B I O C H E M I C A L S T U D I E S

I N

D R U G-I N D U C E D P O R P H Y R I A S I N T H E R A T.

With a review of the literature on experimental porphyria and an investigation of thirteen human cases.

by

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A T H E S I S

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ABBREVIATIONS USED IN THIS THESIS.

AA	-	Aminoacetone
ADP	-	Adenosine diphosphate
AIA	-	Allylisopropylacetamide
ALA	-	Delta-aminolaevulic acid
	-	Delta-aminolaevulinic acid
AMP	-	Adenosine monophosphate
ATP	-	Adenosine triphosphate
B.D.H.	-	British Drug Houses
B.S.A.	-	Bovine Serum Albumin
DDC	-	Dicarbethoxy dihydrocollidine
	-	3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-trimethyl pyridine
	-	Diethyl 1,4-dihydro-2,4,6-trimethyl pyridine-3,5-dicarboxylate
DPN	-	Diphosphopyridine nucleotide
DPNH	-	Reduced diphosphopyridine nucleotide
EDTA	-	Ethylenediaminetetra-acetic acid
G-O.T.	-	Glutamic-oxaloacetic transaminase
G-6-P.D.	-	Glucose-6-phosphate dehydrogenase

HCB	-	Hexachlorobenzene
ICD	-	Isocitric dehydrogenase
L.D.	-	Lactic dehydrogenase
N	-	(Mitochondrial) nitrogen
NAD	-	Nicotinamide-adenine dinucleotide
$NADH_2$	-	Dihydro-nicotinamide-adenine dinucleotide
NADP	-	Nicotinamide-adenine dinucleotide phosphate
$NADPH_2$	-	Dihydro-nicotinamide adenine dinucleotide phosphate

(These 4 abbreviations, as recently recommended by the Enzyme Commission of the International Union of Biochemistry, have been employed in the present manuscript only in those instances where they were used in the work to which reference was made).

O.D.	-	Optical Density
P	-	Phosphorus (Total)
PBG	-	Porphobilinogen
PK	-	Pyruvate kinase
TPN	-	Triphosphopyridine nucleotide
TPNH	-	Reduced triphosphopyridine nucleotide
Tris	-	2-amino-2-(Hydroxy-methyl)-1:3 Propanediol.

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SECTION 1.

INTRODUCTION.

The group of disorders characterised by disturbances of porphyrin metabolism pose many fascinating problems. The drug-induced porphyrias in animals provide a model whereby these problems may be investigated by techniques which cannot be applied to man.

The consumption of alcoholic beverages or other chemical substances has been associated with the development of porphyrinuria and porphyria, but it is controversial whether these agents are of primary aetiological importance, or whether they precipitate the disease in genetically predisposed individuals.

The Turkish epidemic of porphyria has shown conclusively that this disease may occur as an acquired condition in man, and a variety of compounds has been shown to be capable of inducing porphyria in animals.

It is a far cry from the drug-induced porphyrias in animals to the inherited porphyrias in man, and there are obvious limitations to the extent to which the two conditions may be usefully compared. Many points of similarity are nevertheless apparent.

In the so-called "hepatic" forms of the disease - and it is

mainly with these forms that this thesis is concerned - haemotological disturbances have not been significant in either.

Gastro-intestinal and neurological disturbances are prominent in both, although precise manifestations may differ.

Although cutaneous lesions have been described in the rat, photosensitivity has not been a feature, and it is of interest that whereas it was a pronounced symptom in the Turkish epidemic, hexachlorobenzene (HCB)-induced porphyria in animals has NOT had this association. As regards the inherited forms of porphyria, photosensitivity has been noted in certain forms, only, in man, and in cattle, but not in pigs.

Such differences may be as a result of different mechanisms of the disease process, or may reflect species variability. A consideration of the literature on the drug-induced porphyrias reveals marked species differences as regards their biochemical and clinical manifestations, and also a significant influence of the nature of the drug used.

One important common feature is shared. In both experimental and naturally occurring porphyria there is an excessive excretion of haem precursors. This alone is sufficient justification for studying animals with the drug-induced disease in the hope that a pattern of metabolic abnormality would emerge to serve as a basis for a rational approach to research

on the disease in man, and which might contribute towards an understanding of the pathogenesis of the porphyrias.

Early studies in the field of experimental porphyria were directed principally towards documenting the patterns of porphyrin and porphyrin precursor accumulation and excretion, and the clinical and pathological changes which occurred. Other parameters have also been investigated, and biochemical derangements have been demonstrated which have no apparent direct relationship to haem synthesis.

The existence of an inverse relationship between the level of dietary carbohydrate and the clinical and/or biochemical severity of the porphyric state in both allylisopropylacetamide (AIA)-induced (249), and acute intermittent porphyria(340) has been one such observation.

The present investigation was stimulated partly by this finding, and partly by demonstrations of the influence of various substrates of the central glycolytic pathway, the citric acid cycle and certain amino acids on porphyrin metabolism <u>in vitro</u>. The activities of some of the enzymes concerned in carbohydrate, protein, citric acid cycle and porphyrin metabolism have been measured in the livers of rats rendered porphyric with several drugs. These have been compared with levels obtaining in control groups of animals.

A summary of the parameters studied is shown in Figure 1.

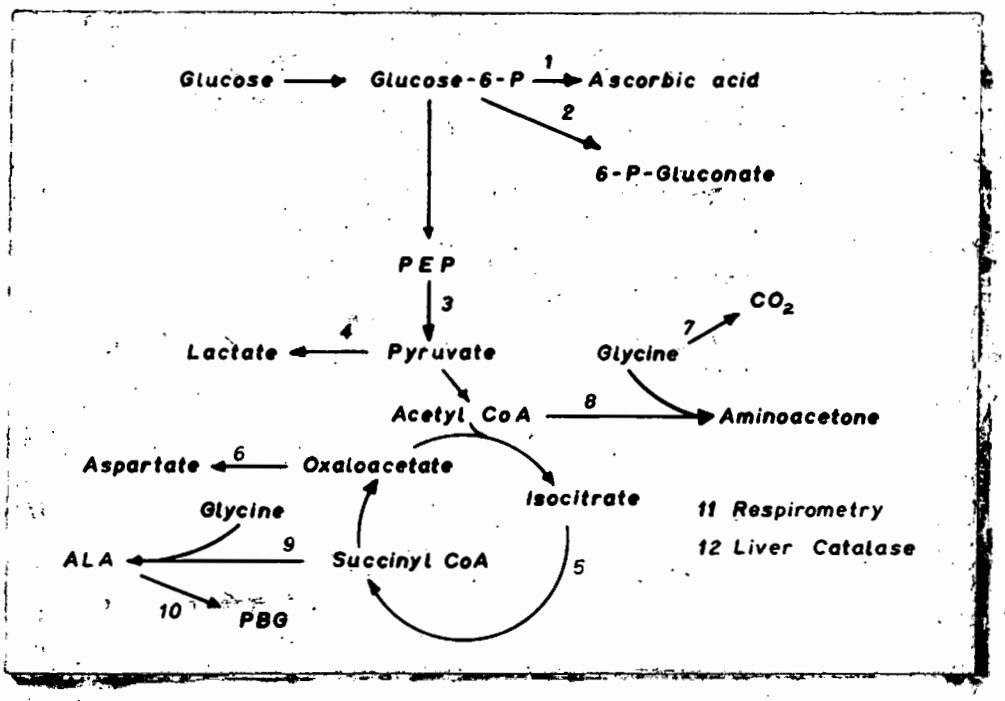


Figure 1. Summary of parameters studied

These comprised a measurement of:-

(1). Urinary excretion of ascorbic acid.

(2). Liver glucose-6-phosphate dehydrogenase (G-6-P.D.) activity.

(3). Liver pyruvate kinase activity.

(4). Liver lactic dehydrogenase activity.

(5). Liver isocitric dehydrogenase (I.C.D.) activity.

(6). Liver glutamic-oxaloacetic transaminase (G-O.T.) activity.

(7). Oxidation of glycine-1-C^{14} and glycine-2-C^{14} by liver homogenates.

(8). Aminoacetone synthesis by isolated liver mitochondria.

(9). delta-Aminolaevulic acid (ALA) synthesis by isolated liver mitochondria.

(10). Liver delta-aminolaevulic acid dehydrase activity.

(11). Oxygen consumption by liver homogenates respiring on endogenous substrate, and by isolated liver mitochondria respiring on exogenous substrate.

(12). Liver catalase activity.

Liver porphyrin concentrations were measured as an index of the development of the porphyric state.

Three drugs known to be capable of inducing porphyria were used initially. These were dicarbethoxydihydrocollidine (DDC), AIA, and HCB.

The levels of activity of a number of these enzymes were also measured in the livers of several patients with porphyria.

The findings are presented in the full awareness that measurement of enzyme activity *in vitro* may in no way reflect the physiological conditions obtaining within the cell. However, there do seem to be grounds for believing that such measurements may be relevant to metabolic processes *in vivo*.

Moog (209) demonstrated that a decreased activity of cytochrome oxidase in embryonic tissues was associated with a decreased oxygen uptake by the intact embryo, and she cited several examples suggestive of a relationship between measurements of enzyme activity *in vitro* and function in *vivo*.

Many of the examples of changes in enzyme activity described consequent upon various dietary regimes seem to be logically predictable on a functional basis. Hepatic G-6-P.D., hexokinase, and the 3-phosphoglycerate-pyruvate span were diminished in activity with fasting, or a low carbohydrate diet (325) - when the supply of glucose was diminished. G-O.T. and glutamic-pyruvate transaminase (G-P.T.) activities, conversely, were increased with fasting (250) - when tissue proteins were being catabolised.

Moreover, increased levels of ALA synthetase activity as measured *in vitro* have been associated with the accumulation of porphyrins in the intact animal.

It is felt that these examples, while by no means conclusive, do justify the presentation of data obtained from measurements of enzyme activity made *in vitro*.

Composition of thesis.

In Section 2 the porphyrin biosynthetic pathway is considered with special emphasis on those of its aspects with which the present investigation has been particularly concerned. Data relating to this pathway which have emerged from this investigation are presented.

Aspects of ascorbic acid and aminoacetone synthesis and metabolism are also described.

In Section 3 the literature relating to the drug-induced porphyrias is reviewed. The various compounds which have been associated with the induction of porphyrinuria and porphyria are considered, and, in particular, the influences of Sedormid, AIA, DDC, HCB and griseofulvin administration on porphyrin metabolism, and aspects of metabolism in general, are analysed.

The experimental methods that have been used are described in Section 4.

Several factors have been shown to influence levels of enzyme activity as measured *in vitro*, and these factors, both as reported in the literature, and as they have manifested in the present investigation are considered in Section 5.

Section 6 comprises a detailed presentation of the data obtained in these investigations.

In Section 7 the significance of the experimental findings

is discussed, with particular emphasis on their contribution to an understanding of the pathogenesis of the porphyrias.

SECTION 2.

A. THE PORPHYRIN BIOSYNTHETIC PATHWAY.

Recent reviews of this subject include those by Watson (329), Shemin (284, 286), Rimington (239), Schmid (262), Eales (86), Gibson et al (113), Bogorad (21) and Mauzerall (201).

1. BIOCHEMICAL PRECURSORS.

In 1945 Shemin and Rittenberg (294) wrote:- "The nature of the precursors employed for the formation of the protoporphyrin of haemoglobin has been the subject of much speculation".

They then demonstrated by experiments with N^{15}- labelled glycine, leucine and ammonia fed in the diet, that glycine was utilised directly for haem synthesis.

In the same volume of the Journal of Biological Chemistry, Bloch and Rittenberg (18) had shown - while investigating the metabolism of acetate and its relationship to cholesterol and fatty acid metabolism - that the haemin isolated from the blood of rats fed deuterioacetate contained significant amounts of deuterium. They noted that condensations involving acetoacetic ester and ammonia had been shown to yield pyrroles (Knorr synthesis).

With this knowledge, Shemin and Rittenberg (294) postulated in their original article on the subject that glycine might condense with an acetic acid derivative in a Knorr type of synthesis to yield pyrroles.

The role of glycine was confirmed by subsequent work from this laboratory (235, 293, 295, 296, 344) and by Muir and Neuberger (210, 211). It was shown that the nitrogen atoms of haem were all derived from glycine and were not contributed to by proline, leucine, ammonia, glutamic acid (295) or ethanolamine (210) except in a non-specific manner; and Granick (134) found that only glycylglycine and ethylglycinate could replace glycine in the synthesis of protoporphyrin.

The initial studies were performed *in vivo*, in man, rats, and rabbits. In 1948 Shemin *et al* (293) reported the development of an *in vitro* system for the investigation of haem synthesis. They incubated red cells from the peripheral blood of ducks - chosen because of their high content of nucleated cells, and, according to legend, because of Shemin's partiality for roast duck - with N^{15}-glycine and demonstrated its incorporation into haem. This experiment provided a prototype for much of the subsequent work on porphyrin metabolism.

Through tracer studies and degradation techniques the alpha carbon of glycine was shown to contribute 8 of the 34 carbon atoms of protoporphyrin, while the carboxyl atom was not utilised for porphyrin synthesis (211, 235, 284, 286). These carbon atoms were always to be found in the methene bridges and in corresponding positions in each of the four pyrrole rings (284, 345).

The remaining 26 carbon atoms were shown to be entirely derived from acetic acid (299) and for this purpose the methyl carbon atom was more efficiently utilised (211, 299). This work, in a negative manner, confirmed the distribution of the carbon atoms derived from glycine.

Radioactive studies and calculations pointed to acetate being utilised through the tricarboxylic acid cycle(347)The distribution of activity eliminated the dicarboxylic acids and pyruvic acid as direct precursors, but was in accord with that theoretically predicted should alpha-ketoglutarate be the precursor. These studies suggested the participation of an unsymmetrical compound arising from both alpha-ketoglutaric and succinic acids. It was necessary to postulate such a derivative in order to explain the pattern of radioactive distribution unless the conversion of alpha-ketoglutaric acid to succinic acid were reversible, and in mammals this had been shown not to be so. Shemin and Wittenberg (299) postulated that this compound was probably a succinyl-coenzyme A complex - an intermediate of the alpha-ketoglutarate-succinate reaction.

Support for this concept was derived from the demonstration of the formation of succinyl-coenzyme A from alpha-ketoglutarate in the presence of coenzyme A and DPN (254) and from studies by Shemin and Kumin (291, 292) who showed by differential labelling of succinate, and inhibition by malonate, that succinate formed succinyl-coenzyme A directly from succinate, and via the

tricarboxylic acid cycle through alpha-ketoglutarate. Several years later, in 1958, Laver et al (191) demonstrated ALA synthesis from succinyl-coenzyme A, and Kikuchi et al (176, 177) and Shemin and Kikuchi (289) showed that the rate of synthesis of ALA in the systems used was greater when succinyl-coenzyme A served as substrate than it was when succinate, coenzyme A and ATP did, from which they concluded that "active" succinate was the coenzyme-A compound.

Thus the concept emerged of an initial step in porphyrin biosynthesis in which there was a condensation of 8 molecules of glycine with 8 molecules of an acetic acid derivative - "active" succinate. However, in the early 1950's there was doubt as to the method of this condensation and several suggestions were put forward to account for the "extra" carbon atoms of glycine - in excess of the 4 nitrogen atoms - in the protoporphyrin molecule.

In 1953, Shemin and Russell (297) demonstrated that the compound delta-aminolaevulic acid could replace the two substrates, "active" succinate and glycine, in porphyrin synthesis.

Morevoer, ALA was shown to be the decarboxylated product of alpha-amino-beta-ketoadipic acid, a substance formed from the condensation of "active" succinate with glycine.

Neuberger and Scott (218) set out in 1952 "to synthesise a number of products formally derivable from glycine condensed with

one or two molecules of succinic acid which might be intermediate in the conversion of glycine and succinate derivatives to porphyrins". One such product was alpha-amino-beta-ketoadipic acid and it was thought that this compound would undergo decarboxylation to yield ALA.

2. ALA SYNTHETASE.

The condensation of "active" succinate and glycine is believed to be enzymatically controlled by the enzyme ALA synthetase, which catalyses the reaction:-

Succinyl-coenzyme A + Glycine → alpha-amino-beta-ketoadipic acid + coenzyme A.

Alpha-amino-beta-ketoadipic acid is then thought to undergo spontaneous decarboxylation to delta-aminolaevulic acid and carbon dioxide. This enzyme is thought to be present in all cells containing either haem-proteins or chlorophyll (113).

Until the work of Laver, Neuberger and Udenfriend (191), evidence for the existence of this enzyme and knowledge of the factors influencing its activity was largely indirect and inferential, as all the available *in vitro* systems produced very little ALA and metabolised it so rapidly that it could not be measured. Work was thus mainly concerned with factors affecting haem and porphyrin synthesis.

In 1958 Laver *et al* (191) showed that the supernate of red cell haemolysates from chickens treated with phenylhydrazine and

acetyl-phenylhydrazine, had, after centrifugation at 3500 g. for 30 minutes, only 10% of the activity of the whole homogenate for haem synthesis. The washed particles separated by centrifugation could synthesise ALA from glycine and succinate, and it was not metabolised further.

In 1954, Shemin, Abramsky and Russell (287) had shown that a soluble, cell-free extract of duck erythrocytes could synthesise protoporphyrin from ALA, but that on homogenisation of the cells the functional activity of the enzymes involved in the condensation of succinate with glycine was lost.

That ALA is actually synthesised in vivo was suggested by the observation of Granick and Vanden Schrieck (143) who isolated ALA from the urine of a patient with acute porphyria - an observation which has since been repeatedly confirmed.

(a). Location of the enzyme.

Granick (134) and Granick and Mauzerall (138) considered that since electron transport systems, oxidative phosphorylating systems, and citric acid cycle enzymes were concerned in ALA synthesis, and since mitochondria had been shown to be concerned with these aspects of metabolism, mitochondria must be active in ALA synthesis.

Granick and Urata (142) were able to synthesise ALA using mitochondria from the livers of guinea pigs acutely poisoned with DDC. Miyakoshi and Kikuchi (208) detected enzyme activity in mitochondria from the livers of AIA-poisoned rats. Ginsburg and

Dowdle (116) demonstrated ALA synthetase activity in mitochondria from DDC-poisoned rat livers, and enzyme activity has been detected in the liver mitochondria of HCB- and AIA-poisoned rats,(this thesis).

Laver et al (191) could find no evidence to show that erythrocytes contained mitochondria, and they did not know whether enzyme activity was associated with cell nuclei, cell membrane or some other cell constituent which might be present in the nuclear fraction. Sano et al (op cit 257) have since described distinct mitochondria near the nuclear membrane of chicken erythrocytes.

Brown (27, 28) demonstrated enzyme activity in the centrifugate of washed, lysed fowl red cells, centrifuged at 1800 g for 15 minutes and Granick (134) showed that when frozen and thawed solutions of chick red cells were centrifuged for 1 hour at 20,000 g, the supernatant solution could not form porphyrins from glycine.

Kikuchi et al (177) concluded that the enzyme system responsible for ALA synthesis in a centrifuged extract of Rhodopseudomonas spheroides was present in very small particles in the supernate, but in other communications this group (176, 289) demonstrated the synthesis of ALA by particle-free extracts of R. spheroides and of Rhodospirillum rubrum. Shemin et al (290) demonstrated ALA synthetase activity in the supernate of an R. spheroides preparation centrifuged at 100,000 g for 30 minutes.

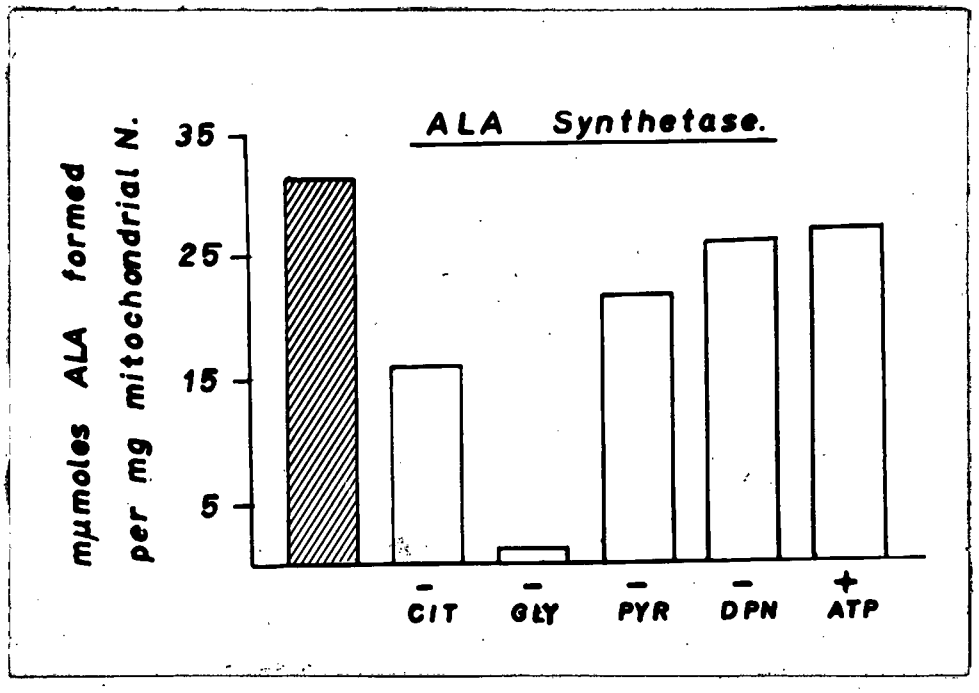


Figure 2. Effects of excluding a variety of components from the incubating medium on delta-aminolaevulic acid synthesis in vitro by intact mitochondria from the liver of a rat treated with DDC for 6 days.

