and changes in hepatic cyclic AMP levels.

(d) The effects of heme and of inhibitors of heme synthesis.

Repression by heme is of central importance in

models for control of ALA synthetase. It also seemed that
the oscillatory changes in enzyme level observed when
preinduced livers were perfused might be the result of
alternating heme repression and derepression of enzyme
synthesis. It was therefore of interest to investigate the
effects of heme on induction by AIA and also the effects of
inhibitors of heme synthesis which might be expected to
prevent build-up of heme and consequent repression of ALA
synthetase.

As shown in Fig. 5-13, heme (10^{-5} M) significantly
reduced the effect of AIA on the level of mitochondrial ALA
synthetase in perfused liver: the increase in activity over
controls observed with AIA was reduced 75% in the presence of
heme. Heme repression of induction by AIA is consistent with
previous *in vivo* findings [143]. However, Kurashima *et al.*
[281] have suggested that heme causes redistribution of
ALA synthetase activity from mitochondria to cytosol in rats
pretreated with AIA. Thus the effect of heme in Fig. 5-13
could conceivably be due to such a redistribution effect.

In further experiments, lead acetate was added to the
perfusate in the hope of selectively inhibiting heme synthesis.
Lead acetate (10^{-3} M) was found to reduce rather than enhance
induction of mitochondrial ALA synthetase by AIA, but lead
alone caused a marked increase in activity (Fig. 5-13). The
former result may reflect toxicity of high levels of lead;
the latter observation was surprising in view of the apparent
lack of inducing effect of lead *in vivo* [150,286, cf. 202]
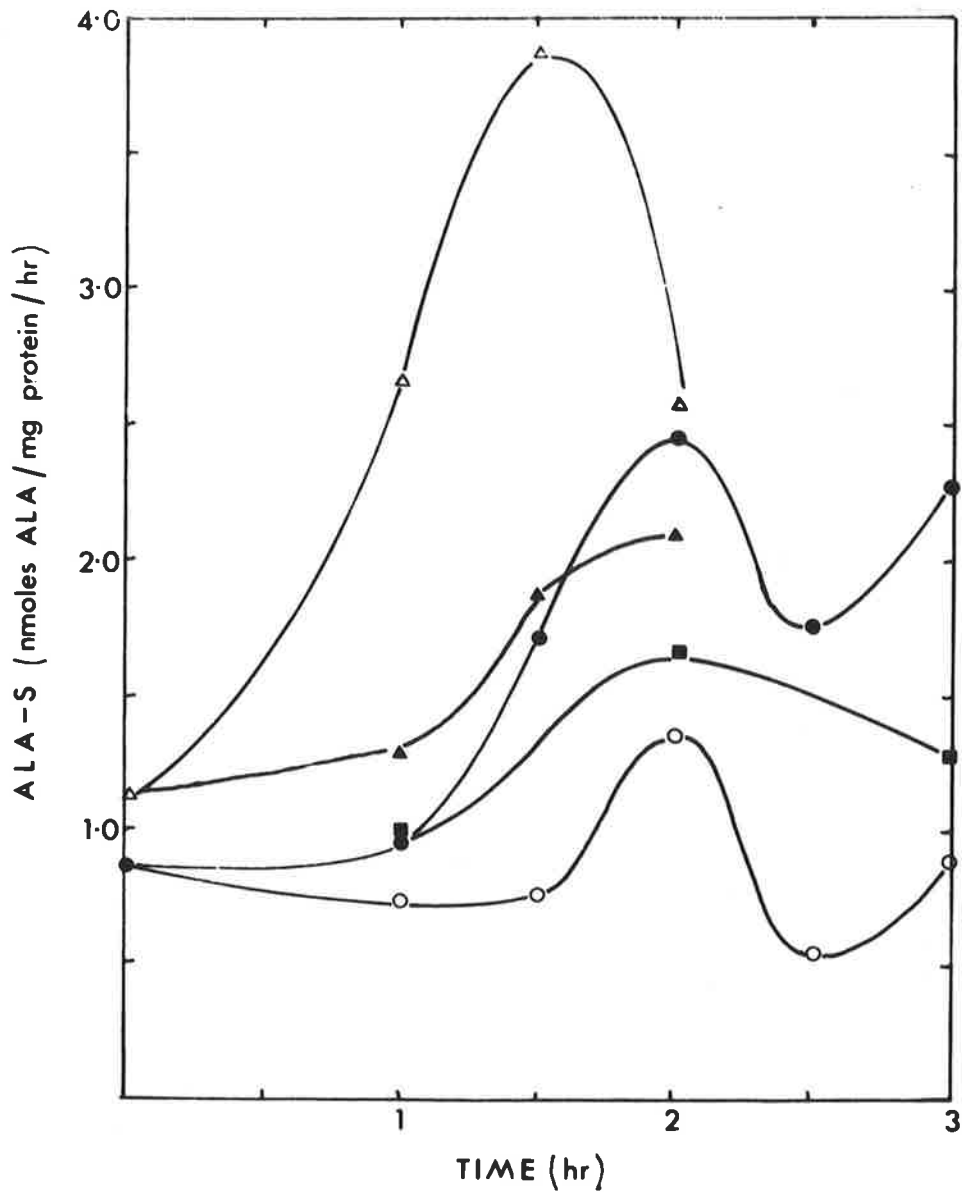
FIG. 5-13. Effects of heme and lead
acetate on induction of mitochondrial
ALA synthetase in perfused liver.
Procedure was as in Fig.5-8 with additions
as indicated.

o————o	no addition [7]
●————●	+ AIA, 550 µg/ml [5]
■————■	+ AIA + hemin, 10^{-5}M [2]
▲————▲	+ AIA + lead acetate, 10^{-3}M [2]
△————△	+ lead acetate, 10^{-3}M [3]

and may be a further indication of altered control in perfused liver. Further investigation would be required to establish whether induction by lead is due to inhibition of heme synthesis.

C. STUDIES OF CHANGES IN THE LEVEL OF TOTAL ALA SYNTHETASE IN PERFUSED RAT LIVER, AS MEASURED IN LIVER HOMOGENATES

(I) Modification of Sampling and Assay Procedure in Perfusion Experiments: Comparison with Previous Methods.

In the experiments described above, ALA synthetase was assayed in isolated mitochondria, firstly because it has generally been considered (see Chapter 1, Section 3) that the biologically important fraction of ALA synthetase is mitochondrial and secondly because the specific activity of the enzyme was much higher in mitochondria than in other cell fractions. However, for preparation of mitochondria it was necessary to take relatively large liver samples, so that a single perfusion yielded only three experimental points. It was therefore necessary to carry out several replicate perfusions to establish the effects of compounds tested, particularly where changes in enzyme level were rapid or followed an oscillatory course. If ALA synthetase were assayed in unfractionated homogenates, it would be possible to assay small liver samples and thus obtain a greater number of samples from each perfusion.

A modified sampling procedure, essentially as described by Seglen and Jervell [315] was adopted; small sections of liver were removed by tying off with surgical silk; homogenates were then prepared and assayed as described in Materials and

FIG. 5-14. Effect of perfusion with and without AIA on total
            ALA synthetase activity as measured in liver
            homogenates; comparison with effects on mito-
            chonrial ALA synthetase.

Perfusions with AIA (a) and without additions, i.e.,
control (b) were carried out as in Fig. 5-9 except that small
liver samples were taken and homogenized as described in the
text. ALA synthetase was then assayed (all incubation times,
60 min.) in unfractionated homogenates as described in
Materials and Methods. Changes in mitochondrial activity
were replotted from Fig. 5-9(a) and changes in cytosol
ALA synthetase activity calculated by subtraction of
mitochondrial activity plus a constant level of activity in
the cell debris fraction, from total activity as measured
in homogenates.

□■————————■□ total ALA synthetase activity

○●————————●○ mitochondrial activity

------------ calculated cytosol activity.
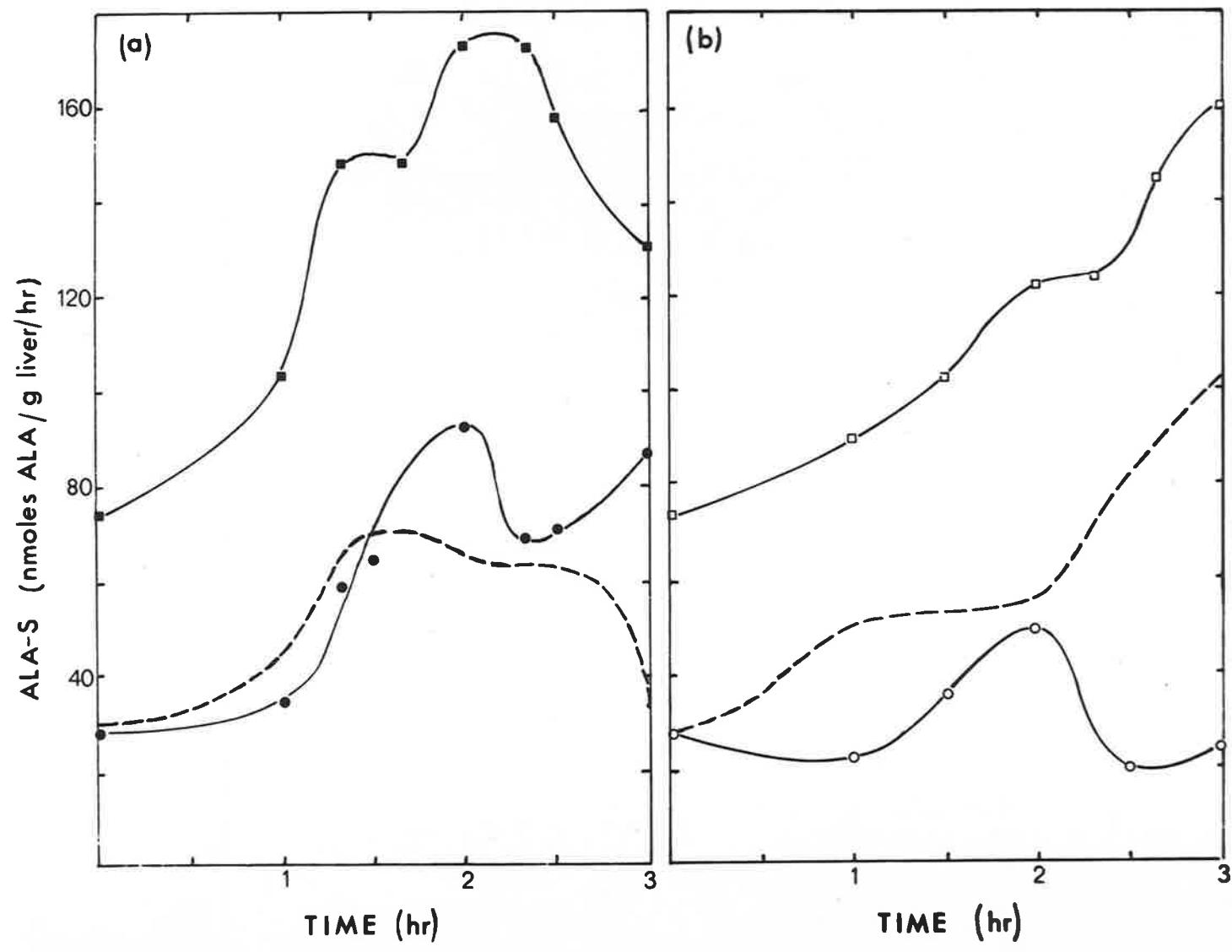
Methods. In Fig.5-14(a) and (b), results (obtained by the
modified methods) for the effect on total ALA synthetase
activity of perfusion with and without AIA are compared with
corresponding changes in the mitochondrial fraction, replotted
from Fig.5-9. In perfusions with AIA (Fig.5-14(a)), there was a
3-4 fold increase in total activity similar to the 3-4 fold
increase previously observed in mitochondria. However, in
control perfusions - i.e., without added AIA (Fig.5-14(b) -
while only a small increase in mitochondrial activity was
observed, a much larger 3-fold increase in *total* activity was
found. The observed changes in total activity are discussed
in the next section.

Comparison of changes in mitochondrial activity with
changes in total activity show that while perfusion with or
without AIA resulted in 3-4 fold induction of *total* activity
over a 3-hour perfusion, the contribution of the mitochondrial
fraction to the total increase at later perfusion times was
much greater in the presence of AIA than in its absence. For
instance, after perfusion for 2 hours the increase in mito-
chondrial activity was 67% of the total increase with AIA but
only 40% in controls. At later times (2-3 hours) the increase
in mitochondria represented an even larger proportion of the
total increase over basal level. It would seem likely that
differences between mitochondrial and total activity represent
enzyme activity in the cytosol fraction of the cell. If
allowance is made for a small, constant level of activity in
the cell debris, removed during preparation of mitochondria

and post-mitochonrial supernatants, then changes in enzyme
activity in cytosol can be predicted by subtraction of mito-
chondrial from total activities: predicted cytosol activities
calculated in this way are shown by the broken lines in
Figs. 5-14(a) and (b). Observed changes in cytosol activity due
to perfusion with or without AIA, measured by assay of post-
mitochondrial supernatants, are shown in Fig. 5-15. It should
be noted that difficulties were encountered in obtaining an
accurate measure of cytosol ALA synthetase activity. For
homogenates and mitochondria, the volumes of samples assayed
were within the range where product formation was proportional
to tissue concentration; this was not the case for post-
mitochondrial supernatants. As discussed in Chapter III,
because of low activity in supernatants, to obtain a reproducible
estimate of ALA synthetase activity it was necessary to assay
volumes of supernatant at the extreme end or somewhat beyond
the range over which ALA formation varied linearly with tissue
volume. The resultant measure of cytosol activity thus
represents an underestimate of true activity. Presumably
because of this, the 'measured' values for cytosol activity
in Fig. 5-15 are lower than the predicted values in Fig. 5-14.
Nevertheless, the changes in cytosol activity approximately
parallel the predicted changes. It therefore appears that the
larger increase in mitochondrial activity in relation to total
increase at later times in perfusions with AIA (in particular
the rise in mitochondrial activity between $2.^1/2$ and 3 hours)
is due to an effect of AIA on distribution of enzyme between
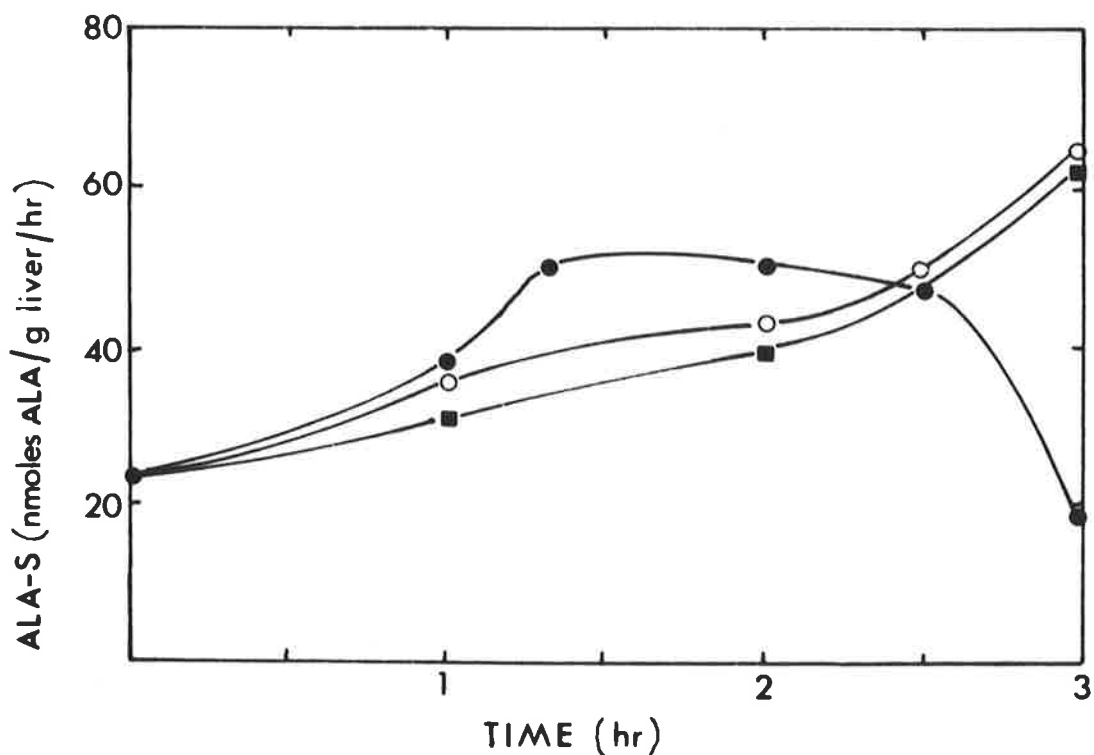
FIG. 5-15. Changes in the level of cytosol
ALA synthetase (as measured in post-
mitochondrial supernatants) in perfused rat
liver. Perfusion procedure was as in Fig.
5-8 with additions as indicated. Post-
mitochondrial supernatants were prepared
and assayed by the colorimetric method
as described in Materials and Methods.

o————o   no additions [4]
●————●   + AIA, 550 µg/ml [4]
■————■   + AIA + hemin, 10^{-5}M [2]

mitochondrial and cytosol fractions.

Since Kurashima *et al.* [281] have suggested that administration of heme to rats in which ALA synthetase was preinduced *in vivo* by AIA caused redistribution of enzyme from mitochondria to cytosol, the effects of perfusion with AIA plus hemin on the level of cytosol activity were examined. The results, compared to the effect of AIA alone in Fig.5-15 suggest that heme in some way reverses the 'redistribution effect' of AIA. This result is consistent with those of Kurashima *et al.* [281] but further work will be required to confirm this effect and also to clearly establish whether heme decreases induction of *total* ALA synthetase in perfusions with AIA. For this further work, attention will have to be given to finding a more accurate assay procedure for cytosol ALA synthetase.

The results above raised the possibility that intracellular enzyme distribution was a variable in regulation of hepatic ALA synthetase. Interpretation of changes in distribution clearly depends on assumptions about the relationship between mitochondrial and cytosol forms of ALA synthetase: however, as a working hypothesis, it seems reasonable to suggest that changes in total enzyme activity might reflect primary controls on the level of ALA synthetase and that changes in enzyme distribution might reflect secondary controls. Thus an understanding of molecular events involved in regulation might best be reached by first studying changes in total activity and later considering intracellular distribution of

the enzyme. Therefore, in further experiments, changes in total ALA synthetase as measured in liver homogenates were investigated.

(II)   Changes in Total ALA Synthetase Activity in Livers Perfused With and Without Added AIA.

The results of perfusion experiments without added inducer (controls) and with AIA, in which changes in ALA synthetase activity were assayed in homogenates are replotted in Fig.5-16*. In the previous section, these were compared to previous findings where isolated mitochondria were assayed and implications with respect to intracellular distribution of ALA synthetase discussed. The significance of the overall changes in total activity will now be considered.

Control perfusion (no inducer) resulted in a significant 3-4 fold increase in total activity over 3 hours. At early perfusion times (2 hours), AIA significantly augmented the induction observed in controls, but over a 3 hour perfusion, maximal levels with and without AIA were essentially the same.

---

*When activity was measured in homogenates, it was possible to compare activity after several periods of perfusion with zero-time activity in a single liver. Results for each per- sion time were expressed as increase in activity over the zero-time value; the means of increases in several perfusions were then calculated for each perfusion time.
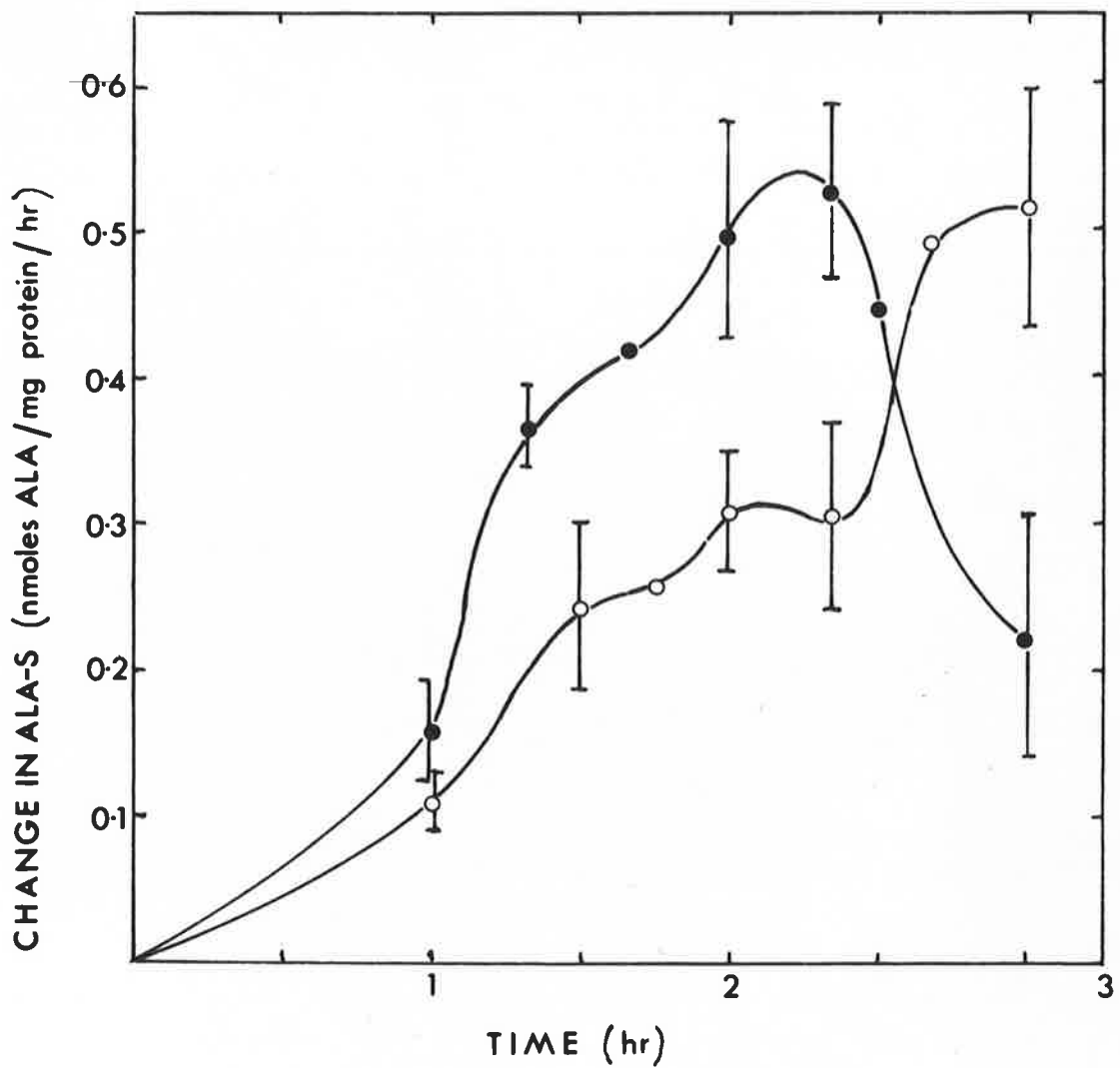
FIG. 5-16. Effect of perfusion with and without AIA on total ALA synthetase activity. The results of experiments in Fig. 5-14(a) and (b) were expressed as change in activity per mg protein and replotted.

o————o no additions (5]

●————● + AIA, 550 μg/ml [4].

117.

In addition to the unexpected increases in activity in controls, these results suggested that AIA was exerting only weak inducing effects in perfused liver. The previous apparently strong inducing effects were seen in mitochondria and as discussed above the principal effect of AIA in perfusion experiments was a redistribution one. Changes in total activity of ALA synthetase in the presence and absence of AIA were further investigated in an attempt to explain the increases in activity in control perfusions and to establish the potency of AIA as an inducer in isolated liver.

(a) <u>Investigation of induction in control perfusions</u>.

Increases in the level of ALA synthetase in control perfusions were unexpected since no known inducers were added to the perfusate. It was, however, possible that some component of the perfusate or compound administered to rats in preparation for perfusions was the agent responsible for the 'control induction'. Table 5-3 lists the effects of such agents on the level of ALA synthetase *in vivo*. Of the compounds to which perfused livers may have been exposed in control perfusions, only the anaesthetic, nembutal (pentobarbitone) caused significant induction when administered to rats *in vivo*.

As shown in Fig. 5-17, control perfusion resulted at later perfusion times ($>$2 hours) in a significantly greater increase in ALA synthetase activity when nembutal was used to anaesthetize rats for perfusion experiments that when ether was used as anaesthetic. (As shown in Table 5-3, ether

TABLE 5-3: EFFECT ON ALA SYNTHETASE ACTIVITY OF *in vivo* ADMINISTRATION OF COMPOUNDS USED IN PERFUSION EXPERIMENTS.

Compound	Dose and method of administration	Time killed hr.	ALA synthetase activity nmoles ALA/mg protein/20 min
None	-	-	0.113
Anaesthetics:			
NEMBUTAL	120 mg/kg injected i.p.	2 3	0.201 0.453
ETHER	inhalation	2 3	0.104 0.120
Anticoagulant:			
HEPARIN	200 units, i.v.	3	0.091
	600 units, s.c.	3	0.074
Pertusate component:			
CASEIN HYDROLYSATE	600 mg stomach tube	3 14	0.099 0.078

The compounds shown were administered to fasted rats as indicated. At the time shown animals were killed; their livers were removed and homogenates prpeared and assayed as described in Materials and Methods. Each value is the average of results from at least three experiments.
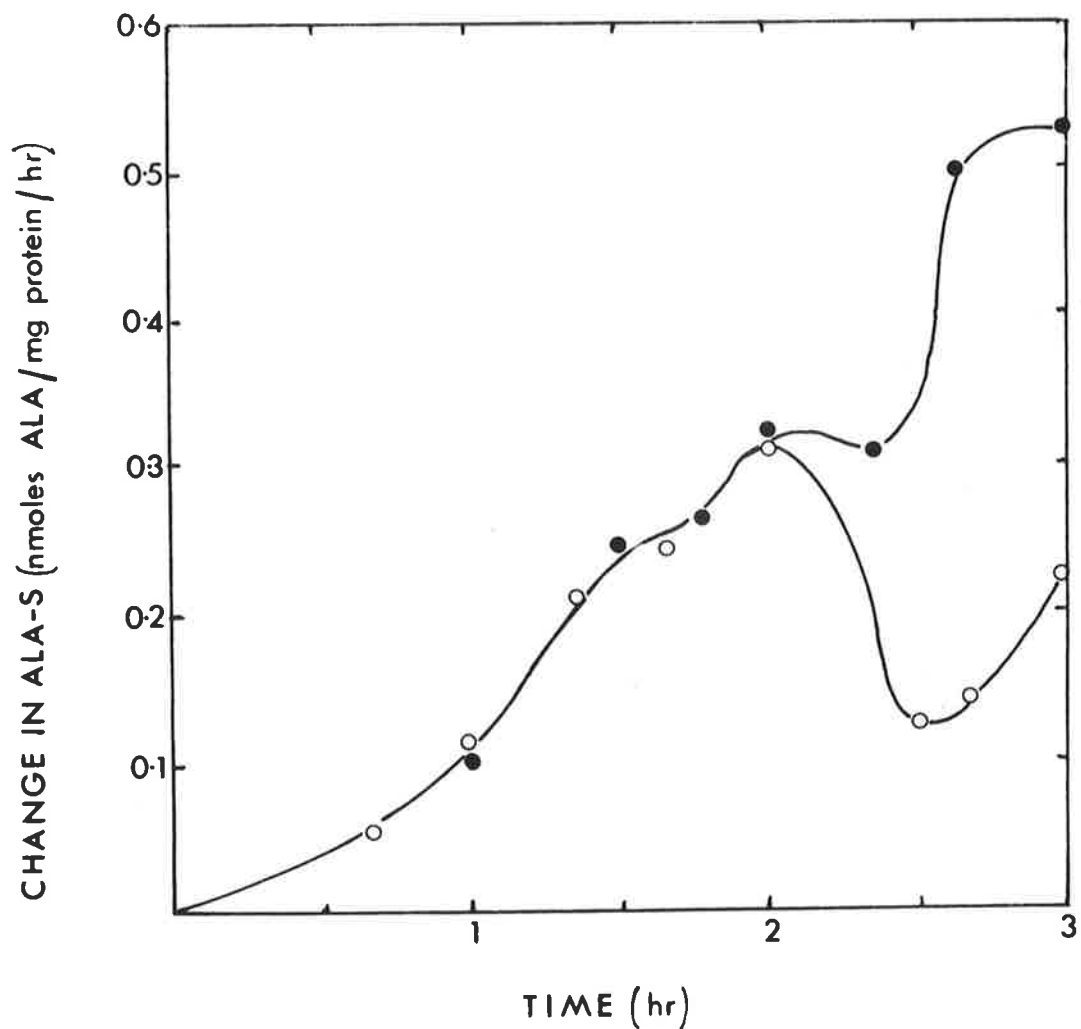
FIG. 5-17.   Effect of control perfusion
on total ALA synthetase activity:
significance of anaesthetic used on blood-
donor and liver-donor rats. Procedure
was as in Fig. 5-14(b) except for variation
in the anaesthetic used.