Factor lacking.	None	Citrate	Glycine	Pyr.ph.	DPN.	ATP **
ALA formed *	31.2	16	1.2	21.6	26	27

* mumoles/2hours/mg. mitochondrial N.

** ATP added to usual medium. (see Section 4).

Lascelles (189) and Gibson (111) also demonstrated enzyme activity in cell-free extracts of R. spheroides.

(b). Nature of Substrate.

In all the in vitro systems employed to study ALA-synthetase activity, certain essential factors have had to be provided. These have included glycine, tricarboxylic acid cycle intermediates, and a variety of cofactors.

(i). Glycine.

Brown (27) in a chick red cell system, showed that maximum synthesis of ALA occurred with glycine at a concentration of 30m-M/L. Dresel and Falk (82) had previously shown that above a concentration of 56mM/L. porphyrin synthesis in a similar system was maximal, and independent of glycine concentration, and in another study (81) 15mM/L. was regarded as the minimum concentration for maximum activity. Laver et al (191) found the optimum glycine concentration for ALA synthesis to be 67 mM/L. and showed no inhibition by excess of glycine. Goldberg et al (125) noted optimum haem synthesis at a glycine concentration of 50 mM/L..

Granick and Urata (142) used glycine in a concentration of 50 mM/L., as did Miyakoshi and Kikuchi (208). In the similar studies described in this thesis, glycine was used in a concentration of 86 mM/L.

In the absence of glycine from the medium, Granick and Urata (142), and Gibson (111) could detect no ALA formation, and

in my own studies none was formed under these circumstances either. (Figure 2).

(ii). Tricarboxylic acid cycle intermediates.

Granick (134) found that in the presence of glycine all members of the citric acid cycle at 10 m M concentrations, except oxaloacetate, enhanced protoporphyrin formation in his red cell system.

Citrate and D-isocitrate were found by Brown (27) to result in the highest yields of ALA, and to be superior to succinate and alpha-ketoglutarate as substrate. Other members of the citric acid cycle studied - fumarate, malate, and oxaloacetate - resulted in the formation of only minimal amounts of ALA.

He used succinate in a 10 m M concentration, and observed no inhibitory effect when it was present in excess. Goldberg et al (125) could detect no influence of succinate upon haem synthesis in a chick red cell system, while added sodium citrate at a 6 m M concentration enhanced haem synthesis by 119%.

Granick and Urata (142) found that succinate in high concentration inhibited ALA formation. When Brown added succinate as a second substrate, an inhibitory action was noted.(27) Fumarate, malate and oxaloacetate also depressed ALA formation when so added - an effect ascribed to their suppression of oxidative decarboxylation of alpha-ketoglutaric acid.

Laver et al (191) found alpha-ketoglutarate to be more effective as a precursor of ALA synthesis than was succinate, but

observed an inhibitory effect at high concentrations. alpha-Ketoglutarate was observed by several authors to inhibit the condensation of succinyl-coenzyme A with glycine (27, 176, 177, 290).

Succinyl-coenzyme A itself had a slightly inhibitory effect when added to an intact particle system (112), but was readily utilised for ALA synthesis by frozen and thawed particles (112, 142) - a system that was incapable of utilising other members of the cycle for this purpose even in the presence of added coenzyme A.

Brown (27) found that when a tricarboxylic acid cycle intermediate was excluded from the system, virtually no ALA was formed. In my own investigations I found that this reduced ALA formation by only 50% (Figure 2) - which presumably indicates the presence of endogenous substrate.

(c). Cofactors.

(i) Pyridoxal phosphate.

In 1957 Schulman and Richert (273) while studying the incorporation of radioactive glycine, succinate, ALA and PBG into haem by avian cells in vitro, showed that blood samples from vitamin B6-deficient ducklings incorporated glycine and succinate into haem at a reduced rate, while ALA incorporation was essentially normal. Addition of pyridoxal phosphate, or pyridoxamine phosphate restored

the rate of incorporation to normal. Other pyridoxal compounds were without effect.

The importance of pyridoxal phosphate in porphyrin metabolism was also shown by Lascelles (188), who studied porphyrin synthesis by cell-suspensions of Tetrahymena vorax (a protozoon), and by Laver et al (191) and Gibson et al (112), who demonstrated directly its influence on ALA synthetase activity in chick haemolysate systems. Its role in chick haemolysate systems was also demonstrated by Granick (134) and by Brown (27), who showed that exclusion of pyridoxal phosphate resulted in a 40% reduction in the yield of ALA. Its importance in bacterial systems has been demonstrated too. (27, 111, 177, 289, 290).

In mammalian systems its significance was demonstrated by Granick and Urata (142). The absence of added pyridoxal phosphate resulted in a 37% reduction in the amount of ALA synthesised. I found a reduction of 29% in ALA formation when pyridoxal phosphate was excluded from the medium. (Figure 2).

The mechanism of participation of pyridoxal phosphate was discussed extensively by Kikuchi et al (175), Neuberger (216) and also by Gibson et al. (112, 113). Added pyridoxal phosphate is so bound that it is not removed by dialysis. It is believed that it combines with a specific enzyme protein - possibly through its phosphate group - and that it can react with the amino group of glycine to form an aldimine (or Schiff's base) which can then

react with succinyl-coenzyme A to form a new C-C bond.

(ii) Other B group vitamins.

These were studied as regards their influence on porphyrin synthesis by Lascelles (187, 188) who showed that the addition of any of nicotinic acid, thiamine, riboflavine, pantothenic acid, folic acid, thioctic acid and pyridoxal, with the exception of pyridoxal, did not increase porphyrin synthesis. However, a deficiency in riboflavine resulted in decreased porphyrin synthesis from both glycine and ALA. Deficiencies in pantothenate, nicotinic acid, thiamine and thioctic acid resulted in some reduction in porphyrin biosynthetic activity from glycine, but not from ALA, - an effect most marked in pantothenate deficiency. No effect was observed in folic acid deficiency.

Biotin was at one stage regarded by Neuberger (216) as a factor exercising an effect on ALA synthetase activity, but decreased porphyrin synthesis by biotin-deficient organisms (187, 190), was subsequently shown to be unrelated to ALA synthetase activity. (113).

Nicotinamide had previously been shown (81) to exert no influence on porphyrin synthesis, and thioctic acid was shown by Brown (27) to increase the synthesis of ALA when alpha-ketoglutarate or isocitrate served as the substrate, but to have no effect when succinate was used.

In 1954 Dresel and Falk (81) had demonstrated an enhanced synthesis of Cu^{++} protoporphyrin ester on the addition of boiled yeast extract to their system, and this may have reflected - in part at least - an influence of B group vitamins upon ALA synthetase activity.

(iii) Adenosine Triphosphate (ATP).

ATP was shown to stimulate the synthesis of porphyrins (81). It was found to stimulate ALA synthesis when succinate served as the substrate, but to produce slight, yet reproducible inhibition when alpha-ketoglutarate or isocitrate were used as substrates. (27). Increased synthesis of ALA by bacterial systems in the presence of added ATP was observed by several authors. (176, 177, 289, 290).

I observed a slight decrease in ALA synthesis on the addition of ATP to the medium, citrate serving as the substrate. (Figure 2).

(iv) Coenzyme A.

Coenzyme A was shown by Brown (27) to stimulate ALA synthesis from each of the Krebs' cycle intermediates and its stimulatory effect was also observed by others. (111, 134, 191, 289).

Granick and Urata (142) did not add it to their system, and I have also not used additional coenzyme A.

(v) Diphosphopyridine nucleotide (DPN).

Dresel and Falk (81) observed no effect on porphyrin formation on the addition of DPN to their chick red cell system.

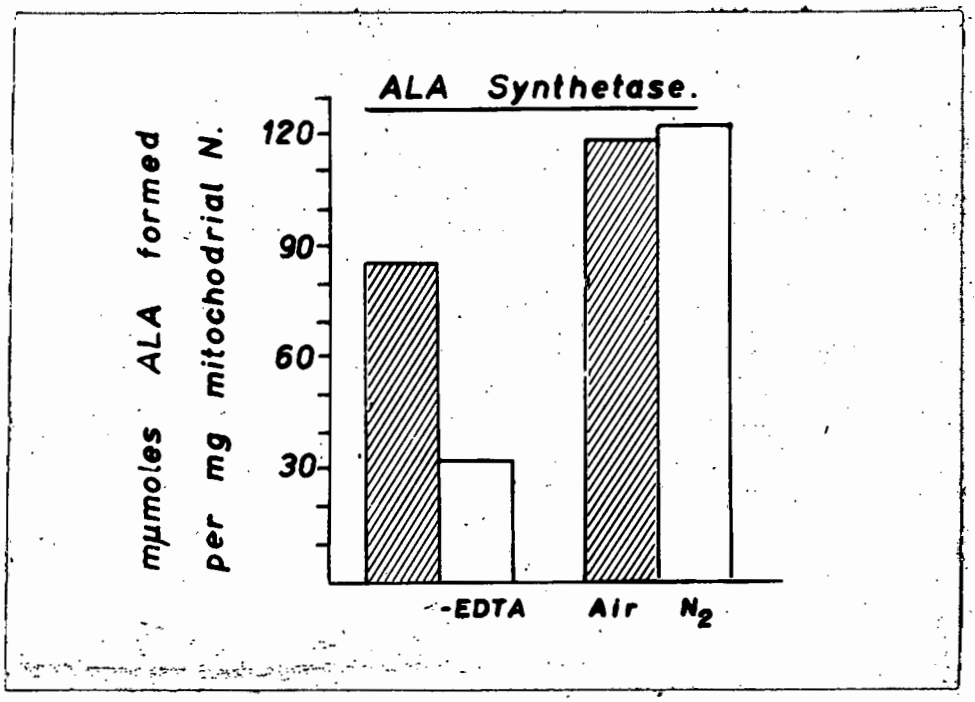


Figure 3. The first pair of columns shows the effect of excluding EDTA from the medium, when the amount of ALA synthesised by intact mitochondria from the liver of an animal poisoned with AIA for 24 hours, was reduced from 86 to 31 mumoles/mg. mitochondrial N. (2-hour incubation period).

The second pair of columns shows the lack of effect of incubating the system in an atmosphere of nitrogen. 119 mumoles ALA were synthesised in air, and 122 in nitrogen - per mg. mitochondrial N/2 hours.

(Two separate experiments).

Gibson et al (112) could demonstrate only a small effect of added DPN, although with frozen and thawed particles it restored somewhat their ability to utilise alpha-ketoglutarate. An enhancing effect was demonstrated by Granick. (134). I was able to demonstrate only a very small reduction in activity on excluding DPN from the medium. (Figure 2).

(vi) Magnesium.

Magnesium chloride was shown by Dresel and Falk (81) to exert no effect on porphyrin synthesis, but Mg^{++}ions have repeatedly been shown to stimulate ALA synthesis(176, 187, 191, 289) and were added to their media by Granick and Urata (142) and by Gibson (111). Additional Mg^{++}ions were supplied to the preparations in the present study too.

Gibson et al (112) could detect no activation by Mg^{++}ions in a frozen and thawed red cell system.

(vii) Ethylenediaminetetra-acetic acid (EDTA).

Laver et al (191) showed EDTA to cause considerable activation of ALA synthesis by an intact red cell system, but only slight activation was observed by Gibson et al (112) in a preparation where the cells had been frozen and thawed. Granick and Urata (142) demonstrated a marked stimulatory effect of EDTA on the ALA-synthetase activity of guinea pig liver mitochondria, and I have demonstrated a similar pronounced effect on the activity of rat liver mitochondria. (Figure 3). Miyakoshi and

Kikuchi (208) found the exclusion of EDTA from a rat liver mitochondrial preparation to reduce the yield of ALA by 70%.

EDTA is thought to act through maintaining the structural integrity of the mitochondria, and experimental evidence in support of this postulate was discussed by Granick and Urata(142).

(viii) Iron.

The role of iron is somewhat contentious. Lascelles (188) demonstrated almost complete inhibition, by iron salts, of porphyrin synthesis from glycine and alpha-ketoglutarate by R. spheroides, while such synthesis from ALA was not prevented. She also showed that iron increased bacteriochlorophyll synthesis but diminished porphyrin synthesis, by R. spheroides grown aerobically in the light (189). With Tetrahymena vorax preparations, iron salts decreased, but did not abolish, porphyrin synthesis from both glycine and ALA.(188).

In a later article Burnham and Lascelles (35) suggested that the inhibitory effects of iron might be mediated through the formation of an iron-porphyrin complex, which, through such an effect on ALA synthetase activity might provide for a biological control mechanism of porphyrin synthesis.

Dresel and Falk (82, 83) showed that below a concentration of 2×10^{-3} M, ferrous sulphate resulted in some increase in haem production, but with an equivalent fall in the amount of free

protoporphyrin formed by an intact chick red cell system. At higher concentrations complete agglutination of the cells occurred.

Brown (28) felt that iron was important and necessary for ALA synthesis and cited several arguments in favour of this point of view, namely:- that Duesberg had shown a decreased porphyrin excretion in patients with iron deficiency; that Heilmeyer and Plottner after intravenous injection of ferrous ascorbate had demonstrated a synthesis of haemoglobin in excess of that calculated for complete utilisation of the iron supplied; that Pappenheimer had demonstrated a stimulatory effect of added ferrous sulphate on porphyrin synthesis by C. diptheriae, although it had been inhibitory in excess; and that iron was known to be necessary for chlorophyll synthesis in plants, although chlorophyll did not contain this metal.

Brown himself demonstrated an <u>inhibition</u> of ALA synthesis by added iron salts in freshly prepared systems, but in aged preparations (stored for more than 24 hours), ferrous salts caused significant stimulation. From these observations he concluded that ferrous iron was used at some stage in the synthesis of ALA - possibly in stabilising the Schiff base formed between glycine and pyridoxal phosphate. He concluded that when in excess these salts proved inhibitory, but when, with aging, the endogenous iron had been oxidised to the ferric form, addition of ferrous

iron - or of a reducing agent - restored the activity of the preparation.

An inhibitory effect of α-α'- dipyridyl, a potent ferrous iron chelating agent, was felt to emphasise the significance of these ions in the reaction.

Patwardhan (231) demonstrated a role for ferrous iron in enzymatic transaminations - reactions also dependent upon pyridoxal phosphate as cofactor.

(ix) Ammonium ions.

These were found to be stimulatory in frozen and thawed preparations (112), an effect believed to have been due to the enhancement of the formation of DPN and glutamate from $DPNH_2$ and alpha-ketoglutarate. Increased amounts of DPN were believed then to favour the conversion of alpha-ketoglutarate to succinyl-coenzyme A.

This may also explain the stimulatory effect of added DPN observed in these preparations.(112).

Ammonium ions were shown to be important by Lascelles.(187).

(x) Buffer.

The nature of the buffer employed in the preparation has been shown to be of very great significance. Laver et al(191) demonstrated optimum formation of ALA when phosphate buffer was used, and showed that replacement by borate, barbitone, or Tris buffers reduced the amount of ALA formed. The superiority of

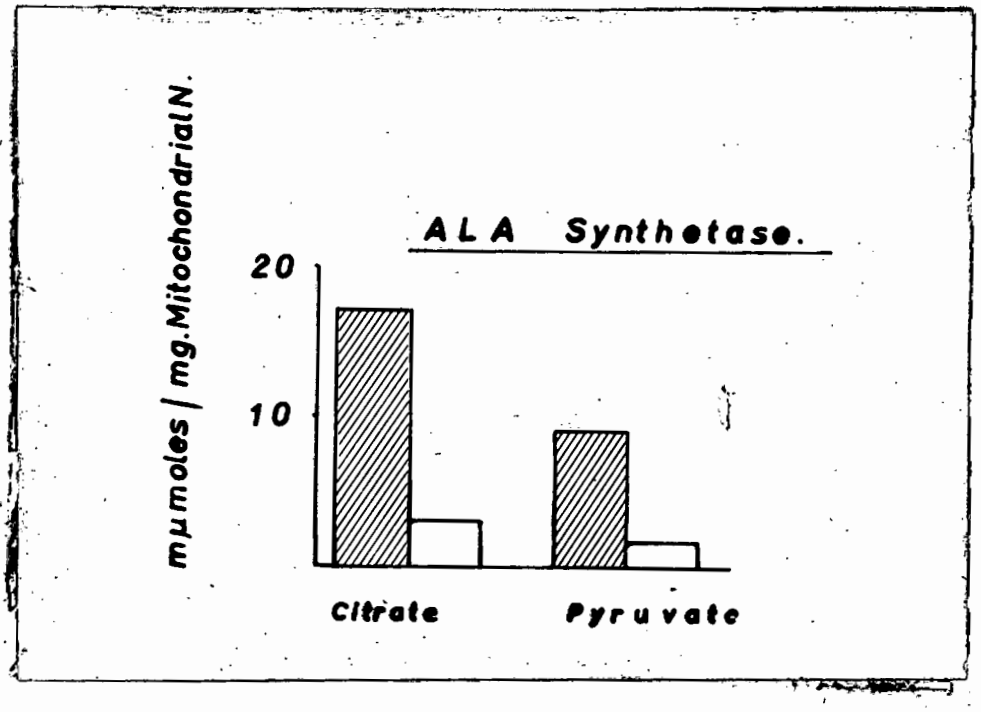


Figure 4. Influence of the nature of the buffer on ALA synthetase activity of mitochondria from a rat poisoned with DDC for 6 days.

The shaded columns show activity in phosphate buffer, and the open columns in Tris buffer.

phosphate buffer was also demonstrated by Gibson et al (112).

I studied the influence which the buffer might have upon ALA synthesis and have confirmed the inhibitory effect of Tris buffer. (Figure 4). Granick and Urata (142) on the other hand employed Tris buffer in all their studies and showed a decreased ALA synthesis - in atmospheres of nitrogen - at high inorganic phosphate concentrations. Miyakoshi and Kikuchi (208) found the exclusion of inorganic phosphate to increase the yield of ALA by 60% under anaerobic conditions, but under aerobic conditions the yield of ALA was highest when inorganic phosphate was present.

(xi) Oxygen.

An absolute requirement for oxygen was demonstrated (112,134, 191) for the synthesis of ALA, and anaerobiosis prevented protoporphyrin formation. (82, 188).

Contrary to these findings, in the preparation that I used, ALA synthesis proceeded normally in an atmosphere of nitrogen (Figure 3), while Granick and Urata (142) demonstrated enhanced ALA synthesis in nitrogen. Miyakoshi and Kikuchi (208) recorded a slight reduction of ALA synthesis under anaerobic conditions. Gibson (112) found that frozen and thawed mitochondria did not require oxygen for ALA synthesis, and Shemin (285) stated that using a crude preparation of duck red blood cells both ketoglutaric acid and succinate were converted to haemin in an atmosphere of nitrogen. Lascelles (190) found that ALA-synthetase

synthesis and activity were inhibited by oxygen in cultures of R.spheroides grown in the light, and Falk et al (94) found that the biosynthesis of protoporphyrin and haem in chicken erythrocyte preparations appeared to be regulated by oxygen tension, and showed increased synthesis with rising tensions to a maximum, and then a diminished synthesis with further increases.

(xii) Freezing and thawing.

The effect of freezing and thawing the mitochondria has been investigated by Gibson et al (112), by Granick (134), by Granick and Urata (142) and by Granick and Mauzerall (138).

Frozen and thawed mitochondria lost their ability to synthesise ALA from glycine and alpha-ketoglutarate or succinate, but were able to do so when succinyl-coenzyme A was employed as substrate. Intact mitochondria were unable to utilise succinyl-coenzyme A for this purpose.

It was suggested (134, 138) that steps in the synthesis of ALA from glycine might require coenzymes and other substances which became diluted out on damage to the cell membrane, or might require enzymes readily damaged in the process of freezing and thawing.

That the inability of frozen and thawed particles to synthesise ALA from glycine, and succinate or alpha-ketoglutarate was due to enzyme defects in the frozen particles was shown by the restoration of activity on the addition to the medium of

either bacterial succinic thiokinase, or alpha-ketoglutaric dehydrogenase, glutamic dehydrogenase and ammonia (to reoxidise reduced DPN). (112).

The inability of intact mitochondria to utilise added succinyl-coenzyme A was attributed to permeability barriers.(142).

(d). Inhibitors.

Studies involving the inhibition of ALA and porphyrin synthesis by various compounds have added to a knowledge of the biochemistry concerned.

Several amino acids have been shown to inhibit the synthesis of protoporphyrin from glycine (134, 138). Granick (134) considered that this might provide for a control mechanism of ALA synthesis. He postulated that a partial block might be removed when these amino acids reached a low concentration in the cell relative to glycine. Such a mechanism might have been a factor in the increased excretion of porphyrins observed by Lascelles(187) in aged cultures of R. spheroides - when amino acids were probably depleted in the medium.

Pyruvate has also been shown to have an inhibitory effect (113, 134, 284, 290) - an effect that was completely overcome by the addition of alpha-ketoglutarate (134) or by other members of the citric acid cycle. (284). It was suggested that the effect might be a competitive one, pyruvate competing with succinate to form aminoacetone; or that there might be competition between

pyruvate and alpha-ketoglutarate for coenzymes. Similar mechanisms may be active in the inhibition observed by alpha-ketobutyrate and parapyruvate.

Inhibition of porphyrin synthesis by inhibitors of the citric acid cycle emphasises the role of this cycle in porphyrin biosynthesis. Granick (134) showed inhibition by transaconitate (which inhibits cis-aconitase), by malonate (succinic dehydrogenase), and by fluoroacetate which is thought to owe its inhibitory action to fluorocitrate formation and inhibition of aconitase. Brown (27) also demonstrated inhibition by fluorocitrate, and Goldberg et al (125) and Wriston et al (347) have demonstrated inhibition by malonate. Arsenite (134) is believed to combine with lipoic acid and so inhibit alpha-keto-acid oxidation - and Lascelles(188) demonstrated a reduction in porphyrin synthesis when the system was deficient in lipoic acid.

Factors which inhibit pyridoxal phosphate activity have been shown to inhibit porphyrin and ALA synthesis also. These include L-penicillamine (112, 113, 142, 191), cysteine (112, 113, 134, 177), cyanide (112, 113, 191, 208) and isonicotinic hydrazide (134). In this context it is interesting that Middlebrook (206) noted that isoniazid-resistant tubercle bacilli were catalase deficient; and that Fisher (99) reported that isoniazid might affect the porphyrin metabolism of the mycobacteria . Talman (316) could demonstrate only a slight effect of isoniazid on porphyrin

metabolism in chick embryos. These compounds are thought to react with the aldehyde group of the coenzyme and so prevent the formation of an aldimine.

Inhibition by 2 : 4 dinitrophenol (81, 82, 134) indicates the necessity for an oxidative phosphorylating system, but such inhibition was not always demonstrated (191), nor inhibition by sodium azide (191).

Necessity for coenzyme A is suggested by the inhibition of ALA- and porphyrin-synthesis by compounds such as p. chloromercuribenzoate and iodoacetamide.(112, 191).

That ALA synthetase may be a sulphydryl enzyme is suggested by the inhibition of ALA formation by o-phenanthroline (111) and p-chloromercuribenzoate. (112).

Lead may also exert an inhibitory effect - at least in part - on ALA synthetase activity but conversely, Chisolm (49) cited Sano as showing that haemolysates from the blood of lead-poisoned animals synthesised excessive amounts of protoporphyrin from glycine.

3. ROLE OF DELTA-AMINOLAEVULIC ACID IN PORPHYRIN SYNTHESIS.

Experimental evidence is available in support of the role of ALA as an intermediate in porphyrin synthesis:-

It could replace "active" succinate and glycine as substrates in the synthesis of porphyrins and served as the source of all the nitrogen and carbon atoms of protoporphyrin. (297). The delta

carbon of ALA was shown to have the same distribution in the porphyrin molecule as did the alpha carbon of glycine (287) and ALA - 1 : 4 - C^{14} resulted in the same C^{14} protoporphyrin distribution pattern as did succinate - 1 : 4 - C^{14} .(260, 261).

ALA exerted a dilution effect upon the radioactivity of haemin derived from labelled succinate or glycine. (78, 84, 297). That this was not due to inhibition was evidenced by the simultaneous use of N^{15} labelled ALA, when there was a large incorporation of N^{15} into the porphyrin. Dresel (78) regarded this observation as fairly conclusive of the role of ALA as an obligatory intermediate in the pathway of haem biosynthesis.

In <u>in vitro</u> systems labelled ALA formed haem containing the radioactive isotope in many times the concentration of that formed from labelled glycine (80, 218, 287, 297), although when given to rats either orally or intraperitoneally, or to man orally, while some labelling of haemin occurred, this was 5 - 7 times lower than with an equivalent dose of N^{15} glycine. (16, 17, 218, 282). In the intact duck, however, ALA was utilised 2½ times better in the synthesis of both haemin and purines than was glycine. (215, 284). Shemin (284) ascribed the poor utilisation of ALA in the intact animal to its possible rapid destruction or to its transformation to compounds not effective as porphyrin precursors; to its failure to penetrate to haemopoietic sites; and to its rapid

excretion. Scott postulated a smaller ALA than glycine pool in the body with a consequent inability to administer a true tracer dose. (282).

Dresel and Falk (82) demonstrated a ready conversion of ALA by chick whole blood and washed cell preparations to protoporphyrin, and traces of uroporphyrin and coproporphyrin. Its conversion to porphyrins and haem in *in vitro* systems has frequently been demonstrated. (80, 82, 83, 91, 134, 186, 188, 273).

Granick and Vanden Schrieck (143) demonstrated that on the day following an intraperitoneal injection of 20 mg of ALA into a white rat, the urine contained a small amount of pigment which appeared to be a zinc-porphyrin complex, and Scott (282) found that within half an hour of administering a dose of ALA to a rat, the bile fluoresced a deep red and contained a large excess of protoporphyrin and a slight excess of coproporphyrin. He also showed an increase of faecal porphyrin excretion following a dose of ALA.

According to Gibson *et al* (113) it is now generally accepted that ALA provides all the carbon and nitrogen atoms of haemoglobin, myoglobin, catalase, peroxidase and the various cytochromes, and probably also of the dihydroporphyrin of chlorophyll and the tetrahydroporphyrin of bacteriochlorophyll.

4. OTHER METABOLIC PATHWAYS OF ALA.

When Shemin and Russell (297) first described ALA and its possible role in porphyrin synthesis they also postulated the

existence of a "succinate-glycine" cycle through which the delta carbon of ALA - and consequently the alpha carbon of glycine - could be utilised for the synthesis of methyl groups, formates, and the ureido groups of purines, and could provide the carbon atom for the beta carbon of serine. They suggested that ALA might be deaminated to form ketoglutaraldehyde, its delta carbon then being utilised as described, while the 4 carbon atom residue was reconverted to succinate. Ketoglutaraldehyde might also be directly oxidised to alpha-ketoglutaric acid.

Shemin and his group (298) subsequently demonstrated that the methyl group of methionine and the beta carbon of serine were radioactive after the injection of ALA-5-C^{14} into a duck; while the injection of ALA-5-C^{14} into a rat resulted in the excretion of highly radioactive formic acid. They also showed that alpha-ketoglutarate contained C^{14} activity after incubation of a duck red-cell haemolysate with ALA-5-C^{14}.

In 1957 this group (215) published results of radioactive studies showing that the delta carbon of ALA was utilised for the synthesis of the ureido groups of purines, and was converted to formic acid, while the succinyl moiety of ALA gave rise to succinate. When rats were injected with ALA-1-C^{14} and the metabolism of succinate inhibited by malonate, succinic acid from the urine was found to be highly radioactive.

Uric acid synthesis from ALA-5-C^{14} was demonstrated in the pigeon.

The role of ketoglutaraldehyde as an intermediate was supported by the finding of radioactive formate in rat urine, and radioactive uric acid in pigeon excreta after injection of ketoglutaraldehyde - 5 - C^{14}. This was greater than when either glycine or ALA were used.

Ketoglutaraldehyde could not be converted to ALA, contrary to the initial suggestion made, when the reversibility of the cycle was postulated as a mechanism whereby formate might be converted to haem.

This cycle was shown to provide a pathway for the conversion of glycine to CO_2 (298) - through decarboxylation of alpha amino - beta-ketoadipic. acid, when the carboxyl carbon was oxidised; while the alpha carbon was converted to CO_2 subsequent to the conversion of ALA to alpha-ketoglutarate, which upon oxidative decarboxylation liberated a molecule of CO_2.

$C^{14}O_2$ derived from glycine - 2 - C^{14} was more radioactive than that derived from ALA - 5 - C^{14} indicating other pathways of metabolism of glycine than through this cycle.

ALA has also been shown to be utilised in the synthesis of cyanocobalamin (55).

5. ALA DEHYDRASE.

Shemin et al (297) and Neuberger et al (218) considered the theoretical possibility that two molecules of ALA might condense

in a Knorr type of synthesis to form a pyrrole having the same structure as that proposed for porphobilinogen (52, 53).

Dresel and Falk demonstrated the *in vitro* conversion of ALA to PBG (80), and Weliky and Shemin (339) showed that administration of ALA gave rise to the urinary excretion of PBG. Berlin *et al* (16, 17) and Scott (282) reported similar findings.

In 1954 Granick (133) isolated an extract from chick erythrocytes that converted ALA to PBG, and Gibson *et al* (114) detected similar activity in aqueous extracts of an acetone powder of ox liver.

Enzyme activity has been detected in chicken erythrocytes(80); in duck erythrocytes (271), in pigeon liver (300), in many micro-organisms - excluding yeast - (114, 115), in plants (115) and in a wide variety of mammalian tissues. (49, 110, 114, 115, 116, 139, 143, 164, 227, 321).

I have demonstrated ALA dehydrase activity in rat and human liver homogenates. (this thesis).

This enzyme has been isolated and partially purified by precipitation techniques (110, 114, 115, 300) and by zone electrophoresis. (139). It has the properties of a euglobulin (115), and is believed to be a sulphydryl-containing enzyme (110, 114, 115, 139) - it was inhibited by p.-chloromercuribenzoate and iodoacetamide (114, 115) and by o.-phenanthroline (164); metals

inhibited roughly in order of the solubility products of their sulphides (115, 139); and reduced glutathione or cysteine were necessary for activation of the purified enzyme. (114, 115, 139).

The inhibition of enzyme activity by lead (49, 83, 115) tends to confirm its thiol nature, but Koike (cited by Chisholm (49)) reported that in lead-poisoned rabbits there was no reduction in ALA dehydrase activity in preparations isolated from liver, spleen, blood and kidney.

The enzyme was shown to be specific for ALA as substrate, and no Ehrlich-reacting compound was formed when ALA dehydrase was incubated with aminoacetone, alpha-delta-diaminolaevulic acid or 6-amino-5-oxohexanoic acid. (114, 115). Its mode of action was discussed by Granick (133) and Granick and Mauzerall (139) who concluded that there was probably a single enzyme only, which catalysed an aldol condensation between two carbon atoms, and that a ketimine condensation then occurred spontaneously. Gibson (110) suggested a similar mode of action.

No loss of activity accompanied dialysis, from which Granick (133) concluded that no loosely-bound coenzyme was involved. Gibson (110) suggested that the enzyme had no requirements for any metal, but Iodice et al (164) showed that it contained copper, and demonstrated that copper-deficiency states resulted in the lowering of the activity of this enzyme in the livers of rats, and

in the blood of ducklings. Anderson and Tove (5) showed that copper was necessary in the synthesis of haem from glycine *in vitro*, but suggested more than one site of action. Burnham and Lascelles (35) recorded a requirement for K^+ ions for activation. Inhibition by EDTA (115, 139, 164) may also indicate a metal requirement.

Enzyme activity was not associated with mitochondria or microsomes (115) and the enzyme has been shown to be water-soluble. (114, 115, 133, 139, 300).

The optimum pH for *in vitro* activity of this enzyme varied between 6.3 and 6.8 (114, 115, 133, 139, 300) according to the tissue preparation studied. My own measurements were made at pH 6.8.

Gibson *et al* (115) found rabbit tissue enzyme to be inactive in Tris buffer, but the preparations could be activated by the addition of inorganic phosphate. Granick and Mauzerall (139) on the other hand, observed inhibition of rabbit red cell ALA dehydrase activity by phosphate buffer, although this did not apply to chicken red cell enzyme. I have used phosphate buffer throughout my experiments.

The enzyme did not lose activity over a short (2 - 3 hours) incubation period (139), and activity over the first hour was found to be directly proportional to enzyme concentration over a wide range. (115, 139). Balance studies showed that the amount of PBG formed was quantitatively equivalent to the amount of ALA that

disappeared. (115, 139).

Gibson stated (110) that even with crude preparations of ALA dehydrase the porphobilinogen that was formed was not metabolised further in vacuo - implying possible further aerobic metabolism, and several authors have demonstrated the conversion of PBG to porphyrins and its disappearance from tissues on incubation in air. (see section 2 : 6). Using a method essentially similar to the one that I have employed, Tschudy et al (321) could barely detect the removal of PBG, and both they and Schwartz (278) stated that PBG production by liver in vitro far exceeded the capacity of the liver to metabolise it. Merchante et al (204) found that increased aeration did not alter the rate of PBG disappearance when incubated with liver homogenates. My own experiments were all performed aerobically, and an experiment comparing activity aerobically with that in vacuo is presented, and shows, if anything, greater apparent activity in air.

Table (i)

Comparison of ALA dehydrase activity* in air and in vacuo.

mumoles

	Normal		Porphyric	
	Formed	Present	Formed	Present**
In air	197	0	408	87
In vacuo	190	0	379	84

* Activity expressed as amount of PBG formed per hour, per mg tissue P.

** This amount of PBG was present after 1 hour's incubation in the absence of added ALA and was presumed to be preformed in the liver.

6. PORPHOBILINOGEN (PBG).

Paula Sachs in 1931 (253) isolated a substance in the urine of a patient with acute porphyria which gave a red colour with Ehrlich's aldehyde which was not soluble in chloroform. The term porphobilinogen was applied to this substance in 1939 by Waldenström and Vahlquist. (328).

Its role in porphyrin biosynthesis is now fairly widely accepted, although slight evidence to the contrary does exist.

In 1953 Falk et al (93) demonstrated the enzymatic conversion of PBG to porphyrins, by a chicken red cell haemolysate, and Bogorad and Granick (23) demonstrated its similar conversion by an extract of chlorella. Goldberg and Rimington (127) detected small, but significant increases in the urinary content of uroporphyrin III and coproporphyrin III after parenteral administration of PBG to rats.

The enzymatic formation of porphyrins and haem from PBG has since been demonstrated on numerous occasions and by many different preparations (19, 20, 24, 26, 78, 82, 83, 91, 103, 133, 139, 151, 162, 194, 195, 204, 225, 271, 276, 279, 281, 300), and porphyrin formation from PBG by non-enzymic chemical methods has also been shown to occur. (49, 53, 80, 113, 234, 278).

Falk et al (93) stressed that although there was no doubt that tissues could bring about the conversion of PBG to porphyrins, this did not prove that PBG was a normal intermediate in the

biosynthesis of haem.

Perhaps more convincing of its role as an intermediate was the demonstration by Dresel (78) that the yield of haem from PBG as substrate was twice that from glycine; and even more convincing was her demonstration of the greatly reduced incorporation of active glycine into haem in the presence of added, unlabelled PBG - which meant that very little could have bypassed the pool - and the detection of radioactivity in the PBG at the end of the experiment. (78, 84). Schmid et al (271) showed that the radioactivity of haem synthesised from ALA, or equivalent amounts of PBG, was similar.

Although probably insignificant in the face of overwhelming evidence to the contrary, there are some indications that PBG may not be an obligatory intermediate of haem biosynthesis:-

(i). Kench (173) demonstrated an alternative route of porphyrin synthesis in yeasts.

(ii). Neuberger (217) emphasised that the condensation of porphobilinogen was unlikely to give rise to naturally occurring porphyrins of types I and III, and to-day the number of postulates as to how this may occur casts just a little doubt as to whether it does.

(iii). Hawkinson in 1952 (154) presented evidence to show that porphobilinogen gave rise only to porphobilin once it had been separated from one or more non-Ehrlich-reacting precursors which

accompanied it in the urine of cases with acute porphyria.

(iv). Porphobilinogen did not cause irritability or hyperaemia in the skin of a rat injected with it and exposed to ultraviolet light, whereas ALA in the same circumstances did do so. (166).

(v). Dresel and Falk (82) showed that whole blood formed only traces of protoporphyrin from PBG, whereas ALA was rapidly converted by such a preparation into protoporphyrin. PBG was not converted to porphyrins by the harvested cells of R.spheroides (156, 162, 186, 187) or by whole cells of Chlorella (23) although cell-free extracts, or damaged cells, were able to do so. These observations have been ascribed to relative cell impermeability to PBG, but it may be possible that intact cells metabolise PBG differently.

7. FURTHER STAGES IN THE PORPHYRIN BIOSYNTHETIC PATHWAY.

As my experiments have not been directly concerned with these later stages of porphyrin metabolism, I shall consider this subject only briefly.

(i). Mechanism of the conversion of PBG to Porphyrins.

The mechanism whereby PBG is converted to porphyrins is not clear, and several alternatives have been postulated to account for its condensation to both the series I and the series III isomers - particularly the latter.

Shemin (284) and Shemin et al (298) suggested that 3 molecules of PBG might condense to form a radial tripyrrylmethane which could

split in two ways to form two different dipyrrylmethane compounds, and that the structure of the final porphyrin molecule would depend upon the subsequent condensation of these dipyrrylmethanes with one another.

Bogorad and Granick (23) postulated the occurrence of a branched "T" tetrapyrrylmethane as an intermediate, the splitting of which would also result in two unlike dipyrrylmethanes. Two possible series of steps by which this intermediate could be synthesised were suggested.

Bullock et al (31) conceived of a linear build up of enzyme-activated PBG molecules, an active $-CH_2$ group migrating from one pyrrole ring to another via the nitrogen atom with cyclisation occurring to yield uroporphyrinogen III. Lockwood and Benson's (194) postulate was similar, but they suggested that PBG molecules might be interpolated between the enzyme-activated $-CH_2$ group and the previously attached molecule.

Carpenter and Scott (43) postulated the enzymically controlled condensation of one molecule of opsopyrrole dicarboxylic acid with a linear tetramer derived from PBG molecules. Rupture of the pentamer at the correct site would result in a series III isomer on cyclisation and the regeneration of opsopyrrole dicarboxylic acid. However, they could not confirm the role of the compound experimentally.

Cookson and Rimington (53) postulated the initial formation

of a symmetrical dipyrromethane , PBG then being condensed in a regular fashion to form linear tetramers which underwent cyclisation.

A possible participation of dipyrromethanes was suggested by Heath and Hoare (155) but this could also not be demonstrated by experiment.

Wittenberg (343) demonstrated the formation of a colourless polypyrrole during haem synthesis and suggested the primary condensation of PBG into linear tetrapyrroles, two molecules of which combining to form a cyclic octapyrrole containing 2 tripyrrylmethane structures. Two molecules of uroporphyrinogen III could then result from the rearrangement of this molecule.

(ii). Enzymes concerned in this condensation.

Two enzymes are thought to be concerned in this process. Bogorad and Granick (23) found that preheating at 55 - 60°C for 30 minutes destroyed the ability of frozen and thawed chlorella cells to convert PBG to the series III porphyrin isomers, while the series I isomers were readily formed. This effect of heat has been repeatedly confirmed. (24, 138, 139, 194, 195).

Heath and Hoare (156, 162) were able to separate two fractions from acetone-dried preparations of R.spheroides by centrifugation, the supernate catalysing the synthesis of the series I isomer only, and the residue, of series III. Bogorad,(19, 20) isolated a fraction from spinach leaf which could convert PBG

to the series I isomer only, and described some of the properties of this enzyme.

(iii). Porphyrins and porphyrinogens as intermediates.

It was at one time thought that the porphyrins themselves might be intermediates in the pathway of haem biosynthesis, and Falk et al (93), and Dresel and Falk (84) were able to demonstrate the conversion of some uroporphyrin III to protoporphyrin, but were unable to demonstrate any role for coproporphyrin III. Salomon et al (254) calculated that 52% of added labelled uroporphyrin was converted to protoporphyrin by a rabbit bone-marrow suspension, but conceded that although uroporphyrin might be synthesised from glycine and could be converted to protoporphyrin, it might not lie on the major metabolic pathway. Eriksen (89) concluded from his experiments that uroporphyrin was successively decarboxylated through coproporphyrin to protoporphyrin which he thought combined with globin, and then ferrous ions, to form haemoglobin.

However, several authors could not demonstrate this role of porphyrins as intermediates(19, 78, 125, 138, 156, 257, 276, 279, 298, 199, 300), and Dresel (78), Bogorad (19) and Shemin (284) suggested that the actual intermediates on this pathway might be colourless precursors of which the porphyrins were oxidation products.

The role of these reduced porphyrins (porphyrinogens) has

since been confirmed. (54, 140, 203, 220).

(iv). Uroporphyrinogen decarboxylase.

The conversion of uroporphyrinogen to coproporphyrinogen is believed to occur in a stepwise fashion through compounds with 7, 6 and 5 carboxyl groups, and to be enzymically controlled by the soluble enzyme uroporphyrinogen decarboxylase - an enzyme which has been partially purified and studied by Mauzerall and Granick (203).

(v). Coproporphyrinogen decarboxylase.

Coproporphyrinogen III has been shown to be oxidised to protoporphyrin by cell particulates of chick erythrocytes or Euglena (140) with a 3 carboxyl porphyrin, being formed in short time incubations. Such enzyme activity is thought to be confined to mitochondrial fractions (256, 257), although Shemin et al (287) were able to synthesise protoporphyrin from ALA by a supernatant solution obtained from haemolysed duck erythrocytes.

(vi). Haem formation.