●————● nembutal as anaesthetic [5]
                    (normal procedure)

○————○ ether as anaesthetic [3].

had little effect on the level of ALA synthetase *in vivo*.)
Thus it appeared that part of the induction observed in control
perfusions was due to *in vivo* administration of nembutal.
However, a further explanation was required for the significant
2-3 fold increase in ALA synthetase activity which was observed,
(Fig.5-17) when ether-anaesthetized rats were used for
perfusion experiments (i.e., no exogenous compounds with known
inducing effect present).

It appeared that the control induction observed when
ether was used as anaesthetic might be due to any of three
causes:

(i)   surgical trauma associated with perfusion experiments

(ii)   dilution of factors in rat blood, in the perfusate

(iii)   accumulation of metabolites or depletion of factors
in blood (e.g., hormones) due to isolation of the liver and
circulation from other organs present *in vivo* (e.g., kidneys,
endocrine glands).

To test possibility (i), the effect of sham operation
on the level of ALA synthetase in liver was investigated. In
sham operations, rats were anaesthetized with ether or nembutal
as indicated, then subjected to surgery (opening of the abdominal
cavity) similar to that in perfusion experiments except that
circulation to the liver was not interrupted, the thoracic
cavity was left intact and the animal remained under
anaesthesia. Small samples of liver were removed after
various times as in perfusion experiments.   This then
represented an *in vivo* experiment (under anaesthestic)
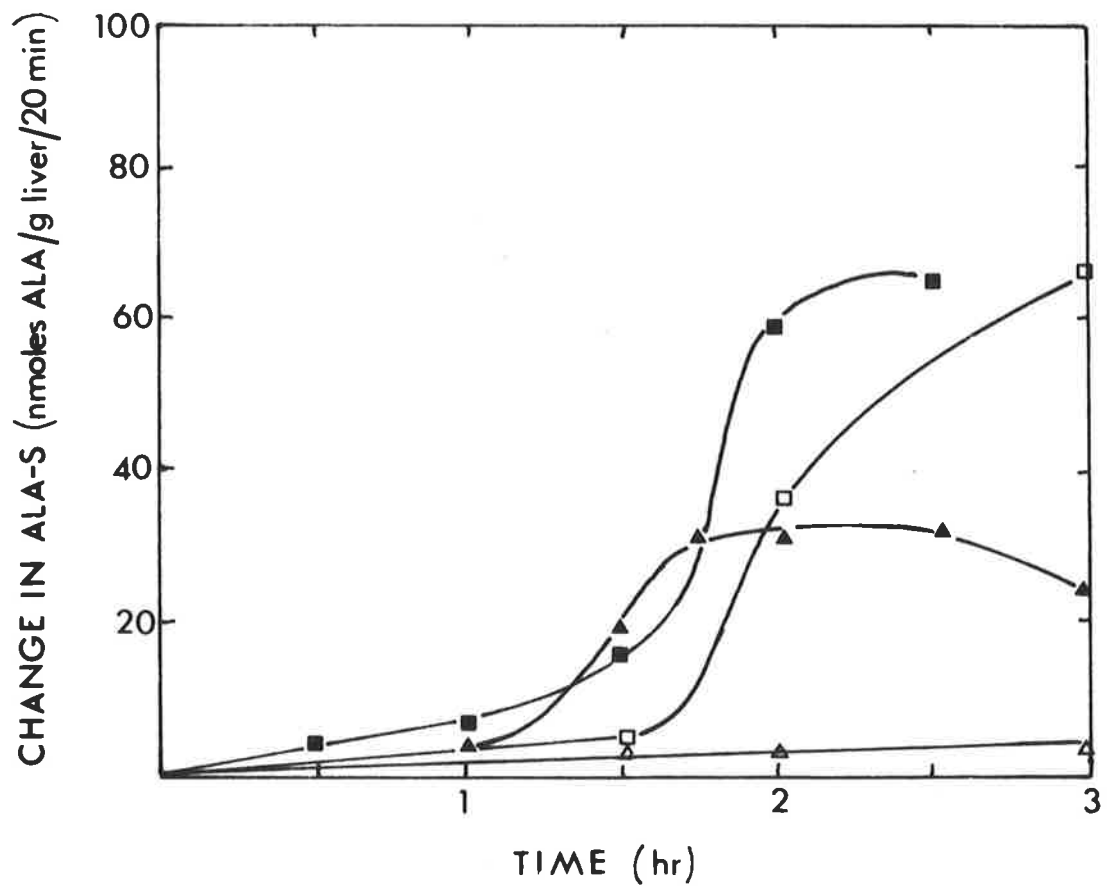
FIG. 5-18. Effect of sham operation on
total ALA synthetase activity in the livers
of anaesthetized rats. See text for
experimental details.

■————■ nembutal anaesthetic, sham
operated [3]

□————□ nembutal anaesthetic, not
operated [3]

▲————▲ ether anaesthetic, sham
operated [3]

△————△ ether anaesthetic, not
operated [3].

in which the perturbation was surgical trauma essentially the same as that involved in perfusion experiments. The effects of anaesthetic plus surgical trauma compared to the effects of anaesthetic alone *in vivo* are shown in Fig.5-18*. In ether-anaesthetized rats, sham operation caused a significant 2-3 fold increase in activity over that in non-operated rats, suggesting that surgical trauma was indeed one cause of induction in control perfusions (see Fig.5-19 for direct comparison of inductions observed in sham-operated rats and in control-perfused livers). In nembutal-anaesthetized rats, induction was also greater in sham-operated, than in non-operated animals.

The possible cause of induction in control perfusions, in particular the possible significance of diluting factors in rat blood, was also checked by perfusing with whole blood and with a perfusate containing 55% rat blood. Changes in ALA synthetase observed in these experiments are compared with those seen in control perfusions with standard perfusate (40% rat blood) in Fig.5-19. That a significant (2-fold) induction occurred in perfusions with whole blood, would suggest that either surgical trauma or isolation of the liver and circulation from other organs contributes to the

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*Twenty minute incubation times were used for assays in these, and most later experiments. These results can be approximately compared with those using 60 minute assays by multiplying the 20 minute activity by 1.45.
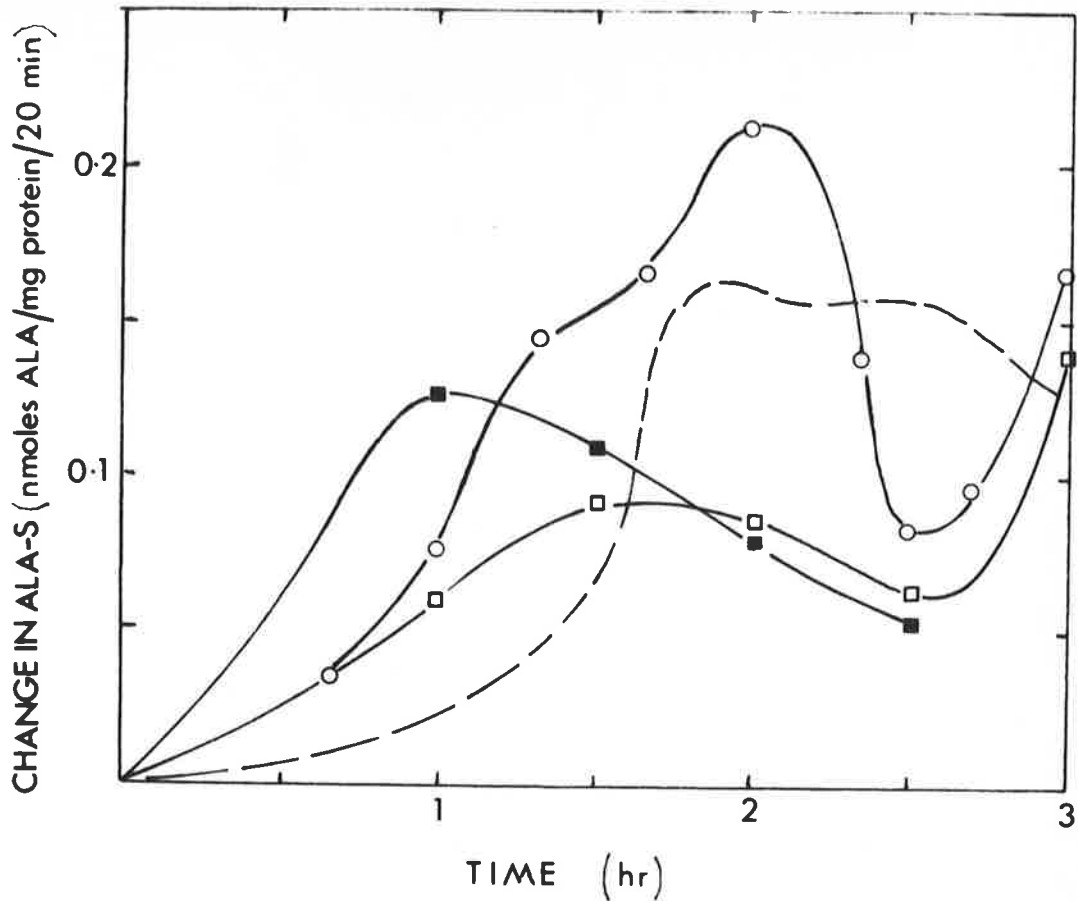
FIG. 5-19. Effect on total ALA synthetase activity of perfusion with media containing different concentrations of rat blood. Ether was used as anaesthetic in all experiments. Homogenates were assayed using a 20 min. incubation time.

○————○   40% rat blood (normal concentration) [3]

□————□   55% rat blood [3]

■————■   100% rat blood [3]

(The effect of sham operation (Fig. 5-18) on activity in a non-perfused liver is shown for comparison —————————.)

TABLE 5-4: EFFECT OF INHIBITORS OF RNA AND PROTEIN SYNTHESIS
ON ACTIVITY OF ALA SYNTHETASE IN RAT LIVER
HOMOGENATES.

Additions		% Activity
None		[100]
Actinomycin D	5 µg/ml	100
	0.5 µg/ml	96
Cycloheximide	20 µg/ml	94
	2 µg/ml	97

A liver homogenate was prepared as described in Materials and
Methods, from the liver of a fasted rat pretreated for 22 hours
with AIA. ALA synthetase activity in the homogenates was then
assayed with additions to the assay mixture as indicated.

control induction. Since induction with whole blood fell between
the increases seen with 40% and 55% blood, it is difficult to
draw conclusions on the significance of diluting components in
rat blood.

The effects of inhibitors of RNA and protein synthesis
on changes in the level of ALA synthetase in control perfusions
are shown in Fig.5-20. Ether was used as anaesthetic in these
experiments. Cycloheximide, at a level shown by other workers
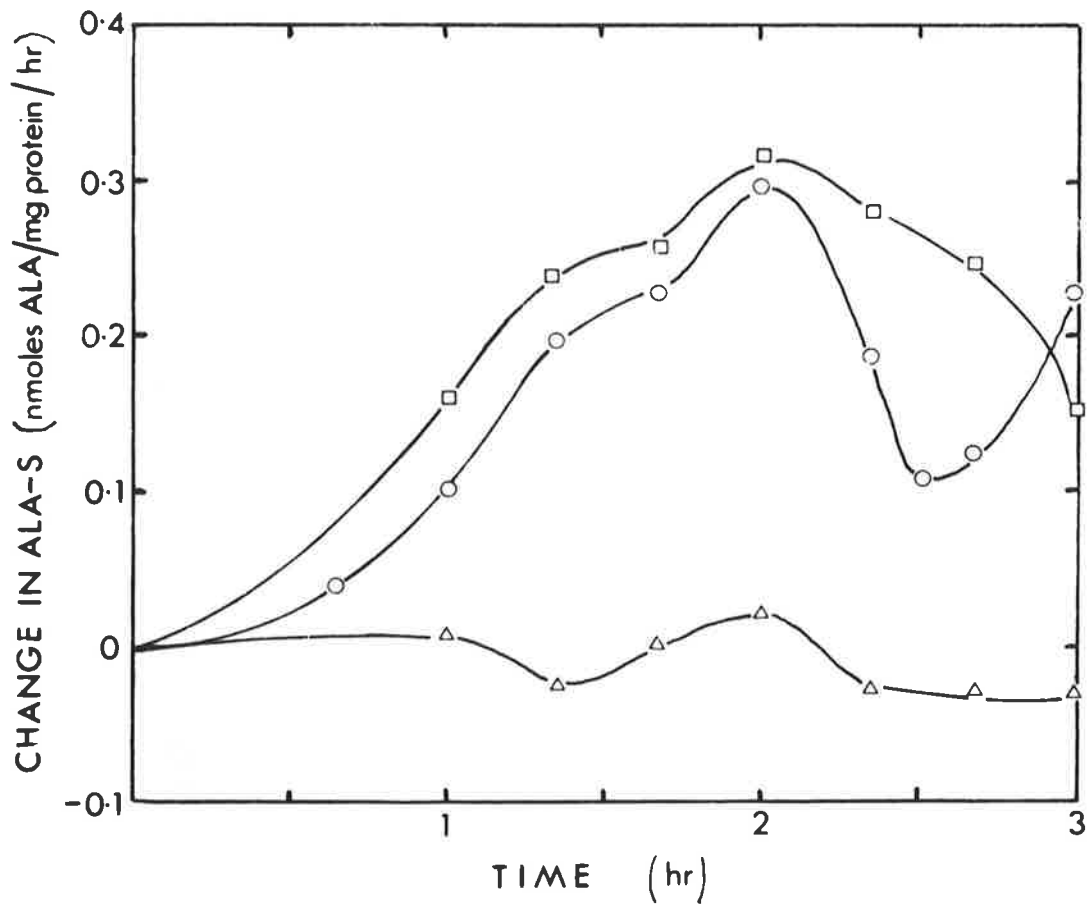
FIG. 5-20.   Effects of cycloheximide and
actinomycin D on induction of total ALA
synthetase in control perfusions.   Ether
was used as anaesthetic in all experi-
ments.   Homogenates were assayed using
a 60 min. incubation time.

○————○   no additions [3]

△————△   + cycloheximide, 10 ug/ml
            [2]

□————□   + actinomycin D, 5 ug/ml
            [3].

[331] to inhibit protein synthesis about 95%, completely blocked increases in enzyme level in control perfusions, suggesting that new protein synthesis was required and that the increase was indeed an 'induction' rather than an activation of existing enzyme. The increase was, however, not prevented by a high level of actinomycin D, suggesting that new RNA synthesis was not required. As shown in Table 5-4, neither actinomycin D nor cycloheximide significantly altered the activity of ALA synthetase *in vitro*.

(b)   The effects of AIA in perfused liver.

As indicated above, where total ALA synthetase activity was measured, perfusion with AIA was found to cause only slightly greater induction than observed in control perfusions (Fig.5-16). The time course of induction by AIA in perfused liver was not signficantly different when ether was used in place of nembutal to anaesthetize the rats for perfusion experiments (Fig.5-21), although in experiments with ether-anaesthetized rats, activity in livers perfused with AIA was relatively greater than in controls because of the lower control inductions observed at later perfusion times.

Regardless of the anaesthetic used, the increased induction due to AIA over that in controls in perfused liver was small. Furthermore, the total induction in livers perfused with AIA was markedly less than that caused by AIA *in vivo*; comparison of perfusion results with induction by AIA *in vivo* in intact rats or sham-operated rats (Fig.5-22), suggests
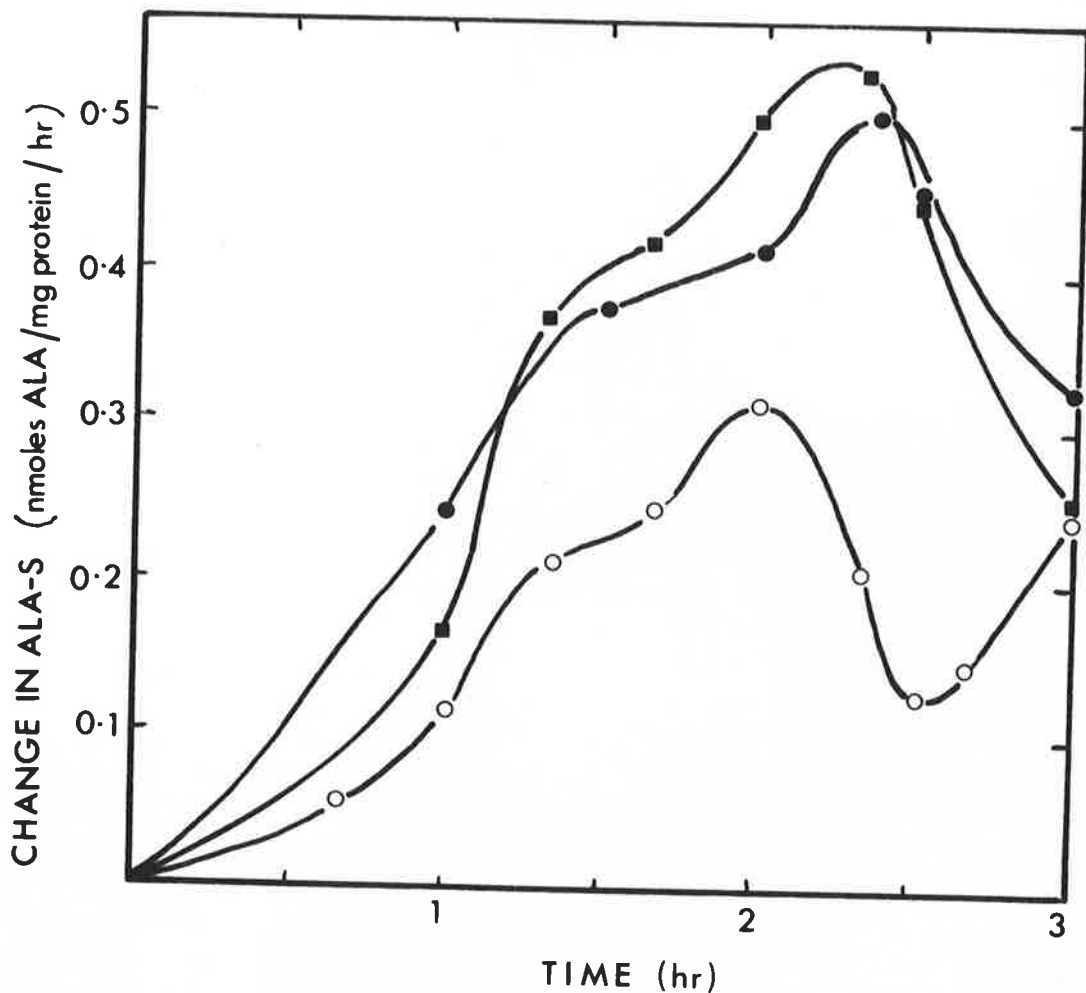
FIG. 5-21.   Effect of AIA on total ALA synthetase activity in perfused liver: significance of anaesthetic used on liver-donor and blood conor rats.   Procedure was as in Fig. 5-14 except for variation in the anaesthetic used.

●————● ether anaesthetic, no addition [3]

○————○ ether anaesthetic + AIA, 550 μg/ml [3]

■————■ nembutal anaesthetic + AIA, 550 μg/ml [4]ˉ
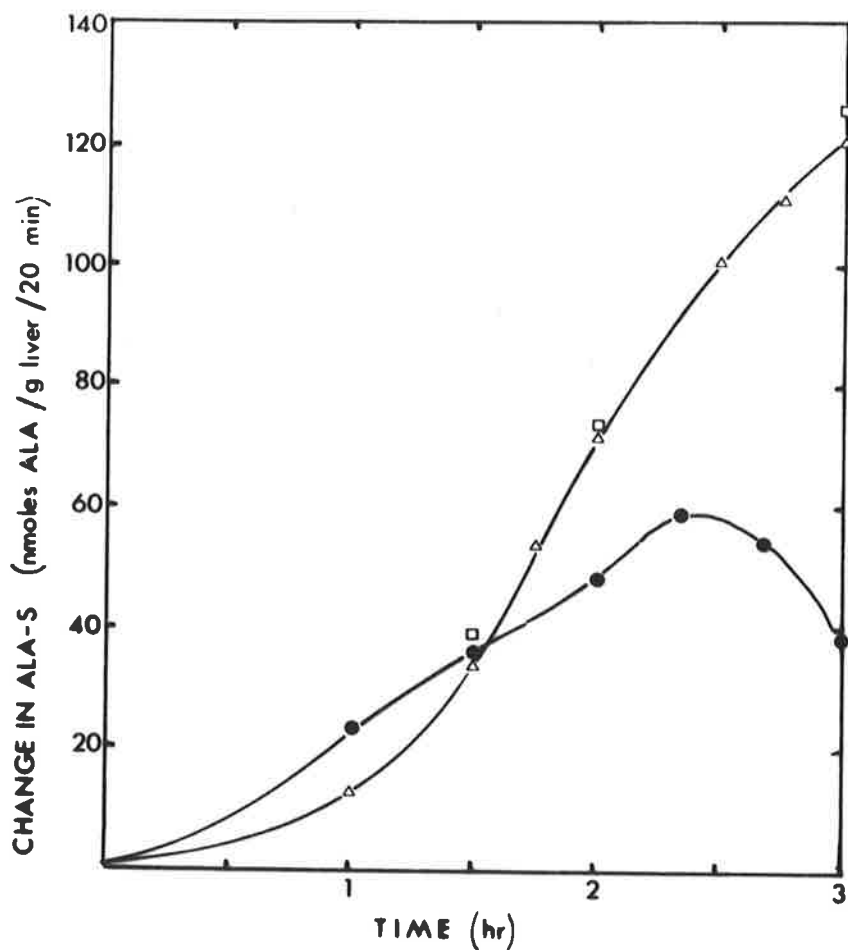
FIG. 5-22. Comparison of the effects of
AIA on total ALA synthetase activity
in vivo and in perfused liver. Two groups
of rats were injected with AIA at zero
time. In one group, the *in vivo* effects
of AIA were measured as in Fig. 5-2(a).
The second group were anaesthetized with
nembutal and a series of liver samples
taken in a "sham operation" as in Fig. 5-18.
The effects of AIA in perfused liver were
measured as in Fig. 5-14(a) except that in
all experiments, 20 min. incubation times
were used in assays.

□———————□ + AIA *in vivo* (not operated)

△———————△ + AIA *in vivo* , sham operated
                  [3]

●———————● + AIA in perfused liver,
                  550 ug/ml [4]

that under the conditions used, AIA fails to exert in perfused liver the potent inducing effect it exerts *in vivo*. The similarity between effects of AIA in intact and sham-operated rats suggests that the relative lack of induction by AIA in perfused liver is, at least, not a result of surgical trauma.

A weak inducing effect in perfusion could be explained if protein synthesis in the perfused liver was reduced because certain amino acids (e.g, tryptophan) were lacking in the perfusate. The effect on induction by AIA of replacing the casein hydrolysate (which lacks tryptophan and certain other amino acids) normally present in the perfusate with a synthetic mixture of all amino acids to a final concentration of 6x serum levels is shown in Fig.5-23. The change in the amino acid compositon of the perfusate did not significantly alter the extent of induction in the presence of AIA.

Since the level of AIA used in perfusions (550 µg/ml) was relatively high compared to the levels used by Granick [80] in tissue culture experiments, another possible explanation for weak induction was that the high concentration of AIA used was toxic to the cells. However, in experiments with lower levels of AIA (100 µg/ml) the change in the level of ALA synthetase was essentially as in control perfusions (Fig. 5-24).

Because these preliminary experiments failed to provide any simple explanation for the weak effects of AIA on total ALA synthetase activity, further experiments were
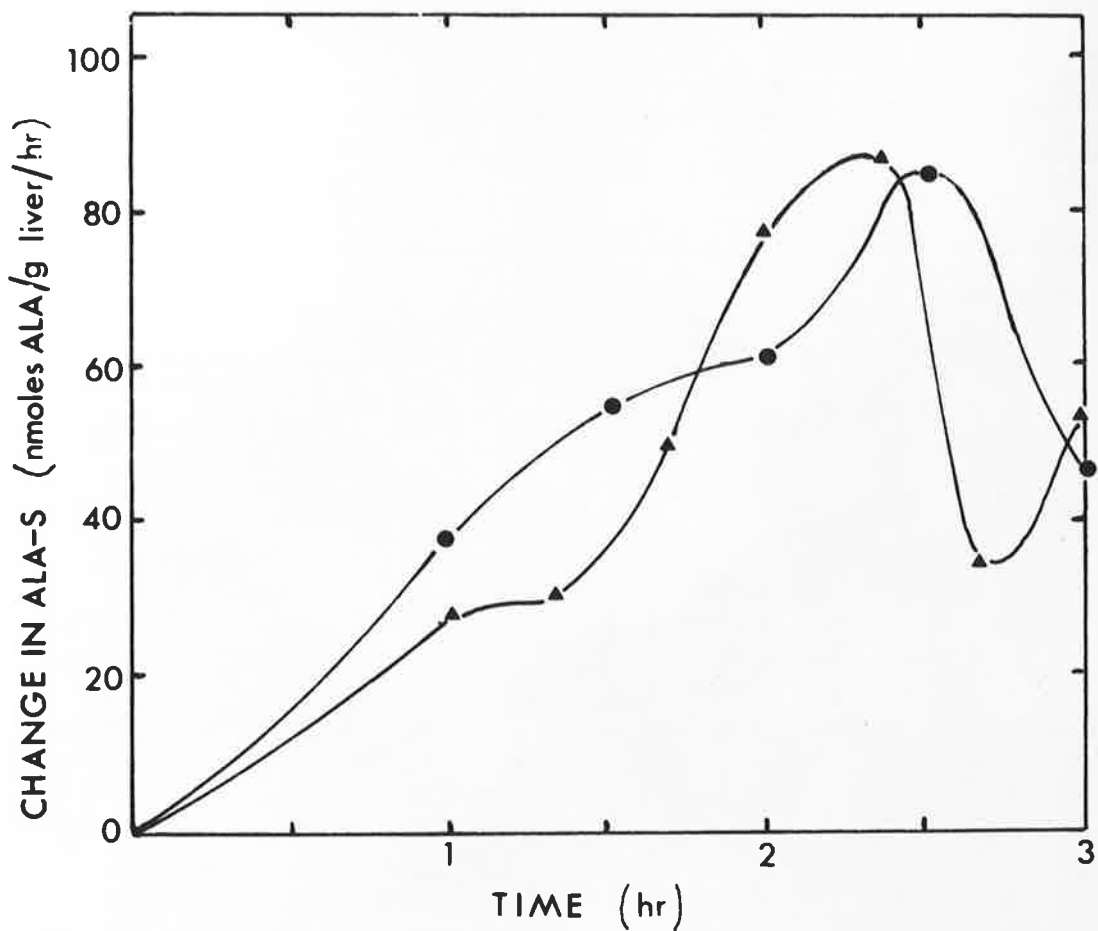
FIG. 5-23. Effect of perfusion with AIA
on total ALA synthetase activity: signifi-
cance of amino acid additions to the
perfusate. Procedure was as in Fig. 5-14(a)
with variations as indicated.

●————● + AIA, 550 µg/ml with normal
amino acid composition, i.e.,
casein hydrolysate [4]

▲————▲ + AIA with synthetic amino
acid mixture, 6x serum levels
[2]

directed towards finding agents with more potent inducing action in perfused liver.

(III) The Effects of Lead Acetate on ALA Synthetase Levels in Perfused Liver.