There is still some controversy as to whether iron combines with protoporphyrin or its porphyrinogen in the final stages of haem synthesis.

Dresel (78) and Dresel and Falk (84) could not demonstrate a role for protoporphyrin in haem synthesis, and suggested that protoporphyrin precursors was the actual haem intermediate.

However, Granick (132) showed that iron was incorporated into protoporphyrin by chicken red cell haemolysates, and its role as a true intermediate has been demonstrated on several occasions. (122, 125, 147, 181, 275).

The enzyme concerned has been found in the particle-free supernate of centrifuged chick red cell haemolysates(122), but Sano et al (257) felt that the mitochondria probably played an important role in the incorporation of iron into the porphyrin; Labbe and Hubbard (181) found the enzyme to be firmly bound in the mitochondria of rat livers, and Nishida and Labbe (225) showed the incorporation of iron into protoporphyrin to be dependent upon the mitochondrial fraction of rat liver cells.

Enzyme activity was shown to be activated by dehydroascorbic acid, ascorbic acid, ergothioneine and glutathione by Goldberg et al (122) who also found dehydroascorbic acid, and ascorbic acid in excess, to have an inhibitory effect. Ascorbic acid was also shown to have a potentiating effect by Labbe and Hubbard (181), as were citrate, adenosine, inosine, thymidine, vitamin B_{12}, DPN and ALA. Activation by glutathione (reduced) and homocysteine, histidine and flavo-adenine-dinucleotide as also by both the reduced and oxidised forms of DPN and TPN was demonstrated by Nishida and Labbe. (225).

Protoporphyrin is also regarded as lying on the chlorophyll biosynthetic pathway. (113).

B. AMINOACETONE SYNTHESIS AND METABOLISM.

Aminoacetone synthesis has been demonstrated from acetyl coenzyme A, or pyruvate, and glycine. (112, 175, 208, 322).

Its synthesis has also been shown from L - threonine. (88, 219, 322). It has been detected in the urine of normal individuals. (253, 319).

Elliott (88) postulated a metabolic cycle for glycine oxidation via aminoacetone analogous to the succinate-glycine cycle proposed by Shemin. In this cycle, glycine and acetyl-coenzyme A are thought to combine to form alpha-aminoaceto-acetic acid which undergoes spontaneous decarboxylation to aminoacetone. Aminoacetone is then deaminated to form methylglyoxal which is converted to D - lactic acid by the glyoxalase system, and D - lactic acid is oxidised to pyruvic acid with the subsequent regeneration of acetyl-coenzyme A.

That aminoacetone might be involved in the oxidative metabolism of glycine was suggested by Nemeth *et al* (215) who showed that in the rat, administration of C^{14} aminoacetone resulted in the production of $C^{14}O_2$, and C^{14} labelled purines. The carbon atom of the aminomethylene group of aminoacetone was shown by Shemin *et al* (288) to play the same metabolic role as did the alpha carbon of glycine - apart from not entering into porphyrin biosynthesis.

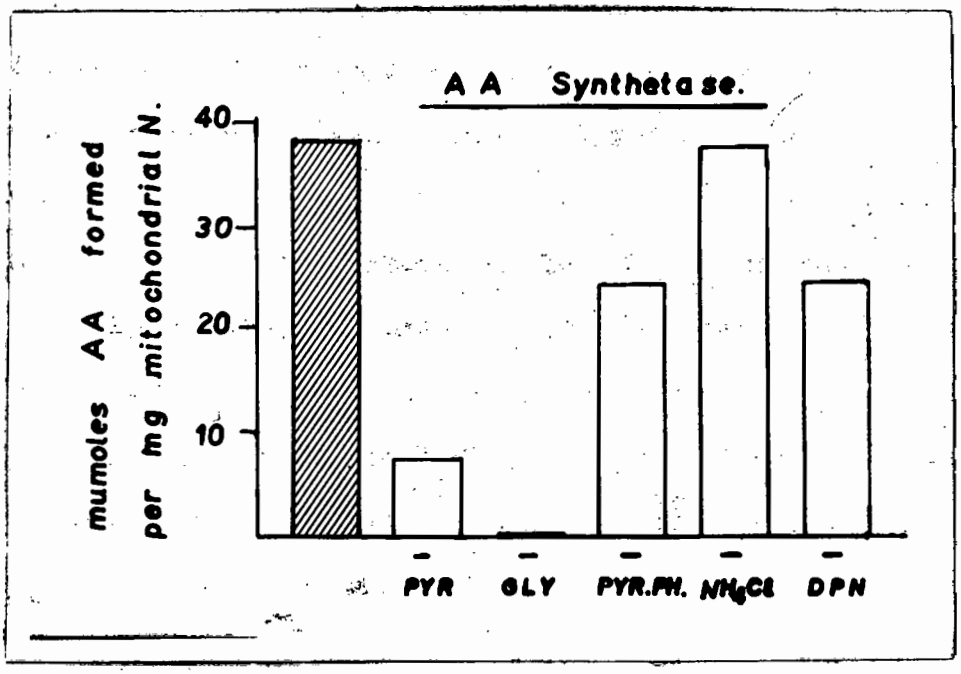


Figure 5. Effects of excluding a variety of components from the incubating medium on aminoacetone synthesis in vitro by intact mitochondria from the liver of a normal rat.

Factor lacking.	None	Pyruvate	Glycine	Pyr.ph	NH_4Cl	DPN.
AA formed *	38.2	7.2	1	24.2	37.6	24.2

* mumoles/2hours/mg. mitochondrial N.

Urata and Granick (141, 322) studied various factors influencing the formation of aminoacetone by guinea pig liver mitochondria, and postulated the existence of a specific enzyme, aminoacetone-synthetase, in these mitochondria.

They demonstrated aminoacetone synthesis from pyruvate and glycine incubated with intact mitochondria. Succinate, malate, alpha-ketoglutarate and a variety of other compounds were shown to be inhibitory. K^{+} and Mg^{++} ions were important, and ammonium chloride increased the yield. Malonate also increased the yield. When employing a frozen-and-thawed mitochondrial system, with acetyl-coenzyme A as substrate, pyridoxal phosphate was found not to be as essential a cofactor as it was for ALA synthetase activity, and in the absence of added vitamin, the yield of aminoacetone was reduced by only 19%.

Miyakoshi and Kikuchi (208) found anaerobiosis to inhibit the activity of rat-liver mitochondrial enzyme, while the addition of ADP had a marked stimulatory effect.

I was also able to demonstrate aminoacetone synthetase activity in rat liver mitochondria.

The effect on the activity of this enzyme of excluding various substances from the basic medium is shown in Figure 5.

Aminoacetone production did not occur in the absence of glycine. Its production was grossly reduced in the absence of added pyruvate.

and that any was produced at all is probably best ascribed to the presence of small amounts of endogenous pyruvate in the preparation. Absence of pyridoxal phosphate, caused a reduction of 27% in the amount of aminoacetone produced.

Exclusion of DPN also caused a reduction in aminoacetone yield, while the absence of ammonium chloride was without effect.

When isocitrate or citrate replaced pyruvate in the medium, aminoacetone synthesis could still be demonstrated. (322). However, the yield was only 18% of that from pyruvate.

In my own studies, pyruvate was also shown to be far superior to citrate as a substrate in the determination of AA synthetase activity. (Section 6).

C. ASCORBIC ACID SYNTHESIS AND METABOLISM.

In 1950 Jackel et al (165a) brought forward direct evidence indicative of glucose as a precursor of L-ascorbic acid synthesis. D-galactose has also been shown to be a precursor (90) and a glucuronic acid pathway of ascorbic acid synthesis has been described by several authors (op cit 39). It is of interest, and possibly pertinent to my findings, that this pathway should traverse the pentose-phosphate shunt.

Enzymes converting D-glucuronolactone and L-gulonolactone into L-ascorbic acid have been demonstrated in liver homogenates of rats and goats and in kidney homogenates of the chick. Such enzymes

are absent from the tissues of the primates, guinea pigs, Indian fruit-bat and red-vested bulbul-species, which cannot synthesise ascorbic acid either. (171).

The enzymes are said to reside in microsomal fractions, although Isherwood et al (164a) demonstrated that the mitochondrial fraction of rat liver homogenates was also active.

SECTION 3.

A REVIEW OF THE DRUG-INDUCED PORPHYRIAS.

The subject of the drug-induced porphyrias has been reviewed by several authors. (48, 129, 170, 264, 329, 332, 334).

A. Historical Background.

Porphyria and porphyrinuria have been induced experimentally in animals, and unintentionally in man, by a variety of chemical compounds.

Shortly after the introduction of sulphonal into clinical medicine in 1888, Stokvis (op cit 129) reported an increased excretion of porphyrins by two patients who had been poisoned with this drug. This observation has subsequently been repeatedly confirmed (264), and a similar abnormality has been noted following the use of the closely related compounds Trional and Tetronal. (op cit:- 129, 264, 332).

Prior to this, MacMunn, in 1880, had reported the occurrence of increased porphyrins in the urine of a patient taking salicylates, and Le Nobel, in 1887, had noticed porphyrinuria as a feature of lead poisoning. (op cit 129).

Stokvis (307) was first to induce an increased porphyrin excretion in animals by means of drug administration. He used both lead and sulphonal, and in 1895 reported instances of what

were both hepatic and erythropoietic forms of experimental porphyria. There was subsequently some controversy as to the nature of the porphyrin excreted following the administration of this compound. Fischer and Duesberg isolated uroporphyrin in the urine of sulphonal-poisoned rabbits, but Waldenström and Wendt, and Stich, could detect an increased coproporphyrin excretion only, in the urine of such rabbits. (op cit:- 170, 264, 332).

In 1937 Brunsting (30) reported an increased urinary porphyrin excretion by patients receiving sulphonamides, and in 1938 Rimington and Hemmings (244) recorded a striking increase in the amount of porphyrin in the urine of nearly every patient investigated while receiving sulphanilamide. They consequently investigated the effects of administering this drug to normal adult white rats and demonstrated a rise in urinary porphyrin excretion to between 2 and 10 times the normal value.

Brownlee (29), because of this observation, and also because of that of Schreus (op cit 29) who identified coproporphyrin III in the urine of patients treated with salvarsan, investigated the effects on the porphyrin metabolism of normal rats, of administering some of the chemically related coal tar antipyretics. Acetanilide, phenacetin, phenazone, amidopyrine, p.aminophenol and aspirin all caused the development of a porphyrinuria in these animals.

Rimington and Hemmings (245) showed that most drugs belonging to the sulphonamide series could induce a porphyrinuria in healthy animals, as could a number of simple aromatic amines and nitrobenzenes.

In 1940 Rimington and Goldblatt (243) found increased porphyrin levels in the urine of workers exposed to aromatic compounds containing nitro- and amino-groups.

In 1940, also, large amounts of porphyrins were found in the excreta of a refrigerator engineer poisoned with methyl chloride. (46).

In 1949, Sumegi and Putnoky found lead, bismuth, mercury, copper, zinc, iron, silver and gold to cause a porphyrinuria in white rats, and 3 years earlier Brugsch had reported increases of urinary coproporphyrin in patients receiving paraldehyde, chloralhydrate, amylene hydrate, and morphine. (op cit 332).

At about this time too, Penew and Tropp (op cit 170, 332) recorded coproporphyrinuria following nitrous oxide and ether anaesthesia and the use of novocaine.

Dent and Rimington (71) in 1947 showed that male Wistar albino rats weighing $\pm$ 100 G, when fed a diet containing oxidised casein, developed a marked coproporphyrinuria.

In 1932 Duesberg (op cit 267) reported the development of the typical clinical and biochemical features of acute porphyria in a patient who had received large amounts of Sedormid over a

long period of time. Stimulated by this report, Schmid and Schwartz (267) induced a disturbance in hepatic porphyrin metabolism in rabbits of both sexes weighing 1.5 - 3 Kg. by administering Sedormid in daily doses of 200 mg/Kg. This work was briefly reported in a lecture to the American Clinical and Climatological Association in 1952.(334).

Goldberg and Rimington (128) investigated the potential to induce porphyria of several drugs structurally related to Sedormid in a search for an active, nonhypnotic compound. Allylisopropylacetamide was shown to induce porphyrinuria in rabbits, fowls and rats; and allylisopropylacetic acid caused a slight increase in coproporphyrin excretion in some of the animals studied. Talman et al (315, 316) investigated a similar group of related compounds for their porphyria-producing properties, using the chick embryo as a test animal. Their conclusion was that the molecular structure of compounds which can induce porphyria must include a dialkyl substituted acetamide, or acetamide derivative, and that one of the substituents must be an allyl group. Stich and Decker (306) concluded that compounds capable of inducing porphyria were substituted derivatives of allyl acetic acid.

Goldberg (119), Kalow (170) and Watson and Larson (332) have reviewed the somewhat controversial literature, both clinical and experimental, of the relationship between the barbiturates and the porphyrias - a relationship which had been noted since the report

by Dobrschansky in 1906 (op cit 119) of a typical attack of acute porphyria following the prolonged administration of diethyl barbituric acid. Goldberg (119) tested the effects of barbiturate administration on urinary porphyrin excretion and concluded that possession of an allyl group appeared to make the barbiturate more effective in this regard. Six of 9 drugs tested were capable of producing some effect on the porphyrin metabolism of normal rabbits.

A purely fortuitous observation by Solomon and Figge (302) was that DDC administration resulted in an intense red fluorescence in ultraviolet light of the liver and gall bladder. At the time they were concerned with investigating the uptake of orally-administered fluorescent substances by the lining of the forestomach of mice. DDC has a blue fluorescence. Investigation confirmed the occurrence of a disturbance in porphyrin metabolism.

The epidemic of cutaneous porphyria in Turkey was traced to the ingestion of the fungicide hexachlorobenzene (Cam op cit 263), and Ockner and Schmid (226) were able to induce porphyria in adult male Sprague-Dawley rats by feeding them a diet containing 0.2% hexachlorobenzene. Rimington and Ziegler (246) were later able to induce porphyria in rats with several chlorinated benzene compounds.

In 1961 Barich et al (7) observed an accumulation of a pigment which did not stain positively for iron in the Kupffer cells of mice given high doses of griseofulvin. Weston-Hurst and Paget (op cit 69) were subsequently able to identify this pigment as mainly protoporphyrin.

Porphyrinuria has also been described following cinchophen administration (op cit 45), poisoning with illuminating gas, (op cit 332) and BAL treatment of hypertension. (op cit 170).

Apart from lead, the photodynamic dye, Rose Bengall(232), and a combination of lead, phenylhydrazine and light (280) have all been shown to induce erythropoietic forms of porphyria.

An association between alcohol consumption and coproporphyrinuria was first noted by Franke and Fikentscher in 1935 (op cit 129). Urinary coproporphyrin has since been shown to be transiently increased following the consumption of small amounts of alcohol by normal adults, and to be high in chronic alcoholics, but to return to normal within a few days of complete abstinence from alcohol. (309, 332). Hoffbauer et al (163) could detect no real change in the porphyrin excretion of rats which were administered 10% alcohol in place of water in their diets. When the diets of these animals were also deficient in choline, a fall in faecal porphyrin levels occurred, in association with a slight rise in urinary levels - but not in excess of normal.

A correlation between excessive alcohol consumption and

acquired porphyria has also been noted. (8, 108, 129, 185).

B. Porphyria induced by Sedormid and AIA, DDC, HCB and Griseofulvin.

Sedormid or AIA have been shown to cause porphyria in rats (44, 65, 67, 70, 121, 127, 128, 145, 180, 204, 208, 246, 249, 265, 268, 269, 278, 321), in rabbits (1, 26, 67, 91, 101, 103, 104, 110, 115, 128, 145, 150, 151, 197, 236, 257, 265-268, 278, 306, 320, 321, 324, 334), in chick embryos (104, 182, 183, 313-317), in fowls (128), in mice (227), in guinea pigs (302), in geese (277) and in dogs. (op cit 264).

DDC has been found to cause porphyria in rats (65, 67, 70, 116, 142), in rabbits (150,151), in mice (227, 302, 303) and in guinea pigs (141, 142, 302).

HCB ingestion has resulted in porphyria in man (41, 45, 48, 62, 263) and has been used to induce disturbances of porphyrin metabolism in rats (32, 42, 66, 70, 75, 104, 226, 259) and in rabbits, guinea pigs and mice (66).

Griseofulvin has been shown to cause a disturbance of porphyrin metabolism in man (245a) and in mice. (69, 228, 342). A similar disturbance could not be induced in rats (311, 342), guinea pigs or rabbits. (342).

Porphyrin synthesis was stimulated in chick-embryo liver cells, cultured on cover slips in the presence of AIA, DDC or Griseofulvin (136, 137), and Cowger et al (57) noted an increased

porphyrin content of Foley mouse leukaemia cells cultured in the presence of AIA.

Case et al (44) emphasised the variability of the response of individual rats to Sedormid administration, and such intra-species variability is apparent in the work of most authors investigating the influences of these various drugs.

In the following sections the effects of the administration of these drugs on various aspects of porphyrin metabolism will be considered. These include:-

1. Patterns of porphyrin and porphyrin precursor excretion in the urine.
2. Patterns of porphyrin excretion in faeces and bile.
3. Patterns of porphyrin and porphyrin precursor accumulation in the liver.
4. Patterns of porphyrin and porphyrin precursor accumulation in other organs.
5. Clinical and Pathological manifestations.
6. Other biochemical studies. In this section some studies in the inherited forms of porphyria will also be considered.

1. PATTERNS OF PORPHYRIN AND PORPHYRIN PRECURSOR EXCRETION IN THE URINE.

(a). Sedormid, AIA and DDC.

Porphyrins.

Coproporphyrin excretion has been found to increase early -

within 1-2 days of the administration of these drugs - and to progress rapidly to a constant level, after 3-8 days, in the urine of rats or rabbits administered Sedormid or AIA(1, 44, 67, 103, 128, 151, 246, 265, 267, 278), or DDC. (67, 150, 151).

Schmid and Schwartz (267) noted a delay of several days before urinary uroporphyrin levels were increased in rabbits treated with Sedormid. A very sharp increase occurred between the 5th and 8th days of such treatment, to levels sufficient to impart to the urine a Burgundy colour, and a brilliant red fluorescence in ultraviolet light. This delay and subsequent marked increase in uroporphyrin excretion by rats or rabbits during Sedormid, AIA, or DDC administration, is also evident in the work of Goldberg and Rimington (128), Schwartz (278), Schmid et al (265), Case et al (44), and Haeger-Aronsen (151).

Gajdos and Gajdos-Török(103), however, detected an increased uroporphyrin excretion within 7-18 hours of the administration of AIA to rabbits, which, nevertheless, succeeded the appearance of increased coproporphyrin levels in the urine of these animals.

De Matteis and Prior (67) found uroporphyrin and coproporphyrin excretion to increase at much the same time, and within 2 days of the administration of a single dose of either Sedormid or DDC to rabbits. The increase in uroporphyrin excretion was greater than was that of coproporphyrin. Abbott and Rudolph (1) found these two substances to reach maximum levels more or less simultaneously in rabbits receiving Sedormid.

In guinea pigs treated with DDC there was a delay of 4 days before coproporphyrin levels increased in the urine, but these, nevertheless, reached maximum levels within 6 days. (302).

Several authors have shown the increased coproporphyrin to be largely of the series III isomer.(44, 128, 183, 246, 267).

De Matteis and Prior (67) found 51% of the coproporphyrin in the urine collected from AIA-treated rabbits to be present in the reduced form, and all occurred as the porphyrinogen in the urine collected during DDC treatment.

The excretion of uroporphyrin in the reduced form was noted by Schmid and Schwartz (267) and by Case et al (44) in the urine of animals receiving Sedormid.

Porphyrin precursors.

Stich (op cit 150) reported that the level of ALA excretion in the urine of Sedormid-intoxicated rabbits was normal. However, Haeger-Aronsen (150) and Abbott and Rudolph (1) observed rabbits to have increased urinary levels of ALA within 24 hours of Sedormid or DDC administration, which progressed to maximum levels over 6-7 days.

De Matteis and Prior (67) noted an early increase in ALA excretion in both AIA- and DDC-treated rats and rabbits, while the rise with Sedormid was very much smaller. Increased urinary ALA excretion by rabbits treated with AIA was also reported by Gray et al. (145).

An increased urinary PBG excretion during the administration

of these drugs has been frequently reported.

The urine of rabbits treated with AIA was shown to contain increased amounts of PBG by Gray et al. (145). Goldberg and Rimington (128) detected PBG in the urine of rabbits after about 7 days of AIA or Sedormid administration, and in the urine of 7 of 9 rats, similarly treated, after intervals varying between 2 and 11 days. They also noted the occurrence of an atypical PBG compound in the urine of AIA- or Sedormid-treated rabbits, but not in the urine of similarly treated rats. A delay in the appearance of porphobilinogen during AIA or Sedormid treatment was also noted by Schmid and Schwartz (267), by Schwartz (278), and by Case et al. (44).

Conversely, increases in urinary PBG excretion beginning within 24 hours of AIA, Sedormid or DDC administration to rats or rabbits, were reported by Haeger-Aronsen (150), by Abbott and Rudolph (1), by Gajdos and Gajdos-Török (103), by Rimington and Ziegler (246) and by De Matteis and Prior.(67).

Solomon and Figge (302) noted the appearance of PBG in the urine of guinea pigs treated with DDC on only a few occasions and in low concentration, and Granick and Urata (142) found that guinea pigs acutely poisoned with DDC (1-2 G in 2 days) excreted traces of PBG in the urine after 7 hours, and maximal amounts after 24 hours, while when the animals were less acutely poisoned,

the urine did not contain PBG.

(b). HCB.

Porphyrins.

HCB administration also resulted in an increased coproporphyrin excretion by rats and rabbits, but this was apparent only after 1-2 weeks and progressed slowly. (33, 66, 75, 226).

Ockner and Schmid (226), and Burnett and Pathak (33) found parallel increases in uroporphyrin and coproporphyrin excretion to occur in rats treated with this drug. In both these studies levels of uroporphyrin were about twice those of coproporphyrin.

De Matteis et al (66) reported a later increase in uroporphyrin than in coproporphyrin excretion in rats treated with HCB. Dowdle and Eales (75) found a very marked increase in urinary uroporphyrin excretion by rats treated with HCB for 8 weeks - but to levels which were slightly lower than were those of coproporphyrin.

Gajdos and Gajdos-Török(104) noted an increased urinary uroporphyrin level in rats fed a diet containing 2% (of) HCB, while in rabbits a moderate increase only, in uroporphyrin excretion occurred. However, the urine of these rabbits showed striking increases in the levels of uroporphyrin precursor.

Guinea pigs treated with HCB showed no significant increase in porphyrin excretion apart from a slight preterminal rise in

urinary coproporphyrin. Mice treated with this compound also showed only a preterminal increase in urinary coproporphyrin excretion. (66). These animals showed no increase in their urinary uroporphyrin levels.

Porphyrin precursors.

Gajdos and Gajdos-Török(104) observed a greatly increased excretion of ALA in the urine of rats treated with HCB in the diet for 2 weeks, but De Matteis et al (66) could detect no increase in urinary ALA excretion by rats or rabbits poisoned with this compound.

Striking increases in the urinary excretion of PBG by rats treated with HCB were observed by Ockner and Schmid (226) and by Gajdos and Gajdos-Török (104). Conversely De Matteis (65) could not detect an increased PBG excretion by rats treated with HCB, although he had observed such an increase in the urine of these animals after poisoning with sulphonal, DDC, Sedormid or AIA. De Matteis and Rimington (66) found such an increase to be a terminal event in rats, and not to occur at all in rabbits, mice and guinea pigs treated with HCB. They noted an early increase of an "ALA-like" substance in the urine of guinea pigs poisoned with this drug.

Human cases.

In persons poisoned with HCB during the Turkish epidemic of porphyria, coproporphyrin excretion was moderately increased

while striking increases in the levels of urinary uroporphyrin occurred. (41, 45, 263). PBG was said to have been consistently absent from the urine of persons involved in this epidemic (45, 62, 263) although Cam and Nigogosyan (41) detected PBG in the urine of some cases.

Related compounds.

Rimington and Ziegler (246) noted an increased urinary coproporphyrin excretion in rats poisoned with various chlorinated benzenes. PBG and ALA excretion occurred late in the course of such intoxications.

(c). Griseofulvin.

Mice of the Alderley Park albino strain, when treated with griseofulvin, showed an early slight increase in urinary coproporphyrin levels. This was transiently more striking after about 9 days of drug administration. Females of the U.C.H. strain showed a more persistent additional increase in urinary coproporphyrin excretion after about 30 days of treatment, which was associated with the development of features of toxicity and liver damage, and a reduction in faecal porphyrin excretion. (69).

Only a late and slight increase of uro-type porphyrins in the urine was noted.

Increased ALA and PBG concentrations were apparent in the urine of these animals after a few days.

Effects of prolonged drug administration; or the cessation of treatment.

Formijne and Poulie (101) found prolonged administration of Sedormid to result in the disappearance of PBG and porphyrin excess from the urine.

A return to normal levels despite continued drug administration was also demonstrated by De Matteis and Prior (67), and by Haeger-Aronsen (150, 151). After withdrawal of the drugs for 1 day, rats responded to their reinstitution with the excretion of large amounts of porphyrins and porphyrin precursors in their urine. (67). Yet despite an irregular dosage scheme employed by Haeger-Aronsen, a return to normal with continued treatment was, nevertheless, observed. (150).

Cessation of drug administration, or a reduction in the dose of drug has led to a rapid return to normal levels of these substances, the decrease in coproporphyrin excretion taking somewhat longer than that of uroporphyrin or PBG. (128, 226, 267, 302).

When HCB administration was continued for too long, the porphyric state became irreversible despite cessation of drug administration, and in many human cases, the disease has persisted despite the patients receiving no further HCB, with exacerbations occurring in the summer. (41, 62, 226, 263).

Factors underlying some of the observed differences.

Apart from obvious species differences, and variations in

the actions of the drugs, other factors may also have contributed to some of these observed differences.

Formijne and Poulie (101) found that during Sedormid administration, the excretion of PBG and porphyrins was very irregular, large amounts being detected on certain days, while on other days they were almost or totally absent. A clear-cut periodicity could be observed in some instances.

Schwartz (277) stated that while no PBG could be detected in the urine of rats during the first two days of Sedormid administration, a marked increase occurred in the urinary content of an Ehrlich-reacting material which differed in several properties from those of PBG itself.

Conversely, Rimington (238) stated that when crystalline PBG was added to the urine of a normal rabbit, inhibition of the colour-reaction with Ehrlich's reagent occurred which could be overcome by the addition of an oxidising agent. He stressed that many substances had been shown to inhibit the primary colour. Prunty (234) found urea to inhibit the rate of reaction of PBG with Ehrlich's reagent. Ascorbic acid was found to have a marked inhibitory effect on this reaction, and to accelerate fading of the colour compound. Glutathione and other sulphydryl compounds have also been shown to interfere with the determination of PBG with Erlich's reagent - an inhibition which could be overcome by

the presence of heavy metals such as mercury (139).

2. PATTERNS OF PORPHYRIN EXCRETION IN THE FAECES AND BILE.

(a). Sedormid, AIA and DDC.

Goldberg and Rimington (128) reported an increase in coproporphyrin excretion in the faeces of rabbits treated with Sedormid or AIA, and a considerable increase in their faecal protoporphyrin content. The levels of the faecal porphyrins were shown to vary pari passu with those of urinary coproporphyrin.

The bile of these rabbits contained strikingly increased amounts of coproporphyrin and protoporphyrin; and uroporphyrin, which did not normally occur, was present in large quantities. They detected small amounts of PBG in the stools of treated rats.

Schmid and Schwartz (267) also reported high coproporphyrin and protoporphyrin contents, with smaller amounts of uroporphyrin in the bile and faeces of rabbits treated with Sedormid. Small amounts of PBG were present in the bile.

Haeger-Aronson (151) showed an increase in protoporphyrin, and a slight increase in coproporphyrin content of the stools of rabbits treated with DDC. Smaller increases occurred in Sedormid-treated animals. Increases in faecal protoporphyrin content preceded by 3-4 days the increases in urinary porphyrin excretion. Gajdos and Gajdos-Török(103) found copro- and protoporphyrin to be increased in the bile within 3-4 days of the administration of 2 G of AIA to rabbits. Although this

increase was considerable after 24 hours, uroporphyrin and porphobilinogen could not be detected.

(b). HCB.

Ockner and Schmid (226) noted slight increases in both the coproporphyrin and protoporphyrin faecal content of rats administered HCB for several weeks, and Burnett and Pathak (33) demonstrated an increased uroporphyrin content, a markedly increased coproporphyrin content, and no change in the protoporphyrin content of the faeces of rats treated with HCB for 7 days. The changes were not progressive over the next two weeks.

In persons with HCB-induced porphyria Cetingil and Özen (45) detected a moderate increase in both the coproporphyrin and uroporphyrin content of the faeces, while in cases reported by Cam and Nigogosyan (41) and by Dean (62) faecal porphyrin content was relatively normal.

(c). Griseofulvin.

Faecal protoporphyrin content increased strikingly, and coproporphyrin content moderately, in mice treated with griseofulvin. The change occurred early, and in a step-wise fashion to reach maximum levels within 5 days. The females of the U.C.H. strain which developed toxic features and died showed a progressive associated reduction in their faecal porphyrin content. (69).

Two fowls treated with Sedormid by Goldberg and Rimington

(128) showed an immediate rise in the coproporphyrin and protoporphyrin content of their excreta. Porphobilinogen was noted on the 4th day, and uroporphyrin on the 7th day. Uroporphyrin and coproporphyrin were of the series III isomer.

The allantoic fluid of chick embryos treated with Sedormid showed a progressive increase in coproporphyrin and uroporphyrin content over the course of an experiment, with the amounts of uroporphyrin being somewhat less than those of coproporphyrin. Porphobilinogen appeared after 4 days, was maximal on the 6th day, and had disappeared from the fluid by the 9th day. (313).

An increase in the allantoic fluid content of both uroporphyrin and ether soluble porphyrins, in equal amounts, was found in fertilised duck and chick eggs treated with AIA. (104).

3. PATTERNS OF PORPHYRIN AND PORPHYRIN PRECURSOR ACCUMULATION IN THE LIVER.

(a). Sedormid and AIA.

Porphyrins.

Goldberg and Rimington (128), and De Matteis and Prior (67) reported a moderate preponderance of protoporphyrin over coproporphyrin in the livers of rabbits treated with these drugs. A marked excess of protoporphyrin was recorded by Schmid and Schwartz (267), by Haeger-Aronsen (151) and by Lottsfeldt et al (197) in the livers of such animals. Gajdos and Gajdos-Török (104) detected an increased liver content of ether-soluble

porphyrins 18 hours after the administration of 2 G of AIA to rabbits.

Sano et al (256) found much of the protoporphyrin which accumulated in the livers of rabbits fed Sedormid or AIA to be present in the mitochondria, while little was found in the supernate, microsomes or nuclei.

Uroporphyrin was present in only 2 of 14 rabbit livers studied by Goldberg and Rimington (128). In one of these, however, it was greatly in excess of both coproporphyrin and protoporphyrin. It could not be detected by Gajdos and Gajdos-Török (103) in the livers of rabbits acutely poisoned with AIA, or by De Matteis and Prior. (67).

Schmid and Schwartz (267) showed that while only a slight increase in uroporphyrin content was apparent in the livers of rabbits treated with Sedormid, heating of these livers resulted in the conversion of much uroporphyrin precursor to uroporphyrin, which was then noted to be present in amounts only slightly smaller than was protoporphyrin.

The work of Goldberg and Rimington (128) revealed only a slight excess of protoporphyrin over coproporphyrin in the livers of rats receiving these drugs and Schmid et al (265) showed an equal increase in both protoporphyrin and coproporphyrin content. Schwartz (278) described a rapid rise in coproporphyrin content to

peak values over 2-3 days, and only a slight increase in that of protoporphyrin. De Matteis and Prior (67) on the other hand, found protoporphyrin content to increase to very much higher levels than did coproporphyrin. Another feature of their observation was the rapid return to normal levels of both these substances despite continued drug administration. Maximum porphyrin content was apparent after 5 days and after 10 days it had returned to normal levels.

Rimington and Ziegler (246) found markedly increased levels of both proto- and coproporphyrin in the livers of rats receiving AIA which were in excess of the uroporphyrin content. In Sedormid-treated rats, the increase in uroporphyrin concentration exceeded that of protoporphyrin or coproporphyrin.

Schwartz (278) found the uroporphyrin concentration to increase only after several days. Schmid et al (265) demonstrated a progressive increase in hepatic uroporphyrin content until in the later stages of their experiments this substance was present in amounts greatly in excess (tenfold) of either coproporphyrin or protoporphyrin.

Goldberg and Rimington (128) also noted the appearance of uroporphyrin after several days of drug administration, and here too, uroporphyrin was present in excess of the other porphyrins from about the 13th day of drug administration onwards.

Onisawa and Labbe (227) reported a preponderance of

protoporphyrin in the livers of mice treated with AIA. There was only a very slight increase in the uroporphyrin content of these livers.

The livers of duck embryos treated with AIA contained increased amounts of porphyrins. Although the major component was comprised of ether-soluble porphyrins, the major relative increase occurred in uroporphyrin. (104).

Schwartz and Ikeda (279) and Schwartz (278) noted the occurrence of considerable amounts of "green porphyrins" in the livers of rats and rabbits treated with Sedormid, and Haeger-Aronsen (150) also reported a 10 - 20 fold increase in "green porphyrins" in the livers of rabbits treated with this compound.

Porphyrin precursors.

Porphobilinogen was detected in 4 of the 14 rabbit livers studied by Goldberg and Rimington (128). Gajdos and Gajdos-Török (103) could not detect any PBG in the livers of rabbits acutely poisoned with a large dose of AIA, while De Matteis and Prior (67) found large amounts of PBG in the livers of such rabbits. They also found the ALA contents of these livers to be markedly increased.

Goldberg and Rimington (128) found rat livers to show a progressive increase in PBG content after 13 days of drug administration. Merchante et al (204) could detect no PBG in the

livers of rats during the first 3 days of drug administration, but found increased amounts between the 5th - 13th days. There was a marked variation in individual livers.

Rimington and Ziegler (246) noted a markedly increased PBG content in the livers of rats treated with AIA or Sedormid for 6-10 days.

No increase occurred in the livers of mice treated with AIA (227).

(b). DDC.

DDC administration was accompanied by a change in porphyrin content very similar to that occurring after AIA treatment in rats studied by De Matteis and Prior. (67). The extent of the increase, and the duration, were somewhat less with this compound, and occurred earlier. They also noted a striking increase in the hepatic protoporphyrin content of rabbits poisoned with DDC, and a marked but less striking coproporphyrin increase. PBG and ALA were also found to be present in large amounts.

Haeger-Aronsen (151) reported a 10-50 fold increase in "green porphyrins" in the livers of rabbits treated with DDC, while Solomon and Figge (303) could detect no increase in the hepatic content of "green porphyrins" of DDC-intoxicated mice. These mice showed a progressive increase in the protoporphyrin and coproporphyrin contents of their livers, with the increase in protoporphyrin being about 10 times greater than that of coproporphyrin. (302, 303). Onisawa and Labbe (227) reported a

similar pattern of change in mice treated with this compound, and also noted a fourfold increase in ALA content, while no change was detected in the amount of PBG present.

(c). HCB.

When hexachlorobenzene was administered to male Sprague-Dawley rats, a fairly marked increase in uroporphyrin content was evident after 14 days, and an even more striking relative increase occurred in the protoporphyrin and deuteroporphyrin contents of the livers. In absolute figures, uroporphyrin was greatly in excess of the latter compounds. (33). Ockner and Schmid (226) also noted a very striking increase in the uroporphyrin content of the livers of rats treated with HCB over 2-8 weeks, with a slight increase in both coproporphyrin and protoporphyrin content, while Gajdos and Gajdos-Török (104) found ether-soluble porphyrins to be in excess of uroporphyrin.

De Matteis and Rimington (66) reported the occurrence of massive quantities of a uro-type porphyrin, with only slight increases in the coproporphyrin and protoporphyrin fractions in the livers of rabbits administered HCB. Qualitative tests for PBG were negative.

Related compounds.

Rimington and Ziegler (246) noted increased liver concentrations of uro-, copro-, and protoporphyrin in rats treated with various chlorinated benzene compounds. In these animals the

increase in uroporphyrin was relatively small, usually being less than that of proto- or coproporphyrin. No increase in the PBG content of these livers was detected.

(d). Griseofulvin.

Griseofulvin administration to mice was shown to result in the accumulation of large amounts of protoporphyrin in the liver, associated with slight increases in the coproporphyrin content, and even smaller increases in the amount of uroporphyrin present. Porphobilinogen also appeared in the livers in increased amounts.(69).

An increased protoporphyrin content in the livers of mice treated with griseofulvin was also observed by Weston-Hurst and Paget. (342).

4. PATTERNS OF PORPHYRIN AND PORPHYRIN PRECURSOR ACCUMULATION IN OTHER ORGANS.

(a). Sedormid and AIA.

Schmid and Schwartz (267) noted increased levels of coproporphyrin, and slightly smaller increases of uroporphyrin and protoporphyrin content in the kidneys of rats treated with Sedormid. This was the only organ in which a coproporphyrin increase predominated. In the spleen, the brain, and the plasma, very slight increases in porphyrin content occurred, with a predominance of protoporphyrin.

Goldberg and Rimington (128) noticed a red fluorescence in

the plasma of some of the rabbits which they had treated with Sedormid or AIA, which was due to the presence of coproporphyrin III and some uroporphyrin I. They also noted an increase in the renal content of uroporphyrin which was in some instances greater than the increase in hepatic uroporphyrin content. Increases in renal coproporphyrin content were of similar magnitude to those occurring in the liver and smaller increases in both protoporphyrin and PBG were also noted. De Matteis and Prior (67) noted a moderate increase in the renal content of copro-, proto- and uroporphyrin of rabbits poisoned with AIA or DDC and also detected increased amounts of ALA and PBG in the kidneys of these animals. No change occurred in the bone marrow.

Case et al (44) noted a brilliant band of red fluorescence at the cortico-medullary junction of the kidney of rats treated with Sedormid.

Chick embryos developed a fluorescence in the mesonephric and metanephric ducts, in the calcified portion of bones, in the stomach, in the intestines and in the cloacae, after 17 days of Sedormid administration. (313).

(b). DDC.

Solomon and Figge (302) noted that after 7 days of DDC administration to mice, a line of intense red fluorescence developed midway between the renal cortex and the pelvis which was as a result of the presence of large amounts of protoporphyrin

and much smaller amounts of uroporphyrin. A pink fluorescence was evident in the serum. The nature of the responsible porphyrin was not elicited.

(c). HCB.

Burnett and Pathak (33) found HCB-administration to be associated with an increased uroporphyrin content in the skin after 14 days.

Ockner and Schmid (226) detected an intense red fluorescence in the cortex of long bones, but not in the marrow, of rats treated with HCB over long periods. A red fluorescence at the metaphyseal region of long bones and along the diaphyseal insertion of muscles, with a lack of fluorescence in the marrow, was also noted by De Matteis et al. (66). Red fluorescence in the epiphyseal line, the shaft of bones, and the marrow cavity was noted by Campbell (42) in HCB-fed rats after two weeks. Cetingil and Özen found an increased protoporphyrin level in the bone marrow and plasma of a few patients with HCB-induced porphyria, and a slight increase in the coproporphyrin content. (45).

Gajdos and Gajdos-Török (104) recorded an increased porphyrin content in the kidneys and lungs of HCB-poisoned rats.

(d). Griseofulvin.

Griseofulvin administration was associated with the appearance of moderately increased amounts of protoporphyrin and coproporphyrin in kidney and spleen, and with very striking

increases of protoporphyrin, and, less striking, of coproporphyrin, in the red blood cells of intoxicated mice, far in excess of the increases which occurred in the liver. A red fluorescence was also visible in cartilage, and less markedly in ephiphyseal bone marrow. (69).

5. CLINICAL AND PAHTOLOGICAL MANIFESTATIONS.

(a). Sedormid and AIA.

Rats.

Rats treated with AIA were noted to show some loss of appetite, to be somewhat constipated, and to lose weight. No cutaneous photosensitivity occurred. For some hours after the administration of each dose they appeared to be dazed, but there was no clinical evidence of paralysis. (128). Case et al (44), on the other hand, noticed the development of lethargy, and weakness which was most marked in the hind legs, and which progressed to paralysis and death in coma, in animals treated with Sedormid. Weight loss was also a feature.

Rabbits.

Gastro-intestinal disturbances were fairly striking in rabbits treated with these drugs. Anorexia, weight loss and constipation were noted by Goldberg and Rimington (128), and a progressively increasing gaseous distension of loops of bowel occurred in one of two rabbits studied radiologically.

Schmid and Schwartz (267) noted rabbits treated with Sedormid to show gastric dilatation on X-ray, with pyloric and upper small bowel constriction. The large bowel was distended with gas. Sudden death often occurred due to rupture of the stomach. Stich and Decker (306) noted the occurrence of abdominal spasms and paralytic ileus in such animals.

Sedormid was found to have a profound hypnotic effect, while no loss of consciousness followed AIA-administration, although a few rabbits appeared dazed for 1 - 1½ hours after each dose. No paralyses or pareses occurred but several rabbits developed tremors of their limbs, and violent convulsions which preceded their deaths. (128). Schmid and Schwartz (267) noted the development of lethargy, and a transient paralysis of the hind legs and bladder during Sedormid administration. A paralysis of the legs in such animals was also noted by Stich and Decker. (306).

Although De Matteis and Rimington (68) did not normally observe neurological symptoms in laboratory animals during Sedormid or AIA administration, when the diet was deficient in pantothenic acid, neurological symptoms followed the administration of AIA.

Pathology. (Rabbits, Rats, Mice).

Schwartz (278) noted an olive-green discolouration of the livers of rabbits treated with Sedormid for a few days, and

hepatic enlargement in rabbits treated with AIA was noted by Tschudy et al. (321). Goldberg and Rimington (128) described areas of necrosis in the livers of rabbits treated with either Sedormid or AIA.

Fenton (95) did not detect histological evidence of myelin degeneration in the peripheral or central nervous systems of rats treated with AIA.