In preliminary experiments (Fig.5-13) in which changes in mitochondrial ALA synthetase were measured, perfusion of liver with lead acetate caused a marked increase in enzyme level. The effects of perfusion with high concentrations of lead acetate on total hepatic ALA synthetase activity are shown in Fig. 5-25. Lead ions caused a small but significant increase over the control at early perfusion times; at later times (2-3 hours) the enzyme level in the presence of lead was not significantly different from that in controls. Induction by lead in the perfused liver contrasts with its lack of effect on ALA synthetase *in vivo*. As discussed in Chapter 1 most previous studies have suggested that lead does not induce hepatic ALA synthetase *in vivo* [150,288], although one group [202] reported that lead acetate injected intravenously caused 3-fold induction over 24 hours. The results of some *in vivo* experiments in which lead acetate was administered by a variety of routes are shown in Table 5-5. When moderate doses of lead acetate were injected intraperitoneally, a small increase in activity over 24 hours was observed but in other experiments lead had little effect. Experiments in which lead acetate was added in enzyme assays (Table 5-6) show lead had little effect *in vitro*, on ALA synthetase activity as
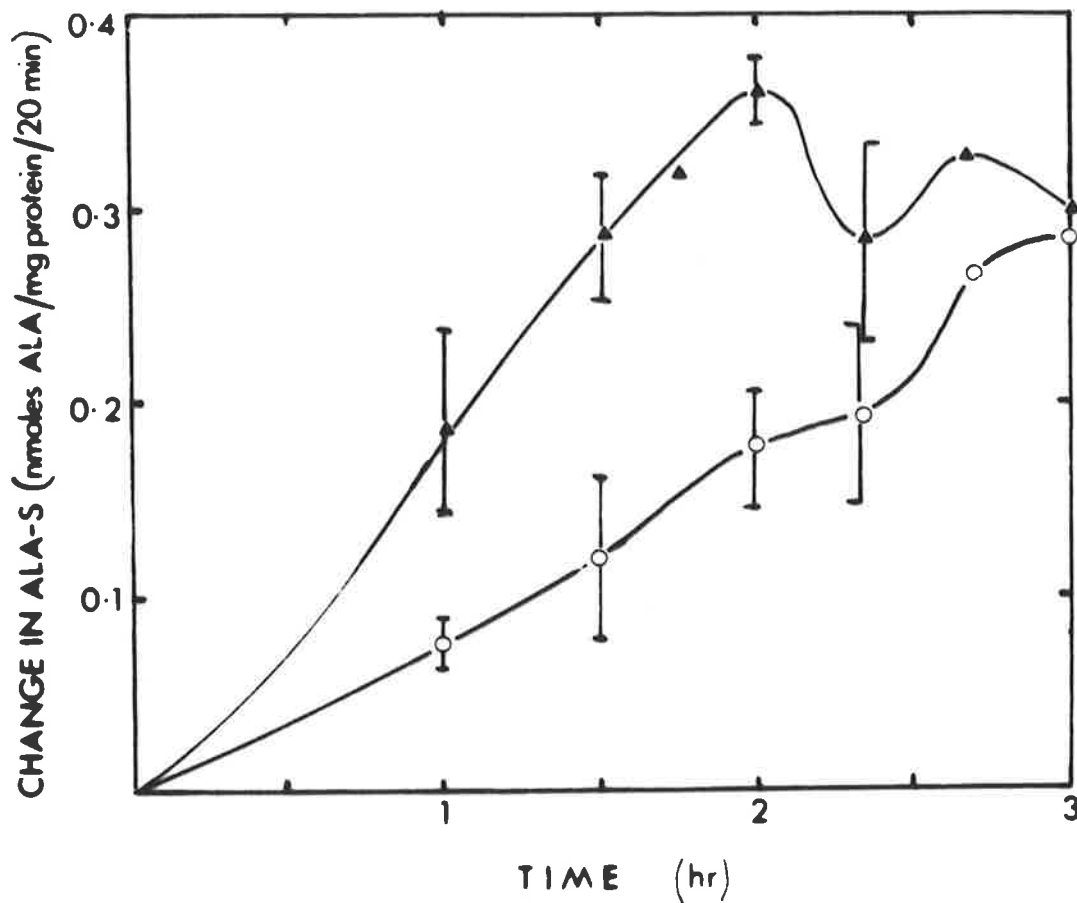
FIG. 5-25. Effect of perfusion with lead acetate on total ALA synthetase activity. Procedure was as in Fig. 5-24.

○————○   no additions [5]

▲————▲   + lead acetate, 10^{-3}M [5]

TABLE 5-5: EFFECT ON ALA SYNTHETASE ACTIVITY OF *in vivo*

ADMINISTRATION OF LEAD ACETATE.

No. of experiments	Method of administration	Dose of lead acetate mg/kg	Time killed hr.	Change in ALA synthetase activity nmoles ALA /mg protein /20 min.	% change
2	i.v.	17	2	− 0.004	NS
2	i.v.	20	24	+ 0.005	NS
3	i.p.	50	24	+ 0.045	+ 27
2	i.p.	100	24	+ 0.038	+ 23
2	i.p.	200	24	+ 0.018	NS
2	s.c.	100	24	− 0.018	NS
3	stomach tube	1000	14	− 0.104	− 57

Experimental procedure was as in Table 5-3.
NS = not significant.

TABLE 5-6: EFFECT OF LEAD ACETATE ON THE ACTIVITY OF ALA

SYNTHETASE IN RAT LIVER HOMOGENATES.

Final concentration of lead acetate	% change in activity
(a) $10^{-4}M$	− 8
$2 \times 10^{-4}M$	+ 2
$10^{-3}M$	+ 1
(b) $10^{-4}M$	+ 12
$10^{-3}M$	+ 7

For (a) experimental procedure was as in Table 5-4. For (b)
procedure was as in Table 5-4 except that the homogenate was
prepared from an untreated rat.

measured in crude homogenates, in the presence of EDTA.

In perfusion experiments, induction in the presence of lead ions was apparently dependent on RNA and protein synthesis since, as shown in Fig. 5-26, induction was prevented by actinomycin D or cycloheximide. That actinomycin D completely prevented induction was surprising since induction in control perfusions was previously found to be insensitive to the antibiotic (Fig. 5-20). Further experiments will be required to clearly establish the effects of actinomycin D.

The effect of varying the amino acid composition of the perfusate was again tested with lead as inducer. In this experiment the casein hydrolysate was supplemented with tryptophan since it has been shown [332, see also Chapter VI] that perfusion in the absence of tryptophan resulted in marked breakdown of liver polysomes. Tryptophan was added to a final concentration of 0.15 mg/ml which corresponded to 10x serum concentration [333]. In the presence of added tryptophan, induction by lead was significantly reduced (Fig. 5-27). This result was surprising, firstly because the addition of tryptophan might have been expected to enhance hepatic protein synthesis and hence possibly the extent of induction observed, and secondly because tryptophan has previously been found in *in vivo* experiments to act as an inducer of hepatic ALA synthetase [334]. Fig. 5-27 also shows the results of perfusions with lead acetate plus AIA. Addition of AIA almost completely prevented the increased induction (over controls) due to lead. In perfusions with AIA plus lead acetate replacement of the
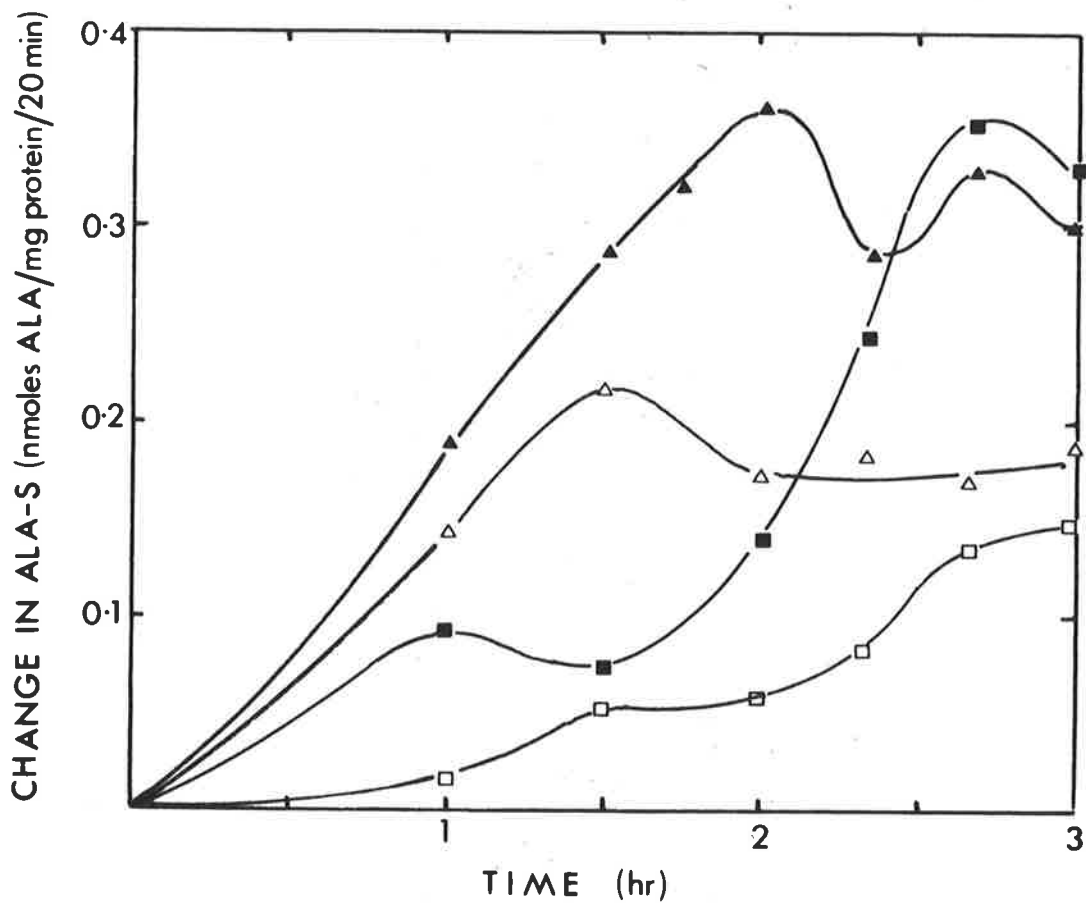
FIG. 5-27. Effects of added AIA and of changes in the amino acid composition of the perfusate on induction of total ALA synthetase in livers perfused with lead acetate. Prodedure was as in Fig. 5-24 with variations as indicated.

▲———▲ + lead acetate, 10^{-3}M; normal perfusate [5]

△———△ + lead acetate + tryptophan, 0.15 mg/ml [3]

■———■ + lead acetate + AIA, 550 µg/ml; normal perfusate [2]

□———□ + lead acetate + AIA; casein hydrolysate replaced by synthetic amino acid mixture, 10x serum levels [2].

(SEE 2 PAGES OVER FOR FIG 26)

casein hydrolysate with a synthetic amino acid mixture, added
to a final concentration of 10x serum levels, resulted in a
further decrease in the induction observed.

(IV)   The Effects of Steroids on ALA Synthetase Levels
       in Perfused Liver.

Previous findings on the role of steroids in control
of hepatic ALA synthetase were discussed in Chapter 1. Studies
on porphyric patients and with chick embryo liver would suggest
that steroids might have an important role in regulation of
ALA synthetase in mammalian liver, but there is little direct
evidence for this from *in vivo* studies with mammals.   In
liver perfusion experiments it is possible to avoid some of the
difficulties in assessing the effects of small doses of steroids
inherent in *in vivo* experiments, since in perfusions the liver
could be exposed to a known concentration of steroid unmodified
by varying rates of absorption, extrahepatic metabolism or
excretion.

As a prelude to investigating the effects of steroids
in perfused liver, the effects of some steroids *in vivo* were
tested.   In short term experiments, only progesterone, of
the small group of steroids tested, caused a marked (4-fold)
induction of ALA synthetase although in this experiment
hydrocortisone also caused a small increase (Table 5-7),
Table 5-8 shows the effects of varying doses of progesterone
on the level of ALA synthetase *in vivo*. The dose which could
be administered was limited by the toxicity of progesterone.

TABLE 5.7:   EFFECTS ON ALA SYNTHETASE ACTIVITY OF

in vivo ADMINISTRATION OF STEROIDS.

No. of experiments	Steroid	ALA Synthetase activity nmoles ALA/mg protein /20 min.
3	None (maize oil alone)	0.126
2	progesterone	0.518
2	17β-estradiol	0.174
2	hydrocortisone	0.282
2	etiocholanolone	0.174

Steroids, dissolved in maize oil, were administered to
fasted rats by i.p. injection. Doses in all cases, were
200 mg/kg. After 3 hours, rats were killed; their livers were
removed and homogenates prepared and assayed as in Materials
and Methods.

The effect of perfusion with progesterone at a final
concentration similar to that used by Kappas and Granick in
tissue culture experiments [86], is shown in Fig.5-28.
Progesterone caused a significant increase in activity over
the control with maximal activity corresponding to a 5-fold
increase over the basal level. Induction by progesterone was
prevented by cycloheximide or actinomcyin D (Fig.5-29)
suggesting that induction required continuing RNA and
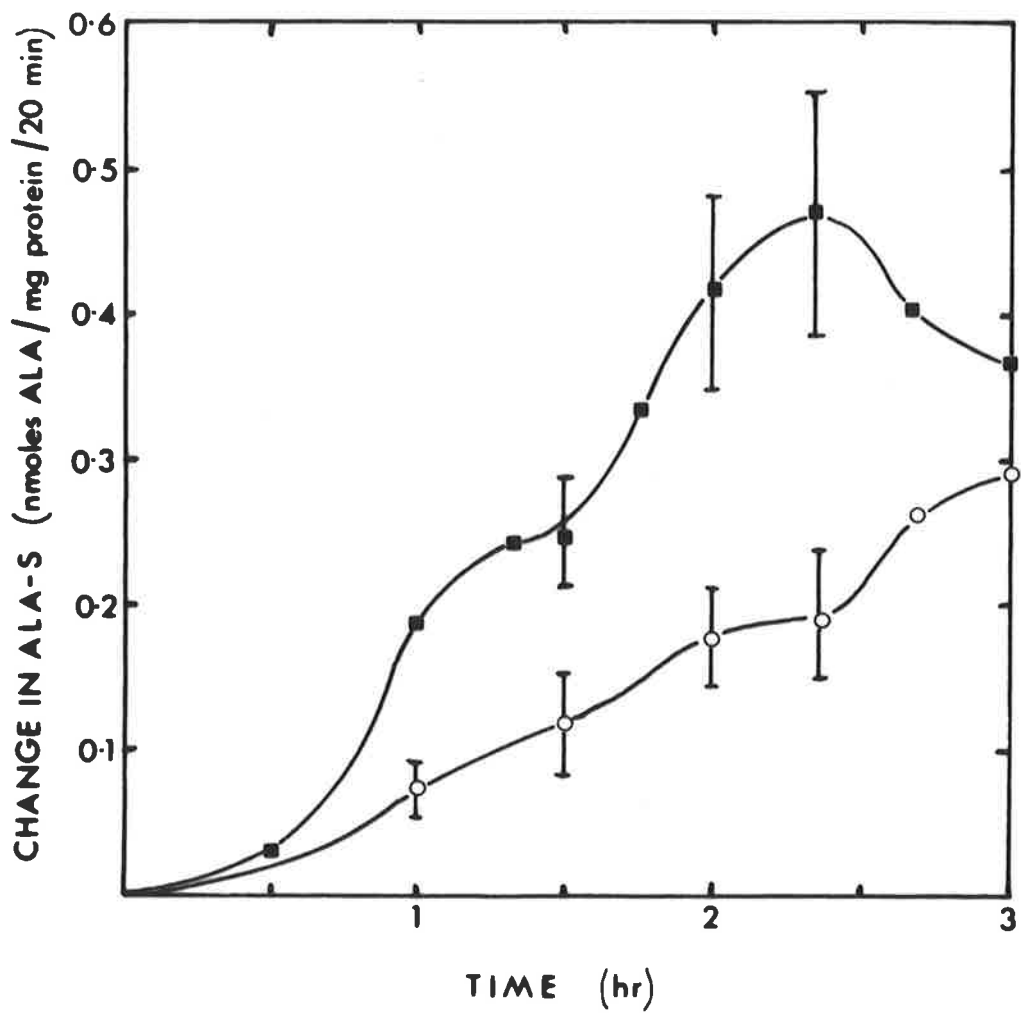
FIG. 5-28. Effect of perfusion with progesterone on total ALA synthetase activity. Procedure was as in Fig. 5-24.