The livers of mice treated with AIA were somewhat enlarged, and fragile, and had a greenish-grey discolouration.(227).

Chick embryos and Fowls.

Chick embryos treated with Sedormid were significantly smaller than controls from the 5th to 9th days of treatment, and a definite retardation of growth occurred for at least 30days after hatching. (313). This retardation of weight gain was also noted in chick embryos treated with AIA. (104).

After 15 days of embryonic life gross external abnormalities appeared in embryos treated with Sedormid - which were reminiscent of those occurring in embryos developing in eggs from riboflavin-deficient hens. (313).

Chicks were unable to leave the shells unassisted. Neurological involvement then manifested in the form of tremors of the wings and an inability to maintain an equilibrium.(313).

The livers varied in colour from olive to dark green. White necrotic areas were apparent. Marked friability was noted. (313).

Fowls treated with AIA showed a loss of weight and became weak and lethargic. (128).

(b). DDC.

Haeger-Aronsen (150) noted the onset of anorexia, and weakness in rabbits irregularly treated with DDC over 3 weeks. Signs of paralysis did not occur.

DDC-administration in mice resulted in a matting and discolouration of the hair after 4-6 days. Loss of weight and abdominal distension also occurred, but no neurological symptoms developed. (227). The livers of these animals were larger than those found following AIA administration. They were very firm to palpation and were dark red in colour.

Guinea pigs acutely poisoned with DDC lost 50-75 G body weight in 48 hours. When less acutely poisoned, the adrenals became enlarged, and cirrhosis of the liver developed subsequently.

Liver mitochondria from such animals were shown to be increased in diameter, and the area of the cristae was also increased. (142).

(c). HCB.

Rats.

Ockner and Schmid (226) noted a high mortality amongst 33 rats treated with HCB. 13 died within one month with tremor, ataxia, weakness and paralysis, without a major disturbance in porphyrin metabolism having occurred. A high mortality was also observed by Gajdos and Gajdos-Torok (104), who described the

occurrence of paresses and generalised tremors in such rats.

Terminal neurological disturbances occurred in rats treated with HCB by De Matteis et al.(66). Baby rats were particularly susceptible to the nervous effects of this poison. The young of HCB-poisoned mothers died with convulsions within 7 - 8 days of birth, and foster rats died after 3 - 4 days. Trembling with being handled (33, 42) and the assumption of a humped posture (33) were other features commented upon during HCB-administration. Campbell (42) did not detect the development of paralyses in rats treated with HCB over 13 weeks.

Burnett and Pathak (33) found rats treated with HCB to be smaller and more docile than control animals, and retardation of normal weight gain in such animals was also reported by Gajdos and Gajdos-Török(104) and by Campbell (42), who commented as well on the absence of significant skin lesions. Burnett and Pathak (33) found that while cutaneous photosensitivity was not a feature, depilation caused cutaneous injury which did not occur in control animals. De Matteis et al (66) found quantitative tests for photosensitivity to be entirely negative, but noted the occurrence of cutaneous lesions in the form of depilated sores with haemorrhagic crusts - often symmetrically placed near the shoulders. They also noted signs of pruritus. Gajdos and Gajdos-Törok (104) reported the development of a cutaneous syndrome comprising poor quality and brownish discolouration of the hair, and the presence of cutaneous eruptions

on the head, feet and back.

Rabbits.

HCB-administration to rabbits also resulted in anorexia, weight loss and constipation. Death occurred after 8 - 12 weeks, and during the last week or so neurological symptoms developed in the form of tremors and paroses. (66).

Pathology.

Hepatomegaly was common; liver cell-degeneration occurred around the central veins; and the size and number of Kupffer cells were increased in rats treated with HCB.(226). Campbell (42) also noted a hepatomegaly in HCB-fed animals, maximum after 5 - 9 weeks of drug-administration. (The livers of animals treated for 13 weeks were often normal, or smaller than normal in size). The livers of these animals were noted to develop a chocolate brown colour. Histology revealed the presence of greatly enlarged liver cells towards the centre of the lobule, while more peripherally atrophy had occurred. Single cell necrosis, focal necrosis and hydropic change were evident. Fatty change and areas of necrosis were described by Gajdos and Gajdos-Török. (104).

Campbell (42) commented upon the appearance of iron granules in parenchymal cells after the 5th week, and upon the absence of wear and tear pigment and cirrhosis.

No demyelination or other histological lesion was observed in the central nervous system. (42).

De Matteis et al (66) recorded the presence of fatty change and patchy necrosis in the livers of rabbits treated with HCB.

Mice and guinea pigs.

Marked neurological symptoms followed 8-10 days of HCB-administration to mice, in the nature of fine tremors involving the whole body inclusive of head and tail, and clonic contractions involving mainly the hind legs (66). Guinea pigs treated with HCB developed a continuous coarse tremor of the extremities with occasional clonic convulsions. Weakness, more pronounced along one side of the body, was a feature. (128).

Human cases.

In persons with HCB-induced porphyria photosensitivity was a marked feature, and skin lesions were prominent in the form of bullous eruptions, with secondary pyodermic infection, ulceration, and extensive scarring, hyperpigmentation, hypertrichosis and alopecia. (41, 45, 48, 62, 263, 264).

Hepatomegaly was common, as wasweight loss.

Neurological and abdominal symptoms were infrequent. (62,263).

(d). Griseofulvin.

Female mice of the U.C.H. strain lost weight, became progressively weaker and died after several weeks of receiving griseofulvin. (69).

The livers of mice treated with this drug were enlarged (7, 69, 342), and acquired a progressive greenish-brown discolouration.

In the later stages hepatomata were common. (342). Microscopically there was extensive cellular necrosis, with round cell infiltration, pigment accumulation in liver cells and Kupffer cells, and bile duct proliferation with bile thrombi in the biliary canaliculi.

6. OTHER BIOCHEMICAL STUDIES.

(a). Haem Proteins.

Because of the high hepatic porphobilinogen and porphyrin concentration in Sedormid-poisoned animals, Schmid *et al* (269) investigated the activity or concentration of some of the haem proteins in the liver - and other organs - of such animals.

Catalase activity was measured in the livers of rabbits given Sedormid orally, and a decrease was noted after 2-3 days, which upon further treatment was progressive to the point where the activity of this enzyme was reduced to less than 5% the normal level.

This effect on liver catalase activity was subsequently repeatedly confirmed - in rabbits treated with Sedormid (103, 151, 265), AIA (320, 321), or DDC (151). Rats treated with Sedormid (246, 265, 268, 269), AIA (67, 246) or DDC (67, 116) also showed a marked reduction in their liver catalase activity, as did mice treated with AIA or DDC (227) or griseofulvin (69), and chick embryos (313) and geese (277) treated with Sedormid.

Ockner and Schmid (226) could not detect a significant reduction in the hepatic catalase activity of rats treated for several weeks with hexachlorobenzene, and no significant effects on the levels of activity of this enzyme were noted in rats treated with various chlorinated benzenes. (246).

An analysis of Haeger-Aronsen's (151) investigations of hepatic catalase activity reveals much variation in the levels of activity which obtained at different intervals after drug administration. This may merely reflect the irregular dosage schema which she employed.

De Matteis and Prior (67) found hepatic catalase activity to return to normal after about 2 weeks despite continued drug administration.

In vivo studies with glycine-2-C^{14} suggested that Sedormid interfered with the formation of catalase. 48 hours after the injection of labelled glycine into rats, no radioactivity could be detected in the prosthetic haem group of the liver catalase of Sedormid-poisoned animals, while significant activity was present in that from control groups. (265, 268).

Catalase activity in the rat kidney was shown to decrease slightly during the first few days of Sedormid feeding, but thereafter the pattern was no longer consistent or progressive, although an overall slight reduction in activity was apparent. (265, 268). Tschudy et al (321), conversely, could detect no

alteration in renal catalase activity in rabbits treated with AIA. Erythrocyte catalase activity was unchanged in Sedormid-treated rats (265, 268) and in griseofulvin-treated mice.(69).

Gray (144) did not detect any change in the hepatic catalase activity of a patient with acute intermittent porphyria. Findlay (98) found a reduction to 10% of control values in the catalase activity of the bullous epidermis of the sun-exposed dorsal surfaces of the forearms of patients with porphyria cutanea tarda. In the normal skin of these patients (back and flexor surfaces of forearms) the catalase levels were normal. Tissue culture cells grown in the presence of AIA showed a 26% reduction in their catalase activity.(57).

Cytochrome C, cytochrome oxidase, and succinic dehydrogenase.

Cytochrome C concentration in rat livers was shown to be reduced (265, 268) during Sedormid administration although it was felt that the effect while apparently significant might have been related to excessive weight loss. Where weight loss was not marked, cytochrome C concentration did not differ greatly from that in control animals.

Vannotti (324) found cytochrome C concentrations in the livers of Sedormid-treated rabbits to be significantly decreased after 3 weeks. He also found daily intravenous protoporphyrin injections into rabbits for 10-15 days to result in an increased cytochrome C

concentration in the liver.

No significant change occurred in the activities of hepatic cytochrome oxidase or succinic dehydrogenase in Sedormid-treated rats. (265).

Haemoglobin.

Haemoglobin levels have frequently been shown to be unaffected in the "hepatic forms" of the drug-induced porphyrias. (66, 67, 128, 265).

(b). Enzymes of the Porphyrin Biosynthetic Pathway.

ALA synthetase.

ALA synthetase activity was shown by Granick (135) and by Granick and Ureta (141, 142) to be increased in the liver mitochondria of guinea pigs acutely poisoned with DDC, while such activity was NOT detectable in the livers of less acutely poisoned or normal guinea pigs. An increased ALA synthetase activity in the liver mitochondria of rats "subacutely" poisoned with DDC was demonstrated by Ginsburg and Dowdle. (116). Miyakoshi and Kikuchi (208) found ALA synthetase activity to be markedly increased in the liver mitochondria of rats poisoned with AIA.

Granick (136, 137) demonstrated that an increase in the activity of this enzyme could be induced in liver parenchymal cells in vitro by a number of chemicals capable of inducing acute porphyria in animals. AIA was most effective, and DDC and

griseofulvin less so. Chloretone and aminopyrine were also found to be effective in this respect.

ALA dehydrase.

ALA dehydrase activity was shown to be increased in the livers of rabbits rendered porphyric with Sedormid or AIA (110, 115, 320, 321), while in rats treated with AIA, Tschudy et al (321) found the activity of this enzyme to be decreased when the results were expressed per unit weight of liver, although the total hepatic activity was greater than that of the control animal livers. Rimington and Ziegler (246) noted an increased level of ALA dehydrase activity in the livers of rats treated with various chlorinated benzene compounds.

Onisawa and Labbe (227) found little change in the hepatic activity of ALA dehydrase in mice treated with AIA, while in mice treated with DDC they found a REDUCTION in enzyme activity to 13% the normal level.

Granick and Urata (142) detected no change in ALA dehydrase activity in the livers of guinea pigs treated with DDC, and De Matteis and Rimington (69) presented evidence suggestive of increased ALA dehydrase activity in liver homogenates from griseofulvin-treated mice.

Levels of activity of this enzyme in the kidney have also been shown to be increased in animals treated with AIA or Sedormid. (110, 115, 321).

Treatment with phenylhydrazine resulted in anaemia, and in an increased activity of ALA dehydrase in blood and spleen; whereas the activity in bone-marrow spleen, blood, brain, heart muscle and small intestine was normal in Sedormid-treated rabbits. (110, 115).

The effects of lead on ALA dehydrase activity have been reviewed by Chisolm (49), who quoted evidence in favour of its having an inhibitory influence.

Scott (282) reported on 2 cases of acute intermittend porphyria who showed an increased rate of conversion of ALA to PBG, and he postulated that this might reflect an increased hepatic ALA dehydrase activity in this condition.

Ferro:protoporphyrin chelatase.

Ferro:protoporphyrin chelatase activity was shown to be greatly reduced in the livers of DDC-treated mice.(227).

(c). Purine Metabolism.

A reduced excretion of uric acid into the allantoic fluid of chick embryos treated with Sedormid or AIA has been demonstrated. (182, 183, 315, 317). That this was not due to impaired oxidative catabolism of purines was shown by the demonstration of normal oxidation and excretion of exogenous adenine. (183, 315, 317). Also, incorporation of glycine-2-C^{14} into purines by these embryos was shown to be reduced (183, 315) suggesting probable inhibition of purine synthesis.

De Matteis and Prior (67), however, did not detect any alteration in the urinary allantoin excretion of rats treated with either Sedormid, AIA, or DDC.

Labbe et al (182) demonstrated a reciprocal relationship between the amounts of uric acid and porphyrin excreted into the allantoic fluid of porphyric chick embryos. It was also shown that the addition of exogenous adenine or adenosine resulted in an increased uric acid excretion, a reduced porphyrin excretion, and a marked improvement in the associated physical signs. (317). Xanthine and guanine were without similar effect.

In DDC- and HCB-intoxicated rats, De Matteis et al (70) could detect no significant change in the liver nucleotide content as compared with normal control animals. AIA and also 2propyl-2isopropylacetamide (a substance that has been reported not to induce porphyria) administration, resulted in a decrease in liver ATP with an increase in liver AMP concentrations - with the result that the ATP : AMP ratio fell, although the total nucleotide content remained within normal limits. Similar changes were noticed in the guanine nucleotides, but uridine nucleotide patterns were unchanged. AIA was found to decrease the ATP concentration of both HeLa cells and "L" cells in tissue culture-preparations. (57).

Merchante et al (204) found no significant alteration in

the concentration of ribo- or desoxyribonucleic acids (RNA,DNA) in the livers of Sedormid-treated rats. Cowger et al (57) found the RNA phosphorus content of tissue culture cells grown in the presence of AIA to be markedly increased. There was only a 4% increase in the DNA phosphorus content of these cells, but this was consistent and reproducible.

Rimington and Ziegler (246) noted a slight decrease in the overall pyridine nucleotide content of the livers of rats treated with various chlorinated benzenes, and an increase in the NAD:$NADH_2$ ratio. The NADP:$NADPH_2$ ratio was relatively unchanged in the livers of these animals, but was increased in the livers of animals poisoned with AIA or Sedormid. The NAD:$NADH_2$ ratio in such animals was if anything, lowered.

Lottsfeldt et al(197) showed that when rabbits were treated with both AIA and inosine, uroporphyrin and coproporphyrin accumulation in the liver did not occur (as did occur with treatment with AIA alone) and the accumulation of protoporphyrin was to only 30% the levels that obtained in the absence of added inosine.

Gajdos and Gajdos-Török (104, 105) demonstrated an inhibitory effect of AMP and inosine on the increased biosynthesis of porphyrins in the drug-induced porphyrias in the rat, rabbit and chick embryo, and demonstrated a beneficial effect of these two compounds on the signs of the disease. They (106) also

show an inhibitory effect of purine nucleotides and nucleosides, but not of the corresponding pyrimidine derivatives, on porphyrin synthesis *in vitro* by Rhodopseudomonas spheroides; and an actual inhibition of ALA synthesis.

AMP administration to humans in attacks of acute porphyria was shown to be beneficial in 8 of 10 cases where it was employed.(104).

De Matteis and Rimington (69) were unable to demonstrate a beneficial effect of AMP upon griseofulvin-induced porphyria in mice.

(d). Protein and Amino Acid Metabolism.

Welland *et al* (340) demonstrated that in patients with acute intermittent porphyria the feeding of a diet deficient in protein resulted in porphobilinogen excretion being increased despite the maintenance of the caloric intake with added fat.

Richards and Scott (237) demonstrated a substantially increased output of ALA and PBG in the urine of 7 of 9 patients with acute porphyria after the ingestion of a large dose of glycine, while the rate of excretion in normal subjects remained within normal limits. Glycine-administration was also shown to facilitate the induction of porphyria by Sedormid in rats. (1). Histidine and beta-alanine were without similar effect.

Richards and Scott (237) also demonstrated an impaired con-

version of glycine to serine in 3 patients with "English" porphyria, while in 3 patients with Swedish genetic porphyria the increase in serum serine concentration following oral glycine-administration was normal, or greater than normal, and its subsequent fall delayed.

Impaired incorporation of glycine-2-C^{14} into purines by chick embryos treated with Sedormid or AIA has been demonstrated (183, 317) but Dowdle (74) could detect no defect in the incorporation of glycine-2-C^{14} into uric acid by 3 porphyric subjects.

The *in vitro* oxidation of the alpha carbon atom of glycine to CO_2 has been shown to be impaired in the livers of rabbits treated with AIA (320,321) and of rats treated with HCB (75) or DDC(116). Dowdle (74) could not detect any defect in the conversion of glycine-2-C^{14} to CO_2, either, in 3 patients with porphyria.

Goldberg and Rimington (128) showed that while a glycine spot could be detected on chromatography of normal rabbit urine, this spot showed at least a fourfold reduction in intensity in the urine from 5 rabbits severely affected by Sedormid or AIA. They also demonstrated that whereas only a glycine spot could be detected from normal rabbit urine, 3 rabbits treated with Sedormid or AIA voided several other amino acids as well.

Changes in the pattern of amino acid excretion could not be detected in the urine of porphyric rats.

Gray et al (145) noted an increased incorporation of C^{14} from labelled acetate or succinate into glutamic acid by the livers of rabbits previously treated with AIA, and also an increased incorporation into alanine when labelled succinate was used.

Elevated blood ammonia levels were shown to be a constant finding in HCB-induced porphyria in guinea pigs and mice. (66).

(e). Carbohydrate Metabolism in Porphyria.

Tschudy's group (249, 340) found increased dietary carbohydrates to diminish the excretion of urinary PBG in both experimental and human porphyria, and De Matteis (65) found glucose administration to repress the development of porphyria in rats treated with AIA. Welland et al (340) cited unpublished work of their group demonstrating an inhibitory effect of oral sucrose upon hepatic ALA synthetase activity which was found to be markedly increased in starved rats given AIA.

Joubert et al (169) found a decreased rate of clearance of exogenous pyruvate from the blood of porphyric Bantu patients as compared with normal controls. Goldberg and Rimington (129) found the pyruvate tolerance test to be frankly abnormal in 1 of 2 patients with acute intermittent porphyria in whom this test

was performed. Blood pyruvate levels were greatly in excess of normal 90 minutes after the ingestion of glucose. Hierons (161) also found an abnormal pyruvate tolerance test in two such patients.

Tschudy et al (320, 321) could detect no alteration in the capacity of liver tissue from porphyric animals to oxidise the 1 and 6 carbon atoms of glucose, and the alpha carbon of pyruvate to CO_2. Lactic dehydrogenase activity in these livers was found to be reduced.

Cowger et al (57), working with several strains of tissue culture cells, found that AIA resulted in a markedly increased excretion of lactate, and a smaller increased pyruvate excretion by these cells. AIA increased their rate of glucose utilisation, and a stoichiometric relationship existed between the excess glucose utilised and the excess lactate produced. They also cited unpublished data indicative of an increased lactic acid concentration of whole chick embryos poisoned with AIA.

Parke and Williams (229) noted an increased excretion of glucuronic acid in the urine of HCB-treated rabbits (signs of porphyria were not sought or commented upon), and De Matteis et al (66) found a prompt and sustained rise in glucuronic acid excretion in the urine of rats receiving HCB. A fall in glucuronic acid excretion was noted in the urine of rabbits receiving this drug.

Granick (136) found that drugs known to be capable of inducing an increased urinary ascorbic acid excretion in animals (chloretone and aminopyrine) caused an increased porphyrin synthesis by chick embryo liver cells cultured in vitro. A pronounced increase in urinary ascorbic acid excretion by rats rendered porphyric with DDC, was demonstrated by Ginsburg and Dowdle. (116).

De Matteis (65) subsequently demonstrated an increased ascorbic acid synthesis in rats as a result of the administration of several drugs known to cause porphyria.

(f). Fat Metabolism in Porphyria.

Schwartz (278) demonstrated a progressive increase in phospholipid and total lipid content of the livers of rats treated with Sedormid. Labbe et al (180) also demonstrated a total lipid increase in porphyric rat livers (AIA). They showed that the incorporation of acetate into fatty acids was more than doubled; and the rate of incorporation of succinate into fatty acids was also felt to indicate a greater fatty acid synthesis in the porphyric rat liver.

(g). Iron Metabolism.

Serum iron levels have been shown to be raised in symptomatic porphyria in the Bantu (172, 184, 185, 259), in coloured subjects (323) and in whites. (op cit 179).

Increased levels have also been detected in subjects with variegate porphyria during the acute attack (25), and in patients with acute intermittent porphyria. (op cit 179). An excess of iron in both the Kupffer cells and the parenchymal cells of the liver has been described in these conditions in a high percentage of cases. (184, 185, 323).

An increased rate of absorption of orally administered iron by subjects with symptomatic porphyria was suggested by Saunders (259), although it has also been suggested that these subjects ingest excessive amounts of iron in the alcoholic beverages to which they are prone. (179, 259).

Vannotti (324) demonstrated a "very real increase" of iron fixation in the bone marrow and spleen of rabbits rendered porphyric with Sedormid, and Lottsfeldt et al (197) demonstrated an increased uptake of Fe^{59} by the livers of such rabbits.

Livers of mice treated with griseofulvin were also shown to have an increased uptake of Fe^{59} - in vivo - (69), as did livers from mice treated with AIA. (227). Conversely, when mice were treated with DDC there was an 81% reduction in the ability of their livers to take up iron. (227).