■————■ no additions [5]

○————○ + progesterone, 12 μg/ml [4]

TABLE 5-8:   EFFECT ON ALA SYNTHETASE ACTIVITY OF *in vivo*
ADMINISTRATION OF PROGESTERONE - DOSE RESPONSE

No. of experiments	Treatment	ALA synthetase activity nmoles ALA/mg protein /20 min.
4	None	0.165
3	Maize oil alone	0.126
2	Progesterone, 100 mg/kg	0.304
2	200 mg/kg	0.518
2	300 mg/kg	0.539*

Experimental procedure was as in Table 5-7 except that the dose
of progesterone administered was varied as shown.

*Several rats injected with 300 mg progesterone/kg, died within
 3 hours.

protein synthesis.

Induction by progesterone in perfused liver is compared
with the effects of perfusing with the 5β-H steroids, pregnano-
lone and etiocholanolone, in Fig. 5-30. In perfusions with
etiocholanolone at a level (6 µg/ml) comparable to that at which
the steroid caused marked induction in chick embryo liver
tissue culture, the changes in ALA synthetase were similar to
those in control perfusions. At higher concentrations, etio-
cholanolone was apparently toxic, since induction was less
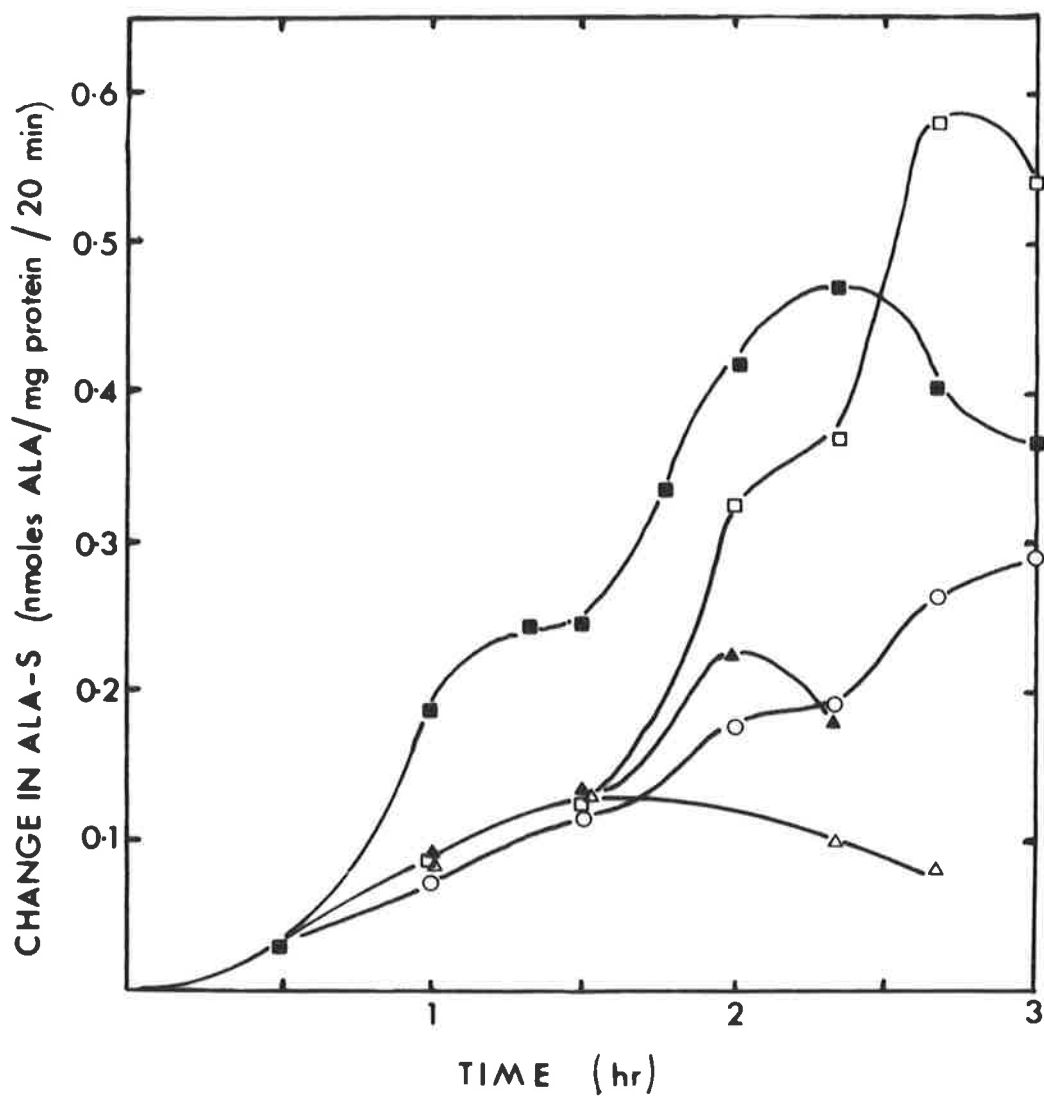
FIG. 5-30. Effect of perfusion with various steroids on total ALA synthetase activity. Procedure was as in Fig. 5-24.

O————O   no addition [5]

■————■   + progesterone, 12 µg/ml [4]

□————□   + pregnanolone, 12 µg/ml [1]

△————△   + etiocholanolone, 12 µg/ml [1]

▲————▲   + etiocholanolone, 6 µg/ml [1]

TABLE 5-9:   EFFECTS OF HEMIN ON THE ACTIVITY OF ALA

SYNTHETASE IN RAT LIVER HOMOGENATES.

Final Hemin Concentration	% Change in ALA Synthetase Activity
10^{-6}M	− 8
10^{-5}M	− 9
2 x 10^{-5}M	− 9
10^{-4}M	− 4

Experimental procedure was as in Table 5-4.

than in controls.   Toxicity of etiocholanolone was previously

noted by Kappas and Granick [86].   Although etiocholanolone

apparently caused no induction, perfusion with the 5β-H

progesterone derivative, pregnanolone, caused a marked increase

in activity (Fig.5-30).   The maximal activity corresponded to

a 6-fold induction.   This preliminary observation (the

result of a single perfusion) raises the important possibility

that at least some 5β-H steroids may be inducers of ALA

synthetase in mammalian liver.

The effect of two agents previously shown to partially

prevent induction of mitochondrial ALA synthetase in perfused

liver (Figs. 5-12 and 5-13) were re-examined with progesterone

present as inducer.   As shown in Fig. 5-31, induction by
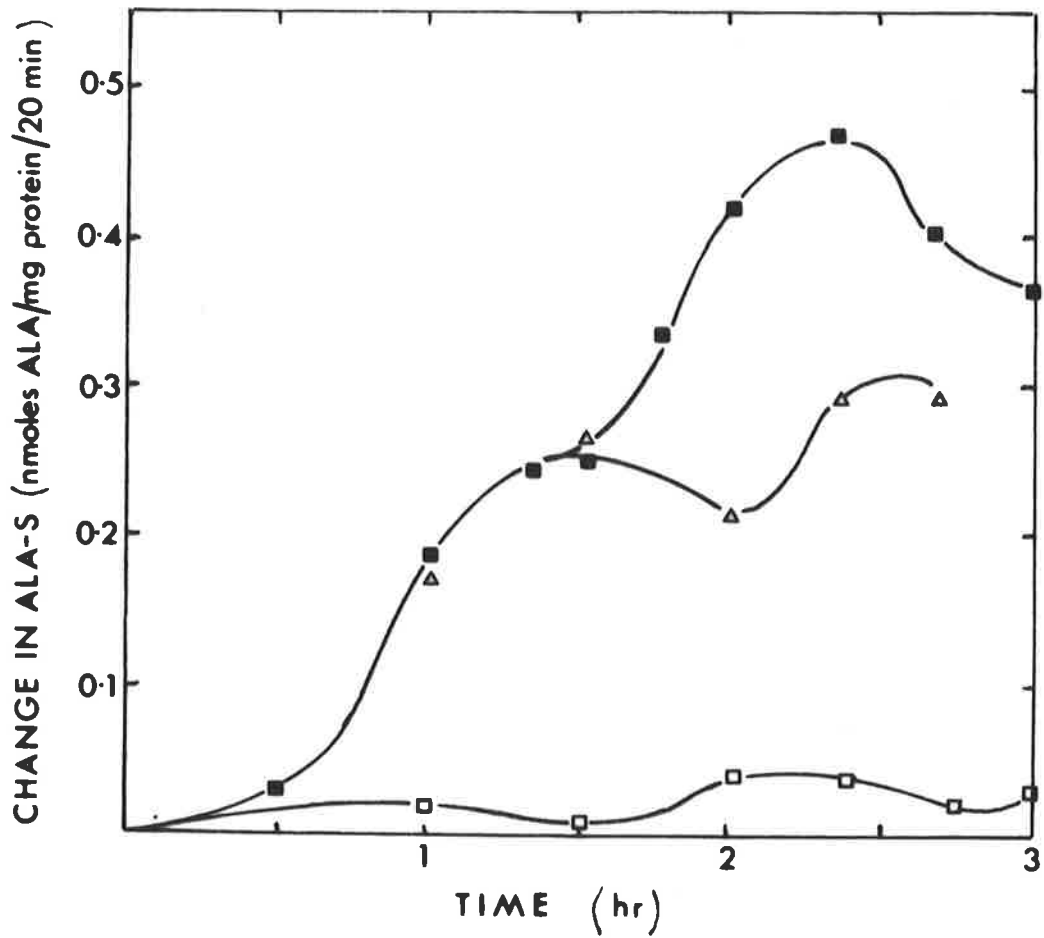
FIG. 5-31. Effects of insulin and heme on
induction of total ALA synthetase activity in
livers perfused with progesterone. Procedure was
as in Fig. 5-24.

■————■   + progesterone, 12 g/ml [4]
△————△   + progesterone + insulin,
                 infused at 50 munits/ml/hr [2]

□————□   + progesterone + hemin,
                 2 x 10^{-5}M [2].

progesterone was completely prevented by addition of hemin
to a final concentration of 2×10^{-5} M. This observation
confirmed that hemin at moderate concentrations acts directly
on isolated liver as a potent repressor of ALA synthetase.
In assays of ALA synthetase in crude homogenates hemin did
not cause significant inhibition of activity (Table 5-9).

In previous investigations of the 'glucose effect'
(see Fig.5-12), it was found that insulin largely prevented
induction of mitochondrial ALA synthetase in perfusions with
AIA. The effect of insulin on induction by progesterone is
shown in Fig.5-31. Overall induction in the presence of
progesterone was reduced about 35% by addition of insulin.
Apparent repression by insulin was thus less in the presence
of progesterone than previously observed with AIA (Fig. 5-12).
ALA synthetase was not inhibited by addition of insulin to
assays of liver homogenates.

(V)   The Effects of Dibutyryl Cyclic AMP on ALA
      Synthetase Levels in Perfused Liver.

The effects of dibutyryl cyclic AMP on the level of
ALA synthetase in perfused liver are shown in Fig.5-32. The
nucleotide caused a significant increase in activity compared
to controls, corresponding to a 5-6 fold induction. Induction
in the presence of dibutyryl cyclic AMP was only partly
prevented by a high level of actinomycin D (Fig. 5-32)
suggesting that induction was at least partly independent
of new RNA synthesis.
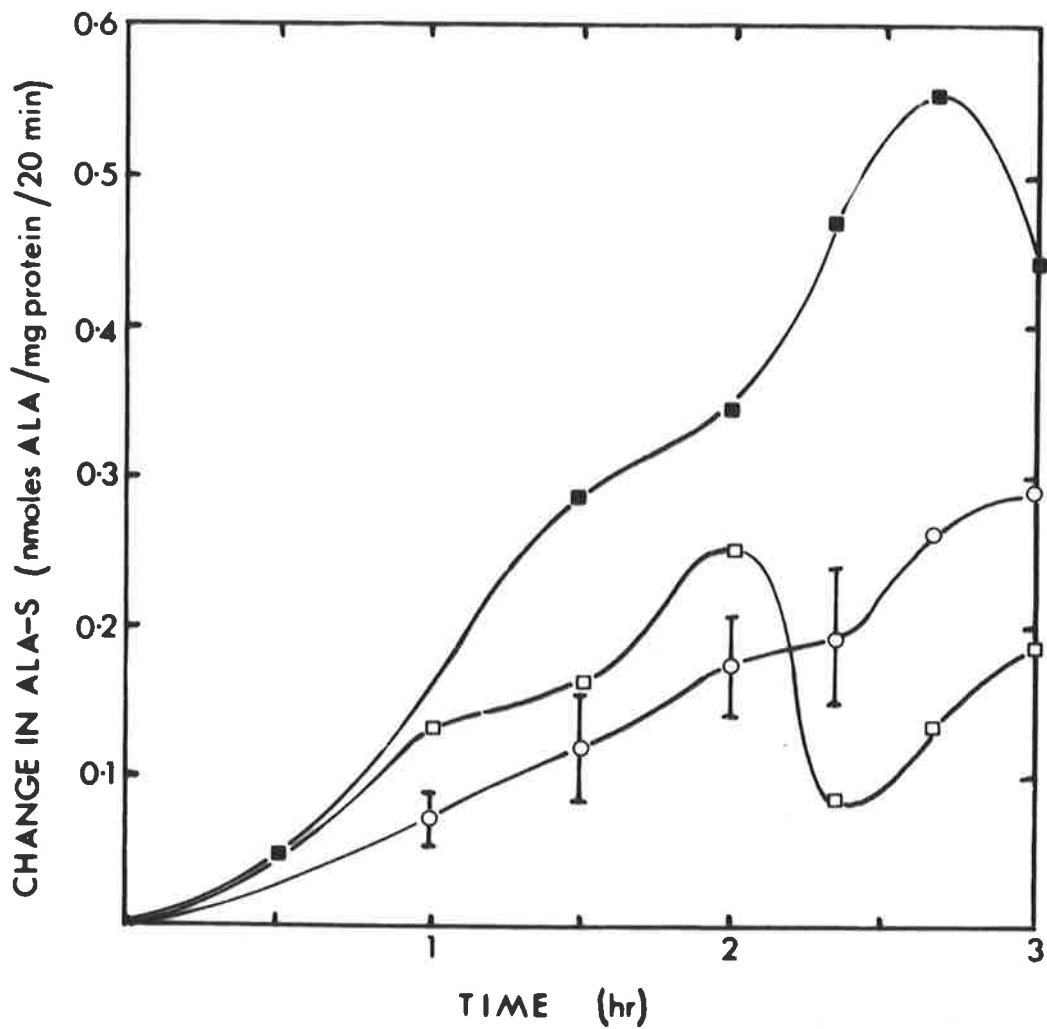
FIG. 5-32.   Effect of perfusion with dibutyryl
cyclic AMP on total ALA synthetase activity; effects
of added actinomycin D. Procedure was as in
Fig. 5-24.

○————○   no addition [5]
■————■   + dibutyryl cyclic AMP, 10^{-4}M
          [2]
□————□   + dibutyryl cyclic AMP +
          actinomycin D, 5 µg/ml [2]

CHAPTER VI

STUDIES ON THE REGULATION OF ALA SYNTHETASE IN
PERFUSED RAT LIVER - DISCUSSION

STUDIES ON THE REGULATION OF ALA SYNTHETASE IN PERFUSED RAT LIVER - DISCUSSION

A. DEVELOPMENT OF PERFUSION METHODS AND CONDITIONS.

(I) Value of Perfused Rat Liver as a Model System for Studies on Control of ALA Synthetase in Mammalian Liver.

The perfusion system described above provides a means of investigating the factors concerned in regulation of mammalian liver ALA synthetase at the level of the isolated intact organ. It might be expected that the features of control in such a system would correspond closely to the controls acting *in vivo*. Studies with the isolated liver are not complicated by interactions with endocrine or other extrahepatic tissues and should establish which agents affect ALA synthetase activity by direct action on the liver. Furthermore the ability to directly control concentrations of potential 'inducers' and 'repressors' without complications of varied absorption, excretion or extrahepatic interaction should simplify attempts to relate clinical findings in human patients and the results of *in vivo* studies with experimental animals, to events at a molecular level.

In turn it is hoped that an understanding of the mechanism of ALA synthetase regulation will suggest methods of treatment for the human hepatic porphyrias. It therefore

seems desirable to use an experimental system derived from mammalian liver: the rat liver perfusion system satisfies this requirement.

Liver perfusion, while eliminating some of the uncontrolled variables of *in vivo* experiments, inevitably introduces new variables. For instance, the replacement of the natural circulation with the partly synthetic perfusate used, results in a change in the environment of liver cells in that all the components of rat blood are diluted and new components are added. Furthermore, during perfusions, the levels of varous serum components (e.g., hormones) must change since they are no longer maintained by the homeostatic mechanisms operating *in vivo*. Such environmental changes may be more or less irrelevant to the metabolic events under investigation (e.g., in studies on control of hepatic tyrosine aminotransferase [314,315,335]). The observation of significant increases in the level of ALA synthetase in 'control' perfusions (Figs. 5-10,5-17) however suggests that such changes are indeed relevant in the present investigation (see further discussion below).

(II)   Choice of Perfusion Methods and Conditions.

(a) General features of perfusion system.

The perfusion methods and conditions used were all based on the methods of other workers [313-5]. With the surgical procedure used, portal blood flow was interrupted for only about 60 seconds. The subsequent flow rate of

perfusate through the liver (15-20 ml/min. or about 1.5 ml/g liver/min.) was rapid and comparable to that noted by other workers [313,315]. No objective assessment of liver 'viability' was made in these studies but perfusate flow rate and appearance of the perfused liver apparently provided a sensitive induction of the success of perfusions. Although flow rate *per se* did not appear to be an important variable in determining the level of ALA synthetase (Fig. 5-5), experiments in which the flow rate was low or where the perfused liver appeared blotchy or dark rather than an even pink-brown colour, have been excluded.

The choice of a standard perfusate composition was subject to a number of limitations since it was based in part on preliminary experiments which aimed only at defining conditions in which a significant and reproducible induction of ALA synthetase could be observed. These limitations are:
(i) The chosen conditions are not necessarily optimal for ALA synthetase induction by AIA or other agents.
(ii) In preliminary studies, AIA was used as 'inducer' on the assumption (based on previous *in vivo* and *in vitro* findings [6,7]) that it would act directly on liver as a potent inducer of ALA synthetase. Subsequent perfusion experiments (Fig. 5-16) suggest this assumption was invalid.
(iii) Because in earlier experiments mitochondrial ALA synthetase was assayed, observed changes in activity may have represented enzyme redistribution rather than true induction. Despite these limitations, some features of the chosen perfusate

are worthy of further comment.

(b) Addition of glucose.

The finding that the increase in the level of ALA synthetase in livers perfused with AIA was greater in the presence of glucose (or lactate) than in the absence of added substrate (Fig.5-7) was interesting in two respects. Firstly, it has previously been suggested [315] that addition of glucose or other substrate in liver perfusions is unnecessary since even when livers from starved rats are perfused, there is a net release of glucose. Greater induction of ALA synthetase in the presence of glucose may reflect glucose utilization to meet energy requirements and/or a 'sparing effect' on certain amino acids which would otherwise be utilized for gluconeogenesis.

Secondly, the results provide evidence that glucose does not directly prevent induction of ALA synthetase: the level of glucose in the perfusate (550 mg/100 ml) is considerably higher than the blood level of glucose in a fully fed (or glucose fed) rat where *in vivo* induction by drugs is almost completely blocked (Table 5-2, refs. 60,98).

(c) Addition of amino acids.

Although extensive amino acid reutilization in liver (e.g., 336) probably allows continued protein synthesis in the absence of any exogenous amino acids [315,324], it seems clear that the amino acid composition of a perfusing medium may influence both general hepatic protein synthesis

[332,337] and the level of specific enzymes [e.g., 335,338, 339[in liver cells. The effects of varying the amino acid composition of the perfusate on induction of ALA synthetase have not been investigated in detail in the present studies. The few results obtained so far indicate that induction by a variety of inducers can be observed when the perfusate contains acid-hydrolyzed casein and that induction by AIA is not enhanced significantly when the casein hydrolysate (which lacks certain amino acids e.g., tryptophan) is replaced by a complete amino acid mixture (final concentrations 6x serum levels) (Fig. 5-23). In perfusions with lead acetate, induction was greater with casein hydrolysate alone than when this was supplemented with tryptophan (10x serum levels) or replaced by a complete amino acid mixture (10x serum levels) (Fig. 5-27). Previous perfusion studies by other workers with rat liver have shown that it is necessary to perfuse with amino acids at final concentrations 10x those found in serum in order to preserve normal *in vivo* polysome profiles [332,335]. At lower amino acid concentrations or in the absence of certain amino acids (particularly tryptophan, asparagine and methionine) polysomes disaggregate to yield largely single ribosomes [332,335]. While no definite conclusions can be drawn from the preliminary observation above, they raise the possibility that induction of ALA synthetase is not only possible but favoured under conditions leading to polysome disaggregation. It has been suggested that disaggregation of polysomes under conditions of amino acid

deprivation reflects decreased initiation of protein synthesis due to some control exerted at the translational level [332, 337,340]. It is conceivable that translation of a short-lived mRNA for ALA synthetase might be favoured in the presence of greater numbers of free ribosomes, even though polysome disaggregation is generally thought to cause a decrease in overall protein synthesis [332,341, cf. 342].

B. INDUCTION OF ALA SYNTHETASE IN CONTROL PERFUSIONS.

When livers from rats which had been starved but were otherwise untreated were perfused with a standard medium containing no added inducer, a significant 3-4 fold increase in total ALA synthetase activity was observed over a 3-hour period (Figs. 5-14, 5-16, 5-17). It was also found that, in livers in which ALA synthetase had been preinduced *in vivo* with AIA, perfusion (without added inducers) led to a marked further increase in mitochondrial ALA synthetase activity (Fig. 5-10). These phenomena, referred to as 'control inductions' apparently result from transferring a liver from an *in vivo* to a 'perfused' environment.

With respect to the primary aim of developing an *in vitro* system where the level of ALA synthetase could be changed at will using known inducers or repressors, the occurrence of this induction in control perfusions was a major disadvantage. Since 'control' conditions *in vivo* and in perfused liver apparently differ, perfused liver is less than ideal as a model system to test the effects of putative

inducers and repressors. Furthermore, because the factors causing the control induction are unknown, it is possible that these might vary from experiment to experiment (in fact, the results in Fig. 5-16 suggest the control induction was relatively reproducible).

On the other hand, the control induction is of potential significance since it must reflect some perturbation of the controls which normally maintain the activity of ALA synthetase at low levels.

The control induction observed in livers preinduced *in vivo* with AIA was not investigated in detail although it might be noted that since perfusion causes further induction in livers already exposed to optimal doses of AIA, the induction in control perfusions may involve regulation at a site different to that at which AIA acts. The nature of and possible reasons for the control induction in untreated liver were examined in some detail in the previous chapter. The increase in ALA synthetase activity in control perfusions was prevented by cycloheximide but not by actinomycin D (Fig. 5-20) suggesting that induction requires continuing protein synthesis and reflects altered control at a post-transcriptional site. However, it will be necessary to check the effects of actinomycin D since in other experiments with added inducers, the antibiotic blocked *all* induction (Fig. 5-26, 5-29).

The control induction presumably results from a change or changes in the medium to which the liver is

exposed when it is removed from its normal *in vivo* situation and perfused. Such changes might be of four types:

(i)   addition of exogenous compounds;

(ii)   dilution of factors in rat blood;

(iii)   changes due to the trauma of surgery; and

(iv)   changes due to the isolation of the liver from other organs.

The results of experiments designed to test these general possibilities (Figs. 5-17, 5-18 and 5-19) indicated that the control induction, particularly at later perfusion times, was due in part to the *in vivo* administration of nembutal to anaesthetize rats before use as blood or liver donors. Nembutal administration *in vivo* caused a 4-fold induction of ALA synthetase in 3 hours (Table 5-3). The control induction could be reduced but not eliminated by using ether (which causes no induction of ALA synthetase *in vivo* - Table 5.3) instead of nembutal as anaesthetic (Fig. 5-17). It remains to be shown whether nembutal (pentobarbital) would cause induction if added directly to the perfusate.

The component of the control induction not due, so far as could be determined, to added compounds may result in whole or part from surgical trauma since sham operations on ether-anaesthetized rats resulted in induction of hepatic ALA synthetase similar to that observed in control perfusions (Fig. 5-18). Trauma may cause increased adrenaline release in the rats used to provide blood and/or the liver for perfusion experiments. The adrenaline could stimulate hepatic adenyl cyclase and thus cause induction mediated by cyclic AMP.

Jefferson *et al.* [343] noted that hepatic cyclic AMP levels
were elevated at the beginning of liver perfusion experiments.

The fact that some induction occurred in perfusions
with whole blood (Fig. 5-19) could indicate that the control
induction results in part from trauma and/or isolation of
the liver. The fact that induction is somewhat greater with
40% rat blood than with whole blood or 55% blood could
indicate that blood dilution is a minor contributory cause
of the control induction. In short, no single factor can
be clearly identified as the major cause of the control
induction in perfusion experiments with ether-anaesthetized
rats. Further work will be required to establish the importance
of serum components (e.g., free of protein-bound heme or serum
factors such as described by Kappas and co-workers [101,102])
and of changes in hormonal levels during perfusion (e.g.,
removal of insulin by degradative enzymes in blood [343]
and liver [345].).

C. EFFECTS OF AIA ON THE LEVEL OF ALA SYNTHETASE IN PERFUSED LIVER.

(I) Effect of AIA on Total ALA Synthetase Activity.

In perfusion experiments where total ALA synthetase
was assayed in liver homogenates, it was found that the
presence of relatively high concentrations of AIA in the
perfusate caused a significant but quite small increase in

141.

enzyme activity over that in controls in the first two hours

of perfusion (Figs. 5-16 and 5-21); at later times induction

with AIA was the same as (Fig. 5-21) or less than (Fig. 5-16)

that in controls, depending on the anaesthetic used. A lower

concentration of AIA had no effect compared to controls (Fig.

5-24).

Comparison of the weak inducing effect of AIA in

perfused liver with its potent inducing action *in vivo*

(Fig. 5-22), would suggest either that AIA does not act

directly on the liver or that the particular conditions

prevailing in the perfused liver block the inducing action of

AIA. While it cannot be totally discounted, the former

explanation seems unlikely in view of the direct and potent

induction by AIA in chick embryo liver cells in tissue culture

[80]. It is possible that prerequisite conditions for

induction by AIA are lacking in the perfused liver. As

already noted the 'control' environment differs significantly

from that of the liver *in vivo*. It seems unlikely that a non-

specific inhibition of protein synthesis is primarily involved

since induction by other agents (e.g., lead acetate, steroids)

is as great as, or greater than that observed *in vivo*. It

is known that induction by AIA *in vivo* can be prevented under

certain circumstances (e.g., by administration of heme [143]

or SKF-525A [53], by feeding glucose [98] or by prior

adrenalectomy [141,142]): comparable circumstances may

prevail in the perfused liver.