An increased incorporation of iron into liver haem was shown in livers from AIA-treated mice (227), from Sedormid-treated rabbits (197), from AIA-treated rats (180) and from griseofulvin-treated mice. (69). When mice were treated with DDC there was a

marked reduction in the ability of their livers to incorporate iron into haem. (227).

Cowger et al (57) found that cells grown in tissue culture in the presence of AIA showed a 63% greater uptake of Fe^{59} than did control preparations. When these cells were grown in the presence of AIA, there was a 14% increase in their iron content.

Theron et al (307) showed that rats fed an iron-enriched maize-meal diet had a high content of iron in the liver parenchymal cells after 16 days, which later affected the Kupffer cells also. In the livers of these animals there was a reduced activity of isocitric dehydrogenase, malic dehydrogenase and L-glutamate dehydrogenase, and of DPNH diaphorase, while DPNH-cytochrome C reductase and DPNH oxidase activities were considerably increased.

Dowdle and Ginsburg (75a) fed weanling rats a similar diet but were unable to detect any increase in the porphyrin content of their livers.

(h). Miscellaneous data.

Rimington and Ziegler (246) showed that the induction of porphyria by chlorinated benzenes was prevented by the administration of glutathione, and Owens (228) cited the unpublished work of De Matteis showing that glutathione was not effective in reversing the effects of an established intoxication with HCB.

Owens himself (228) demonstrated a retardation in the rate of development of porphyria in two strains of mice treated with glutathione and receiving griseofulvin in the diet. Porphyrin precursor excretion increased less quickly, but eventually reached the same levels as those in the urine of control animals not receiving glutathione. No reduction in the initial excretion of protoporphyrin in the faeces was observed, but glutathione lessened the amounts excreted after 100 hours.

Rimington and Ziegler (246) could not detect any change in the hepatic glutathione concentration of rats receiving Sedormid, AIA or HCB. Trichlorobenzene resulted in a definite fall in hepatic glutathione concentration.

Burnett and Pathak (33) found the levels or uroporphyrin to be markedly increased in the urine of some HCB-intoxicated rats on the day following a 20 minute ether anaesthetic. They also found a period of exposure to radiant energy to result in an increased excretion of coproporphyrin by HCB-poisoned rats, and also of uroporphyrin by some of the animals. An increased uro- and coproporphyrin excretion in the faeces was noted in 3 of 4 such rats, and of protoporphyrin in 1 of these animals. The uroporphyrin content of the skin and liver of such HCB-poisoned rats was found to decrease following this exposure.

Runge and Watson (251) could demonstrate porphyrins in

the skin of the hands of only 3 of 6 porphyric patients. In skin from areas not usually exposed to sunlight, porphyrins were demonstrable in all 6.

Hellman et al (160) detected an elevation of the protein-bound iodine in the serum of patients in various stages of acute intermittent porphyria. Levels wereusually highest during the active phase of the disease. In 1939 Sumegi and Putnoky (op cit 332) had reported intense thyroid hyperfunction in rats rendered porphyric with various heavy metals or injected with porphyrins.

A disturbance of zinc metabolism in the acute phase of acute intermittent porphyria has been demonstrated in the form of an increased urinary excretion of zinc and zinc-porphyrin complexes, an increased zinc content of erythrocytes, and an increased activity of the zinc-containing enzyme carbonic anhydrase. (op cit 170). A derangement of tryptophan metabolism under these circumstances has also been demonstrated.

Cowger et al (56) detected an inhibitory effect of various compounds on beef heart DPNH oxidase activity. They were struck by the structural similarity of these compounds and compounds able to induce porphyria. They investigated the influence of these compounds on the activity of this enzyme in cultured cells, and found AIA, and also amytal and seconal to be inhibitory.

None of these compounds inhibited succinate oxidase activity.

These compounds were also found to inhibit oxygen utilisation by the cells respiring on endogenous substrate.

SECTION 4.

MATERIALS and METHODS.

Wistar albino rats, of both sexes, were used throughout the investigations.

They were treated with a number of compounds known to be, or suspected of being, capable of inducing porphyria.

The following compounds were administered in the manners, and to the groups, indicated:-

(1). Dicarbethoxy-dihydrocollidine (DDC). This was prepared according to the method described by De Matteis and Prior (67) and given to female rats, (i), as a 12½% w/v emulsion in 1% Edifas (I.C.I.) by twice daily (8 a.m. and 4 p.m.) intragastric administration, in a dose of 250 mg/Kg. body weight, and (ii), as a 0.2% mixture in the diet.

(2). Allylisopropylacetamide (AIA), which was received as a gift from Roche products, was given to male rats as a 12½% w/v solution in propyleneglycol by twice daily intragastric administration in a dose of 250 mg/Kg body weight.

(3). Hexachlorobenzene (HCB). This compound was bought from British Drug Houses (B.D.H.) and given to female rats (i), as a 0.2% mixture in the diet and (ii), by twice daily intragastric

administration as a 12½% emulsion in 1% Edifas in a dose of 250 mg/Kg body weight.

(4). Chloretone (Chlorbutanol). This compound was received as a gift from the Groote Schuur Hospital dispensary and was given to rats of both sexes as a 0.5% mixture in the diet.

The animals were bred in the animal house of the University of Cape Town Medical School, and were transferred to an animal room adjacent to the laboratories as required. They were housed in iron and zinc cages with steel bottoms. The temperature and humidity were maintained at constant levels.

The rats were fed a diet of rat-biscuit cubes (Vereeniging Consolidated Mills) and tap water ad lib. When the drugs were to be administered in the diet, the cubes were crushed and the drug admixed in the proportions indicated.

Animals from some groups were normally fed until their removal to the laboratory just prior to being killed, while those from other groups were placed in metabolism cages at 4 p.m. on the day before their being killed. These animals were starved, but allowed free access to water. Their urine was collected over an 18-hour period, and they were then transferred to the laboratory and killed.

Control animals were matched for sex, weight, dietary status

and season. The importance of these factors is discussed in Section 5.

They were housed in the same animal room, but in separate cages, and received the same diet as the test animals.

The parameters which were studied are summarised in Figure 1.

The animals were killed by guillotine, and their livers immediately removed, washed in the appropriate ice-cold homogenising solution, placed in a Petri dish surrounded by cracked ice, and cubed.

In order to investigate the levels of activity of hepatic pyruvate kinase, G-6-P.D., I.C.D., G-O.T., lactic dehydrogenase, and catalase, the livers were homogenised in ice-cold 0.1 M Tris buffer (2-amino-2-(Hydroxy-methyl)-1:3 Propanediol), buffered to pH 7.4 at room temperature in 1:5 w/v ratio. Homogenisation was obtained by means of a power driven Teflon homogeniser (Gallenkamp) followed by the use of a Potter-Elvehjiem glass homogeniser. The homogenates were kept in cracked ice whenever possible.

Aliquots were removed for determinations of catalase activity, and total phosphorus concentration, which was measured according to the method of Fiske and Subba Rau. (100).

Enzymes of intermediary metabolism.

The homogenates were centrifuged in a Heka model centrifuge, set to position 3 for 10 minutes. The supernates were then transferred to tubes where they were suitably diluted with cold 0.1 M Tris buffer (pH 7.4).

Dilutions of 1:1 with this buffer were used for the measurements of the activities of pyruvate kinase, and G-6-P.D. For determinations of the levels of activity of G-O.T. and ICD a 1:3 dilution was used, and of lactic dehydrogenase a 1:79 dilution.

All these enzyme activities were studied by following the change in optical density at 340 mu of a DPN:DPNH system in coupled reactions.

The methods are based on those described in "Methods in Enzymology" (51), as modified by Metcoff (205). G-6-P.D. activity was measured according to the method of Marks (200a). The composition of the reagent mixtures is detailed in the appendix. 1(a).

After an equilibration period of 3 minutes the reactions were initiated by the addition of homogenate to the reagent mixtures in quartz microcuvettes maintained at 37°C in a Beckman model DU Spectrophotometer equipped with a circulating water bath.

The percentage light transmitted was automatically recorded every 2.4 seconds by a YSI model 80 laboratory recorder. (The recorder was built to respond every 2 seconds to an

alternating current of 60 cycles per second. South African current alternates at 50 cycles per second).

The rate of change of optical density was calculated, and enzyme activity is expressed as the number of μmoles of substrate converted per minute per mg. total tissue phosphorus. Stoichiometry of the reactions was assumed as was an extinction coefficient for DPNH of 6.22×10^6 for a solution of 1 mole in 1 ml in a light path of 1 cm at 340 mu.

Catalase.

Catalase activity was determined by a modification of the method of Sumner and Dounce. (308). Details are described in Appendix 1 (b). In principle, the rate of destruction of hydrogen peroxide after the addition of homogenate was measured.

Activity is expressed in arbitrary units, 1 unit being that amount of enzyme which, under the experimental conditions, would give a first order velocity constant of 1.

ALA dehydrase.

For measurement of ALA dehydrase activity 1 ml of a 1:5 w/v homogenate of liver in 0.25 M sucrose (pH 7.4) was added to 0.5 ml of potassium phosphate buffer (0.11 M, pH 6.8) and 0.2 ml of 0.6 M reduced glutathione. After 30 minutes of pre-incubation in a water bath at 37°C, 0.2 ml of 0.02 M ALA were added and the contents of the tubes were incubated for a further

60 minutes at 37°C in air. The reaction was stopped by the addition of 2 ml of 0.3 N trichloro-acetic acid (TCA), and the PBG concentration in the protein-free supernatant was determined by the method of Mauzerall and Granick. (202). The PBG concentrations of the liver homogenates were measured in control tubes to which ALA was not added, and whose volume was completed by additional phosphate buffer.

PBG concentration is expressed as mμmoles/mg total tissue phosphorus, and ALA dehydrase activity as the number of mumoles of PBG formed per hour per mg total tissue phosphorus.

ALA and AA synthetase.

Liver was homogenised in 10 volumes of 0.25 M sucrose (pH 7.4) in the cold, and mitochondria were isolated by refrigerated centrifugation techniques. (Details are described in the Appendix 1 (c).

Mitochondria from approximately 0.75 g of liver were incubated in Warburg flasks containing 250 μmoles of sucrose, 86 μmoles of glycine, 0.4 μmoles of EDTA, 0.8 μmoles of $MgCl_2$, 1.7 μmoles of each of pyridoxal phosphate, DPN, and ammonium chloride, 47.4 μmoles of potassium phosphate (pH 7.4) and either 172 μmoles of sodium pyruvate, or 86 μmoles of sodium citrate, in a final volume of 2cc.

The flasks were incubated for exactly 2 hours in air at 37°C, after an initial period of 8 minutes allowed for equilibration purposes. Oxygen consumption was recorded

manometrically over the first 20 minutes of the 2-hour incubation period, carbon dioxide being absorbed by one drop of 15% KOH on a square of Wattman No. 3 filter paper in the centre well of the flasks.

The reaction was stopped by the addition of 2 ml of 0.3 N TCA, and the aminoacetone and delta-aminolaevulic acid contents of the protein-free supernatants were determined according to the methods of Urata and Granick (322) - by separation on ion exchange columns, conversion to pyrroles by boiling with acetylacetone, and measurement of the optical density of the colour compound formed on addition of Ehrlich's mercury reagent.

Aliquots of the final mitochondrial suspension were also taken for determination of total nitrogen content. The results are expressed as the number of mumoles of ALA (or AA) formed in the 2-hour incubation period, per mg mitochondrial N, or as the number of uliters of oxygen consumed per hour per mg mitochondrial N.

Urata and Granick (322) had reported a molar extinction coefficient for the compound formed between ALA pyrrole and Ehrlich's mercury reagent of 5.3×10^4 at 552 mu. I adopted an essentially similar technique for its measurement, and found it to be markedly different. It was, therefore, considered important to establish the molar extinction coefficient of ALA in our laboratories; to investigate possible reasons for the difference; and to investigate the influence of various

environmental factors upon the measurement of this substance. The details of these investigations are shown in the Appendix 1(d).

The molar extinction coefficient as measured was 2.87 $x10^4$, and this is the figure which has been used in all measurements of ALA concentration.

ALA pyrrole was shown to be fully formed within 10 minutes of boiling ALA with acetylacetone at buffered pH 4.8.

The colour-compound formed between an aqueous solution of ALA pyrrole and Ehrlich's mercury reagent was found to be relatively stable over 15 minutes and then to lessen gradually in intensity over a 2-hour period. When the ALA pyrrole was dissolved in a methanol:glacial acetic acid solution, the intensity of the colour of the compound formed with Ehrlich's mercury reagent was less (65%) than was that formed by ALA pyrrole in aqueous solution. Moreover, whereas the intensity of the aqueous solution colour-compound lessened on standing, that of the methanol:glacial acetic acid solution increased steadily with standing, the maximum increase occurring in the first 10 minutes after the addition of the Ehrlich's reagent. In the experimental studies, the optical density of the colour-compound was always determined exactly 15 minutes after the addition of the Ehrlich's reagent.

The intensity of the colour-compound was less when freshly prepared Ehrlich's mercury reagent was used than when a month-

old preparation was used. In all the present experiments, Ehrlich's mercury reagent was never more than one week old, and was kept in a bottle, surrounded by aluminium foil to protect it from the light.

ALA pyrrole was found not to be completely eluted from the Dowex-1-acetate column, and the optical density of equivalent amounts of ALA pyrrole before and after passage through the column differed by about 28%.

Washing with water, or with 1 N acetic acid did not result in the loss of appreciable amounts of ALA pyrrole from the column.

The optical density of the colour-compound was shown to be proportional to the amount of ALA pyrrole present whether or not it was passed through a Dowex-1-acetate column.

Glycine oxidation.

Glycine oxidation by homogenates of liver was studied with C^{14} labelled glycine by the method of Dowdle and Eales. (75).

These results are expressed in terms of micro-atoms of glycine converted to CO_2 per mg tissue phosphorus, per hour.

Urinary ascorbic acid.

Urinary ascorbic acid content was measured by the method of Roe and Kuether. (247). The urine was collected over an 18-hour period, and the results have been extrapolated so as to be expressed as the number of mgs. of ascorbic acid excreted in

the urine per 24 hours.

Liver porphyrins.

Liver protoporphyrin and total porphyrin concentrations were measured according to the unpublished methods of Sweeney and Eales. These are described in detail in the Appendix 1 (e).

Total nitrogen content was measured by the method of Kanchukh. (170a). This was preferred (as a reference base) to total protein content because the protein was found to be unstable if kept too long.

Total tissue phosphorus as reference base.

A very significant article on the subject of reference bases is one by Weber and Cantero (337), who emphasised that results expressed on a wet weight basis do not reflect the total enzyme activity of the whole organ, and that activities which are apparently increased when expressed on such a basis may actually be decreased when considered per total weight of organ, should the organ have undergone a reduction in weight. The reverse would also apply.

In the present investigations total organ weights were not used because of the delay that would have been entailed in weighing the liver, and because it was also planned to investigate activities in human livers.

The choice of a reference base in these studies was

directed towards enabling the influence of metabolically inert substances on the apparent concentration of parenchymal cell enzyme activity to be reduced, and towards permitting of an accurate assessment of the actual amount of tissue homogenised.

It was felt that wet weight or dry weight references, both obvious choices, would be inadequate. These are satisfactory for tissues in which the ratio of active tissue to storage material remains reasonably constant. (40). However, in liver, variations in glycogen, in fat, and, particularly in these experiments, in porphyrin, and perhaps also in iron, content were to be expected - all factors which might be expected to alter this ratio. Furthermore, it was anticipated that necrotic and fibrous tissue might spuriously lower the apparent enzyme activity as expressed on a weight basis.

It was felt, too, that errors might arise during the preparation of the homogenate - especially as far as the completeness of the process was concerned. Biopsy specimens from patients with cirrhotic livers homogenised with difficulty and never completely, and a wet weight basis would, therefore, again have proved inadequate - but has been employed initially because of difficulties in establishing a micro method for total phosphorus determinations.

In 1946, Hoagland, (op cit 193) wrote:- "The problem of

adequate bases of reference for constituents of diseased tissue in general is one of the principal barriers to the development of a rational discipline of chemical pathology". The extent of this problem is emphasised by the many reference bases that have been employed. (15, 40, 85, 193, 337). The choice in these experiments is not ideal.

Total tissue phosphorus presented certain advantages and enzyme activities have been expressed on the basis of the activity obtaining in relation to the amount oftissue containing one mg. of phosphorus.

It was chosen because of its ease and accuracy of determination. Expected variations in glycogen content were well reflected and collagen was shown to have a lower phosphorus content than did normal liver (1.9 mg/G wet weight as opposed to ± 4.0 mg/G wet weight).

Human material.

Several of the parameters described have also been measured in the livers of 13 patients with porphyria admitted to Groote Schuur Hospital during 1963/4.

These included 4 cases with variegate porphyria of whom 2 were females; 8 with acquired porphyria of whom 6 were males, and 1 female with congenital porphyria.

3 control subjects were also studied.

In the majority of instances liver was obtained by needle biopsy, but from C.P., C.K., and S.P., larger portions of liver were obtained at abdominal operation.

Specimens of liver weighing between 15-30 mg were obtained by needle biopsy. They were blotted dry on filter paper, and immediately placed in weighed glass homogenisers kept in ice. The homogenisers were reweighed to determine the exact weight of liver obtained, and this was homogenised with 5 volumes of cold 0.1 M Tris buffer (pH 7.4). 25 lambda were utilised for determinations of catalase activity, and in experiments performed towards the end of 1963 10 lambda were kept for determinations of total phosphorus concentrations.

The remainder was diluted so that the activities of pyruvate kinase and G-6-P.D. were measured in a 1:1 dilution of this original homogenate; of I.C.D. and G-O.T. in a 1:3 dilution; and of lactic dehydrogenase in a 1:19 dilution.

Where larger specimens ($\pm$ 3 G) were available at operation, the liver was immediately washed in cold 0.25 M sucrose (pH 7.4), cubed in a small beaker surrounded by ice, and rapidly transported from the theatre to the laboratory.

$\pm$ 300 mg were homogenised with 10 volumes of cold sucrose (0.25 M; pH 7.4) and utilised for ALA dehydrase determinations. An aliquot was kept for total phosphorus determinations.

± Fifty mg were homogenised with cold 0.1 M Tris buffer (pH 7.4) and used for measurements of the activity of catalase, the enzymes of intermediary metabolism, and total phosphorus concentration.

The remainder was homogenised with 10 volumes of cold 0.25 M sucrose (pH 7.4) and mitochondrial suspensions were prepared in the usual way(Appendix 1(c)), which were utilised for the measurement of ALA and AA synthetase activities, for oxygen utilisation and for total nitrogen content.

SECTION 5.

FACTORS INFLUENCING LEVELS OF TISSUE ENZYME ACTIVITY.

Physico-chemical environmental factors, and the metabolic status of animals being studied, have been shown markedly to influence *in vitro* measurements of tissue enzyme activity.

A consideration of these factors is felt to be prerequisite to the consideration of changes occurring in levels of enzyme activity in the drug-induced porphyriae, and in persons with porphyria.

This subject has been extensively reviewed by Knox, Auerbach and Lin (178).

1. PHYSICO-CHEMICAL ENVIRONMENTAL INFLUENCES (IN VITRO).

In vitro determinations of enzyme activity are subject to a number of environmental influences. These include temperature, pH, buffer system, substrate and cofactor concentration, electrolyte concentration and the presence of activators or inhibitors.

It was attempted to eliminate these variables and to standardise the experimental conditions as far as possible.

Temperature was maintained at 37°C by thermostatically controlled water baths.

pH was in most instances buffered to 7.4 with freshly prepared Tris buffer. The activities of certain enzymes were

studied in phosphate buffer and at different pH's, but this was constant for a particular enzyme.

Stock reagents were kept in the deep freeze (-15°C) until required and were made up into solution at frequent, but irregular intervals. These solutions were also kept in the deep freeze and were thawed just prior to the preparation of the reagent systems on the day of the experiment.

The final solutions were kept in melting ice until they were finally transferred either to cuvettes in the Beckman spectrophotometer where two minutes were allowed for temperature equilibration before liver homogenate was added to initiate the reaction, or to the Warburg apparatus where an 8-minute period was allowed for equilibration purposes.

In this way it was hoped to avoid variations as the result of deterioration of the reagents; and each reagent system was prepared according to a standard technique whenever required, so that the nature and the amount of factors present were constant as far as they could be controlled.

A possible cause of nonspecific variation in the measured levels of enzyme activity is evident in that the Tris buffer was adjusted to the desired pH at room temperature while the determinations were made at 37°C. Variations in room temperature over the several months during which the experiments were performed might have resulted in slight differences in the pH of the buffer at 37°C.

2. PHYSIOLOGICAL FACTORS AFFECTING THE INTACT ANIMAL.

Factors of particular importance which are operative *in vivo* and which have been shown to influence *in vitro* measurements of enzyme activity include:-

(i). Nutritional status.

(ii). Sex.

(iii). Hormonal function.

(iv). Age and body weight.

and (v). Seasonal and climatic conditions.

The relevant literature as it relates to the enzymes which were studied in the present investigation will be briefly reviewed.