If induction in control perfusions and by AIA occurs

by distinct mechanisms as suggested above, it is conceivable (if unlikely) that under certain circumstances the two effects may be antagonistic. In this respect it is interesting that the presence of AIA in the perfusate apparently partly prevents further induction due to perfusion of preinduced liver (Fig. 5-10).

(II) Effect of AIA on Distribution of ALA Synthetase Between Mitochondria and Cytosol.

The following observations suggest that AIA may increase the proportion of total ALA synthetase localized in the mitochondria.

(i) In preliminary *in vivo* studies of induction by AIA (Fig. 5-3), the ratio of mitochondrial:cytosol activities was higher between $1^1/2$ and 6 hours after AIA administration than in untreated liver (no AIA) or at later times after AIA (AIA removed by metabolism?).

(ii) In experiments where ALA synthetase was assayed in isolated mitochondrial and cytosol fractions (Figs. 5-9 and 5-15)comparison of the effects of perfusion with and without AIA suggested that at later perfusion times (2-3 hours) AIA caused loss of enzyme from cytosol with a corresponding increase in mitochondrial activity.

(iii) Comparison of induction with and without AIA as measured in mitochondria and in homogenates (Fig. 5-14) suggested that apparent 'induction' in the mitochondrial fraction reflected

mostly redistribution rather than an increase in total activity.

(iv) Perfusion without added inducers (i.e., control perfusion) caused induction in both untreated liver (Fig. 5-17) and in liver preinduced *in vivo* with AIA (Fig. 5-10) but only in the latter case was there a marked increase in mitochondrial activity. This observation would be consistent with the proposal that AIA favours enzyme accumulation in mitochondria although it remains to be shown that in preinduced liver, control induction in the mitochondrial fraction represents a greater proportion of the total increase than in control induction in untreated liver.

The effect of AIA on the intracellular distribution of ALA synthetase may have escaped notice previously since other studies on distribution of the enzyme [65,70,281] have all depended on AIA as inducer. In the present case control perfusion caused induction but AIA had little effect on total enzyme level so that its effects on enzyme distribution were more apparent.

The relationship between the effects of AIA on total ALA synthetase activity observed *in vivo* and its effects on enzyme distribution remains to be established, although the present results suggest these could represent distinct actions of AIA.

Interpretation of the redistribution effect is clouded by present uncertainty on the relationship between mitochondrial and cytosol enzymes. AIA may enhance some transport or

modification process necessary for incorporation of enzyme into the mitochondrion e.g., the chloramphenicol-sensitive reaction(s) necessary for an increase in mitochondrial ALA synthetase activity [70].

D. EFFECTS OF OTHER AGENTS ON THE LEVEL OF ALA SYNTHETASE IN PERFUSED RAT LIVER

Further investigations with the rat liver perfusion system have been of a preliminary nature, designed to establish some of the elements of control of ALA synthetase operating in the perfused liver. Unfortunately, most of these experiments were carried out before the effects of nembutal as anaesthetic in control perfusions was recognized, so that effects discussed below were viewed against a marked induction in controls. It would be desirable to carry out further experiments with ether as anaesthetic so that the control induction is minimized; there has not been time to do this work to date.

(I) Effects of Lead Acetate.

The relatively high concentration of lead acetate tested in perfusion was found to cause a small but significant increase (total 4-fold increase) in ALA synthetase over that

in controls (3-fold increase) (Fig. 5-26). Preliminary experiments with isolated cell fractions showed that perfusion with lead caused a marked increase (4x) in mitochondrial activity (Fig. 5-13). By contrast, administration of lead by a variety of routes *in vivo* caused at the most only small increases (20 - 30%) in hepatic ALA synthetase (Table 5-5). The relative lack of effect of lead *in vivo* was consistent with previous findings [150,288]. A reported 3-fold induction of ALA synthetase by intravenously administered lead [202] could not be reproduced.

Induction in perfused liver in the presence of lead could be almost completely prevented by cycloheximide or actinomycin D (Fig. 5-26) suggesting that induction involved *de novo* enzyme synthesis dependent on continuing RNA synthesis.

The observed induction by lead in perfused liver is surprising in view of the failure of lead to induce *in vivo*. It may be that the liver is exposed to high levels of lead in perfusions without complication from extrahepatic toxic effects. Alternatively, lead may act synergistically (perhaps by inhibition of heme synthesis) with those effects causing induction in control perfusions. *In vivo* experiments have shown that ferric citrate (but not lead acetate) acts synergistically with drugs such as AIA in causing induction of ALA synthetase [150]. The

action of lead in perfused liver might involve a similar but distinct effect. Addition of AIA with lead in perfusions reduced rather than enhanced induction: while this might result from non-specific toxicity, it could reflect antagonistic actions of lead and AIA.

The finding that lead ions cause induction, at least under special circumstances in perfused liver raises the possibility that lead may also induce *in vivo* under some circumstances. Such an effect could help explain the great similarities between lead poisoning and acute intermittent porphyria discussed in Chapter 1.

(II) Effects of Steroids.

(a) Hydrocortisone.

In preliminary experiments with AIA present in the perfusate, addition of hydrocortisone had little effect on the observed changes in the level of mitochondrial ALA synthetase (Fig. 5-8). Previous *in vivo* experiments suggested that hydrocortisone exerted a permissive [141] or potentiating [142] effect in the induction of ALA synthetase by AIA. For investigation of this effect in perfused

liver it may be necessary to perfuse livers from adrenalecto-
mized rats with a fully synthetic perfusate or with perfusate
containing blood from adrenalectomized animals, since in the
work described the endogenous glucocorticoid level is unknown.

(b) Sex steroids and their derivatives.

Progesterone caused an increase in total ALA
synthetase activity both *in vivo* (Tables 5-7 and 5-8) and
in perfused liver (Fig. 5-28). The total increases were
similar *in vivo* and in perfused liver (4-5x in 2-3 hours)
although in perfusions, roughly half the total increase
occurred in controls without progesterone. Induction by
progesterone apparently required continuing RNA and protein
synthesis since it was prevented by actinomycin D or cyclo-
heximide (Fig. 5-29).

The observed induction by progesterone was consistent
with the results of previous *in vivo* experiments [163,168],
and may help explain increased occurrence of porphyric symptoms
associated with pregnancy or preceding menstruation in certain
patients with hereditary hepatic porphyria (see Chapter 1,
Section 4B IV). In this respect, however, it should be noted
that the level of progesterone used in perfusion experiments
(12 μg/ml) was approximately two orders of magnitude greater
than progesterone levels in human serum during pregnancy [255].

Perfusion with the 5β-H derivative of progesterone,
pregnanolone, was also found to cause significant induction
of ALA synthetase over that in controls (Fig. 5-30). This
observation may indicate that its metabolites, rather than

progesterone itself are the active inducers of ALA synthetase; it also suggests that certain 5β-H steroids previously shown to exert potent inducing effects in chick embryo liver ['7,86, 89] may also be active in mammalian liver. Previous failure to detect induction by these steroids *in vivo* in mammalian liver [89] could reflect purely technical difficulties - for instance, since many 5β-H steroids are more polar than their sex steroid precursors, they may be conjugated and/or excreted relatively rapidly so that only small amounts of steroid accumulate in the liver.

It may be that some 5β-H steroids which induce in chick embryo liver are inactive in mammals, since etiocholanolone, an active inducer in chick liver [86,89,92], failed to cause significant induction when tested in the perfusion system (Fig. 5-30).

Further investigations of the effects of 5β-H and possibly other steroid metabolites may establish whether elevated levels of ALA synthetase in acute intermittent porphyria could result directly from disturbed steroid metabolism observed in patients with the disease [83,240].

(III) <u>Effects of Glucose, Dibutyryl Cyclic AMP and</u> <u>Insulin in Perfused Liver and Possible Relevance</u> <u>to Glucose Repression of ALA Synthetase *in vivo*.</u>

In preliminary perfusion experiments, the effect on induction of mitochondrial ALA synthetase of supplementing perfusate containing AIA with various substrates was investigated

(Fig. 5-7). As discussed above, the fact that the greatest inductions were observed in the presence of glucose suggested that glucose does not directly prevent induction in liver and that glucose repression of induction by drugs *in vivo* may result from indirect effects. The role of glucose will require further investigation but if the conclusion above is correct, it would seem unlikely that the 'glucose effect' involves increased glucuronidation of steroids as postulated by Kappas and Granick [97], increased drug detoxification [20] or elevation of ATP levels [262] as have also been suggested.

As indicated in Chapter 1, there is evidence from studies on glucose repression of other enzymes in mammalian liver that the effects of glucose may be mediated by pancreatic hormones and/or changes in hepatic cyclic AMP levels (e.g, 272,274). Observations in perfused liver suggest that this may also be the case for glucose repression of ALA synthetase:

(i) Perfusion with dibutyryl cyclic AMP alone increased ALA synthetase activity more than control perfusion (Fig. 5-32); the nucleotide also enhanced induction of mitochondrial ALA synthetase by AIA (Fig. 5-12). The concentration of cyclic nucleotide used in these experiments (10^{-4}M) was high compared to hepatic concentrations of cyclic AMP reported by Jefferson *et al.* [343]: it may be that *in vivo*, lower concentrations of cyclic AMP have essentially a permissive effect. Thus starvation or glucagon administration (which *in vivo* cause at the most, small increases in ALA synthetase (Table 5-2, refs.

98,327)) may raise the cyclic AMP levels to an extent which permits induction by drugs but causes only a small induction *per se*. Conversely, glucose feeding, by raising insulin and lowering glucagon levels could cause the concentration of cyclic AMP to fall below that necessary to permit induction.

Induction by dibutyryl cyclic AMP was only partly prevented by actinomycin D (Fig. 5-32). Because of the short half lives of ALA synthetase and the mRNA which codes for it [98,170], it might be expected that induction of the enzyme would be particularly sensitive to inhibition of RNA synthesis; thus the partial insensitivity of induction by cyclic AMP to actinomycin D could indicate a post-transcriptional effect. However, further investigation will be required to establish the level of action of the cyclic nucleotide. Studies on induction of other enzymes in mammalian liver by cyclic AMP have provided tentative evidence for effects at both transcriptional and translational levels [for review, 278; also 274,346-8].

(ii) It was found that addition of high levels of insulin to the perfusate prevented induction of mitochondrial ALA synthetase by AIA (Fig. 5-12) and partly blocked the increase in activity due to perfusion with progesterone (Fig. 5-31). There are several possibilities for the mechanism of this effect: insulin is known to lower cyclic AMP levels in liver, although it is unclear whether this results from decreased adenyl cyclase activity, increased phosphodiesterase activity, or both [278,343,349]. Alternatively, insulin may

alter the specificity of hepatic RNA or protein synthesis by
a direct effect e.g., on histone phosphorylation [350] and/or
chromatin template activity [351] or on ribosomal activity
[337].

(IV) Effects of Heme.

Induction of ALA synthetase resulting from perfusion
with progesterone was completely prevented by addition of
hemin (2×10^{-5} M) to the perfusate (Fig. 5-31).   This
observation suggests that at moderate concentrations, heme
represses ALA synthetase by a direct action on the liver as
would be expected from previous reports that heme prevents
induction by drugs [80] and steroids [97] in chick embryo
liver cells in tissue culture.

Hemin (10^{-5} M) also reduced the increase in mitohcondrial
ALA synthetase caused by perfusion with AIA (Fig.5-13).   While
this observation alone could be interpreted as further evidence
for direct heme repression, preliminary experiments in which
the effects of heme on changes in cytosol activity were
investigated (Fig. 5-15), provided tentative evidence that
heme reversed the effects of AIA on the intracellular distrib-
ution of ALA synthetase, seen at later perfusion times.   Such
an effect could be due, when heme is present, either to loss
of existing enzyme from mitohcondria or prevention of an
AIA-stimulated increase in mitochondrial activity.   An
effect of heme on enzyme distribution would be consistent
with the report of Kurashima et al. [281] that administration

of heme to rats, in which ALA synthetase had been preinduced
with AIA, caused loss of mitochondrial ALA synthetase
activity with a corresponding increase in cytosol activity.
Further studies will be required to establish the relative
importance of effects by heme on total activity and enzyme
distribution.

(V) General Conclusions; Further Studies.

Perhaps the most important conclusion from the work in
this thesis is that rat liver perfusion is a potentially
valuable tool for studying control of ALA synthetase in
mammalian liver. The studies with perfused liver to date
must be regarded as relatively preliminary: they have
provided some information on the important variables in ALA
synthetase regulation as a basis for further work; they have
confirmed some findings of previous *in vivo* studies and
extended others, as well as suggesting new questions and
possible answers on the effects of agents such as AIA
or glucose, previously shown to affect the level of ALA synthet-
ase *in vivo*.

It is not possible, on the basis of work in this
thesis, to propose definite mechanisms of control of ALA
synthetase. This remains the long term aim of further
work. Immediate plans are the development of a defined
perfusion medium (containing washed red blood cells), followed
by a systematic investigation of steroid induction of ALA
synthetase. It is hoped that such studies will contribute

to an understanding not only of the molecular basis of ALA
synthetase regulation but of the human hepatic porphyrias
and of the nature of specific gene regulation in higher
cells.

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APPENDIX I.

During the final stages of preparation of this thesis, a report appeared, on regulation of ALA synthetase in isolated perfused rat liver [Bock, K.W., Krauss, E., and Frohling, W., *Eur. J. Biochem.*, *23*, 366 (1971)]. The authors found that perfusion of livers from starved rats, with a medium containing either 50% rat blood or washed red cells plus albumin, caused a decrease in the level of mitochondrial ALA synthetase. The decrease could be prevented by addition of hydrocortisone (but not testosterone or etiocholanolone) to the perfusate. Addition of AIA alone partly prevented the decrease, addition of AIA plus dexamethasone resulted in a 3-4 fold induction of mitochondrial ALA synthetase which could be blocked by cyclo-heximide. The authors concluded that glucocorticoids have a role in regulation of heme synthesis and suggested that glucocorticoids might induce ALA synthetase when heme repression was released by AIA or other drugs. Some aspects of these results are difficult to reconcile with the present studies where lower basal levels of enzyme tended to increase rather than decrease during perfusion and a 3-4 fold induction by AIA of mitochondrial ALA synthetase could be observed without addition of glucocorticoids. Further investigations with a defined perfusion medium may provide information necessary to reconcile the apparently different requirements for gluco-corticoids observed by us and by Bock *et al.*