As the effects of several of these variables have been apparent in this investigation, such data will also be included.

In the studies on the drug-induced porphyrias, control animals were matched for sex, weight, and nutritional status and were studied over the same period as the corresponding experimental group.

Somewhat fortuitously, individual groups differed as regards some of these variables, which has permitted of statistical analyses concerning their influences on the parameters studied. Since this was not a planned part of the investigation these groups are not always strictly comparable. The conclusions

drawn must, therefore, for the most part, be tentative, and these findings are presented and discussed in this light.

Many of these factors have been shown to influence the manifestations of the various porphyrias, and such influences will also be briefly considered.

(i). Nutritional Status.

Niemeyer et al (221-224) have investigated the effects of variations in the nature of the diet and its composition, and also the effect of starvation, upon various rat liver enzyme levels of activity.

Amongst other enzymes studied, they demonstrated, in the livers of male albino rats weighing ± 200 G, a decrease in the activity of glucose-6-phosphate dehydrogenase of 56% after a 48-hour period of fasting. Glock and McLean (118) also showed a significant reduction in the activity of this enzyme in the livers of rats fasted for 48 hours.

Vaughan et al (325), working with male Sprague-Dawley rats weighing 100 - 125 G, demonstrated a depressed activity of hepatic glucose-6-phosphate dehydrogenase, and of the 3-phosphoglycerate-pyruvate span, in animals maintained on a high-fat, carbohydrate-free diet for 40 days. A high-protein, carbohydrate-free diet resulted in increased activity of these enzymes.

Glutamic-oxaloacetic transaminase activity was shown by Rosen et al (250) to be very slightly increased in the livers of

rats fasted for 48 hours, while glutamic-pyruvate transaminase activity was doubled under these conditions. Bartlett and Glynn (11) showed that in normal rats maintained on a greatly restricted food intake there was an increase in hepatic transaminase activity, while that of renal transaminase was unaffected.

Conflicting reports are available concerning the influence of fasting on liver catalase activity. Some of the differences may be ascribed to species variation, and the influence of the sex of the animals has also been prominent.

Greenstein et al (146) reported that a three-day period of starvation caused no decrease in the activity of rat liver catalase, and Schmid and Schwartz (265, 268), could demonstrate no change in the activity of this enzyme in the livers of rabbits starved for 3 days. Miller (207) demonstrated no significant alteration in the level of liver catalase activity in male rats, but showed a fairly marked decrease in activity in female rats starved for seven days. Cline et al (50) reported a decrease in hepatic catalase activity of 25% in two strains of mice of both sexes following a 24-hour period of food and water deprivation.

Appleman et al (6) found that rats fed a protein-free diet for 7-10 days had a liver catalase activity of 50% the normal level; and a reduction in activity on such a diet was reported by Borgoni (op cit 178), while no change was observed by Begg et al (14) or by Weil-Malherbe and Schade. (338).

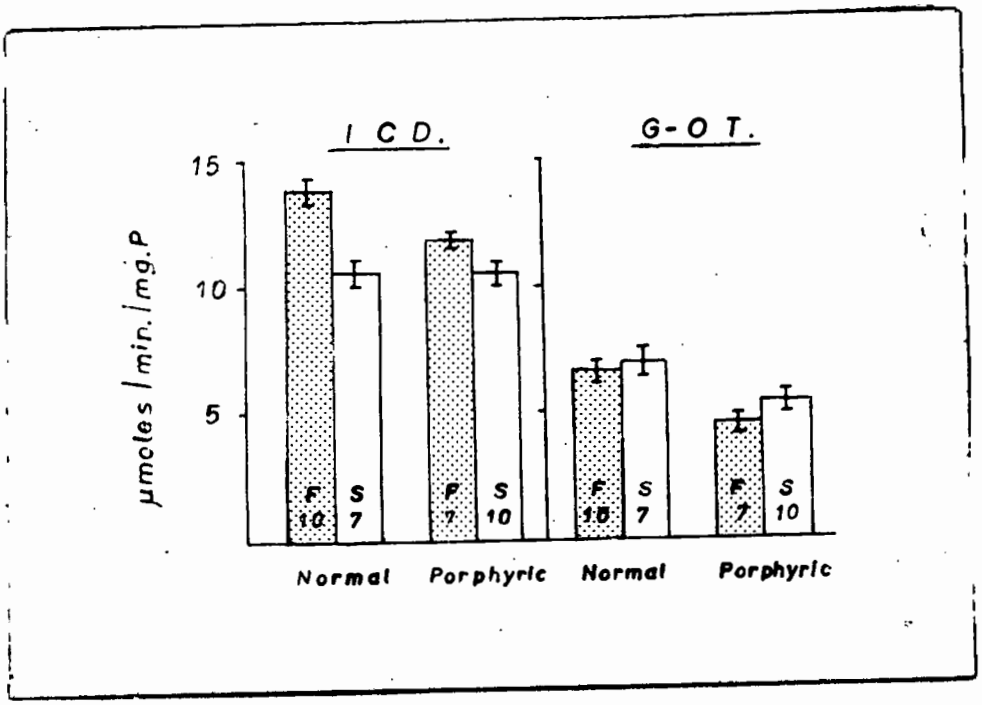


Figure 6.

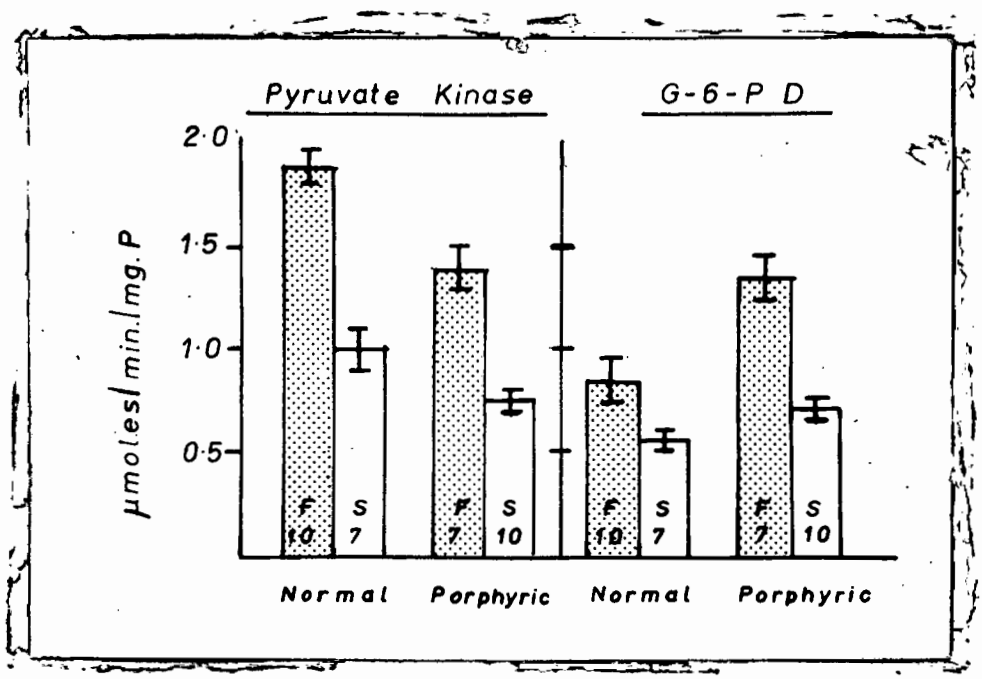


Figure 7.

The influence on liver catalase activity of iron, copper, manganese and several amino acids has been studied. (4, 274, 305).

My findings as regards the influence of an 18-hour period of starvation upon the mean level of activity of various liver enzymes are summarised in the accompanying figures, (6 - 9), which also indicate values one standard error on either side of the mean. The number of animals in each group is shown at the base of the columns. Fed animal groups are indicated by stippling.

The effects of fasting are shown in both normal and DDC-treated animals. All the animals were females and weighed between 164 and 242 grams. All investigations were undertaken over the period 21st May, 1963 to 16th July, 1963. Detailed results are given in the tables indicated in brackets.

Pyruvate kinase (Table 1) and Glucose-6-phosphate dehydrogenase (Table 8).

Normal rat livers showed a reduction in the activity of pyruvate kinase of 47% ($p = < 0.001$) and of glucose-6-phosphate dehydrogenase of 34% ($p = < 0.05$). In the DDC-treated animals the reduction in pyruvate kinase activity was 46% ($p = < 0.01$) and in G-6-P-D activity, 36% ($p = < 0.001$).

Isocitric dehydrogenase (Table 27) and glutamic-oxaloacetic transaminase (Table 34).

Isocitric dehydrogenase activity was reduced by 24% ($p = < 0.001$)

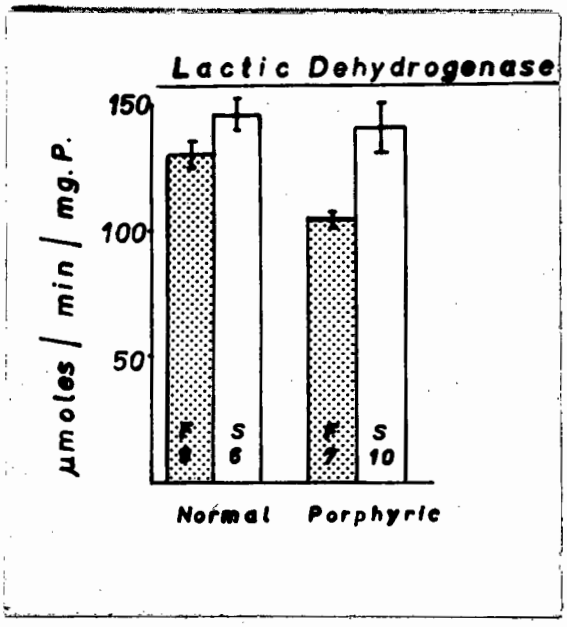


Figure 8.

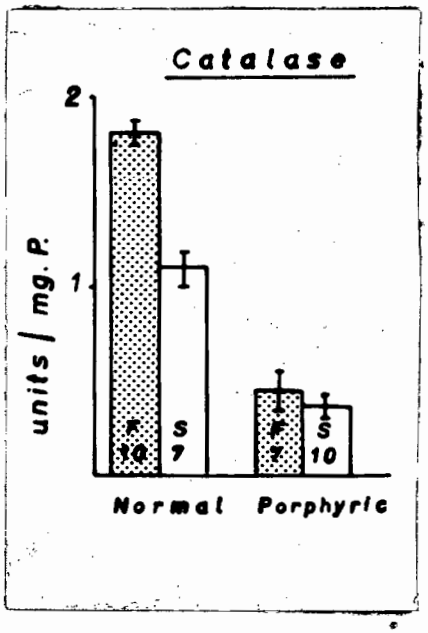


Figure 9.

in normal rat livers, but by only 12% ($p = < 0.02$) in DDC-treated animal livers. This difference may be meaningless and due to experimental artefact, but a possible interpretation is that the drug itself caused a reduction in the enzyme activity which was aggravated only slightly by starvation.

Glutamic-oxaloacetic transaminase activity showed a slight and non-significant increase in both groups of animals, which is in accord with the findings of Rosen et al (250).

Lactic dehydrogenase (Table 20).

Normal rat livers showed an increase in lactic dehydrogenase activity of 12% (not significant) and the porphyric animal livers showed a significant increase in the activity of this enzyme of 32% ($p = < 0.05$). The reason for this difference is not clear, but it is probably best ascribed to the very wide range in activity obtaining in individual livers, and, as such, not important.

Catalase (Table 54).

Catalase activity was reduced by 31% ($p = < 0.001$) in normal rat livers, and by 29% ($p = < 0.001$) in the DDC-treated animal livers.

Protoporphyrin (Table 48).

There is a suggestion of a greater protoporphyrin content in starved animal livers, both normal and drug-treated, than in the

livers of normally fed animals. However, this is not supported by statistical analysis in either instance - possibly because of the very wide range of protoporphyrin concentration in the individual livers. Moreover, in normal livers the protoporphyrin content was so low that the accuracy of the method was probably exceeded.

Vannotti found that in rabbits starved for 48 hours there was a significant increase in the hepatic cytochrome c content.(324).

Nutritional status and the Porphyrias.

Waldenström (op cit 340) in 1944 suggested that porphyrin precursor excretion in acute intermittent porphyria might be influenced by the diet. Welland et al (340) studied the effects of variations in the diet on urinary ALA and PBG excretion in patients with acute intermittent porphyria, and found an acute reduction in caloric intake, an isocaloric diet deficient in carbohydrate and protein, and an isocaloric diet deficient in protein alone, all to result in an increased excretion of these precursors. They also reported briefly on unpublished work of their group which had shown that the feeding of sucrose to rats given AIA prevented the marked rise in hepatic ALA synthetase activity which occurred in starved animals given this drug.

Rose et al (249) found that when AIA-treated animals were starved there was a greater ease of induction of porphobilinogenuria.

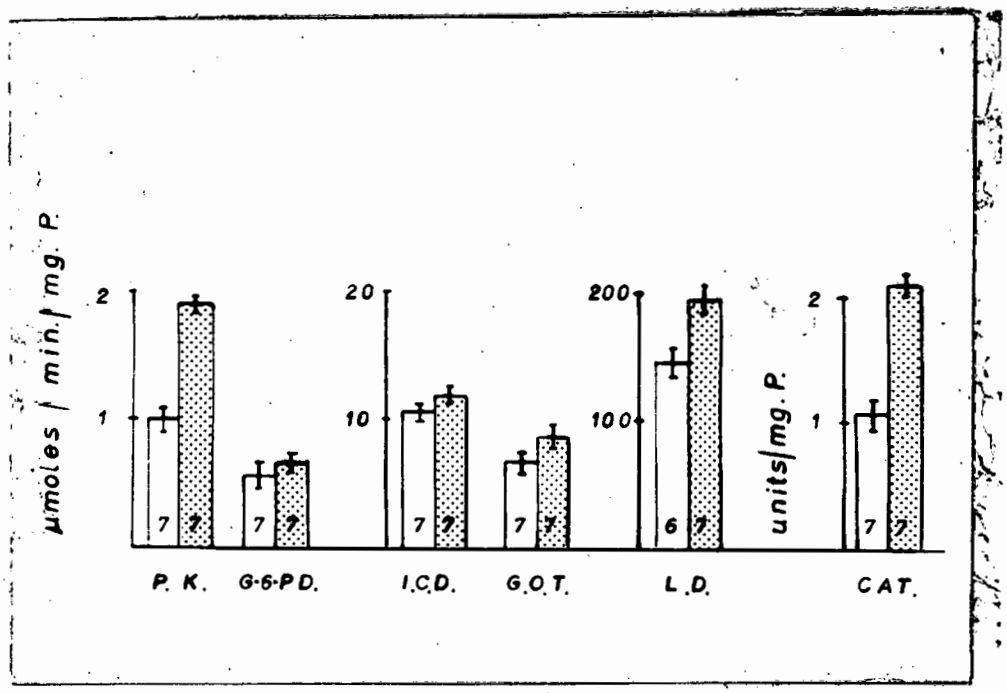


Figure 10. Influence of sex on the levels of activity of several hepatic enzymes in the rat.

Each pair of columns compares mean levels of enzyme activity in groups of male and female animals.

Male animal groups are indicated by stippling.

Values one standard error on either side of the mean are shown, as also the number of animals in each group.

Weight loss has been a feature of all the drug-induced porphyrias (see Section 3(7)), and the majority of patients in the Turkish epidemic of porphyria showed signs of malnutrition. (41, 45, 48, 62, 263).

(ii). Sex.

Variations in enzyme activity according to the sex of the animal have been well documented.

Glock and Mclean (117) reported a mean level of G-6-P-D activity of 104 units in the livers of adult female rats, and of only 46 units in the livers of their male counterparts.

The level of liver catalase activity has been demonstrated by a number of authors to be about 30% higher in the male. (3, 207, 274).

A variety of other enzymes having this sex differentiation are reviewed by Knox et al. (178).

I have recorded definite and significant differences in the liver activities of pyruvate kinase and catalase. Both enzymes had greater mean levels of activity in male then in female rats. That of pyruvate kinase, was 94% higher ($p = < 0.05$) and of catalase, 95% higher ($p = < 0.001$). Male animal livers also showed a greater but statistically non-significant I.C.D. activity, while the differences apparent for G-O-T and G-6-P-D activities are not statistically significant either. There were no difference in mean hepatic lactic dehydrogenase activities in the two sexes.

These results are shown graphically in Figure 10, which is a composite graph summarising data from Tables 1 and 2 (pyruvate kinase), 8 and 9 (G-6-P.D.), 20 and 21 (Lactic dehydrogenase), 27 and 28 (ICD), 34 and 35 (G-O.T.) and 54 and 55(Catalase).

All animals were normal, and were starved for 18 hours before being killed. Male animals weighed 162 - 218 G, and female animals weighed 164 - 198 G.

It should be noted that these findings are not strictly comparable, as the male animals were studied during the period 15th October, 1963 to 15th November, 1963 and the females during the period 10th June, 1963 to 5th July, 1963. This may account for some of the observed differences.

Sex differences and the porphyrias.

Acute intermittent porphyria has been shown to affect more females than males in a ratio of about 3 : 2 (107, 123, 152, 326) and variegate porphyria was found to be more common in the female (87) or to affect the sexes equally. (62).

Symptomatic porphyria has been found to affect males predominantly (87, 152, 323, 326) except amongst the Bantu races where the sexes have been equally affected. (87, 185). Barnes, however, noted a marked female preponderance.(8).

The Turkish epidemic showed a marked male predilection. (41, 45, 263).

Few workers in the field of the drug-induced porphyrias have

commented upon a sex difference in relation to the induction of the disease, or its manifestations. De Matteis and Rimington (69) noted a different response to griseofulvin administration in female mice of the U.C.H. strain to that occurring in their male counterparts. (See Section 3).

(iii). Hormonal function.

The variations according to sex may probably be largely attributed to oestrogenic and androgenic hormones, and the effects of the administration of these hormones, as of castration, have been studied. Adams (3) investigated their influences on catalase activity and demonstrated a fall in activity in castrated young adult mice, which was restored by the injection of testosterone. Testosterone injected into females resulted in an elevation of the level of catalase activity to male levels.

Adrenalectomy in both sexes resulted in a lowering of catalase activity. Conversely, Begg et al (14) could detect no significant alteration in the hepatic catalase activity of adrenalectomised rats, while administration of cortisone resulted in a lowering of activity. Stilboestrol administration also caused a decrease in catalase activity.

Rosen et al (250) demonstrated a marked increase in hepatic glutamic-pyruvate transaminase activity in rats treated with hydrocortisone and showed a similar rise in the activity of this

enzyme in alloxan-diabetic rats. Glock and Mclean (118) studied the influence of hormonal control on hexose monophosphate shunt activity. Hepatic G-6-P-D activity was reduced in alloxan diabetes, and after administration of thiouracil. Thyrotoxicosis resulted in a twofold increase in the activity of this enzyme.

Niemeyer et al (223) suggested that some of the dietary effects on enzyme activity might be mediated through an endocrinal influence.

Hormonal Factors and the porphyrias.

A correlation between menstruation, mittelschmertz and pregnancy, and attacks of acute intermittent porphyria has been reported. (123, 124, 152, 333, 341).

Adverse effects following the administration of oestrogens to patients with acute porphyria have been reported by Redeker (235a), and Levit et al (192) reported on a case of acute intermittent porphyria in whom exacerbations had been caused by the administration of progesterone. Welland et al (341) noted an increased urinary excretion of ALA and PBG by patients with acute intermittent porphyria in remission when they were administered ethinyl oestradiol. Watson et al (333) showed that 5 mg. of stilboestrol daily resulted in a significant increase in the urinary excretion of PBG and uroporphyrin by a patient with latent hepatic porphyria.

They also reported on 6 cases of hepatic cutaneous porphyria in whom manifestations of the disease first appeared after the administration of stilboestrol.

Theologides (op cit 333) studied the effects of prolonged stilboestrol administration on a number of non-porphyric individuals and in none did serial determinations of urinary ALA, PBG, uroporphyrin and coproporphyrin show any change.

Goldberg (124) described inhibition of exacerbations of acute porphyria, coinciding with menstruation, by the administration of testosterone.

Haeger-Aronson (152), however, reported the prevention of such exacerbations by treatment with "Anovlar".

Other hormonal factors have also been shown to be concerned. Diabetes has frequently been reported in association with the porphyrias - especially the symptomatic form. (op cit 86, 248).

Acute exacerbations of porphyria have been reported in association with an "inappropriate secretion of antidiuretic hormone" (130, 159) and M.S. a patient with variegate porphyria (studied during an acute attack) had a serum Na^+ concentration of 118 m. eq/L. (Appendix 2).

Figge and Davidheiser (96) showed that the amount of porphyrin formed by liver homogenates from hypophysectomised animals was double that formed by homogenates from control animal livers.

(iv). Age and body weight.

Changes in the level of enzyme activity are said to occur during the embryonic period, and further changes to occur during the post-embryonic period, when the general tendency is for enzyme activity to increase with age until a plateau is reached. (102, 178). Innumerable factors may be operative.

Glock and Mclean (117) showed a higher level of hepatic G-6-P.D activity in adult than in young female rats. Adult male rats, however, had a lower level of activity of this enzyme than did young males.

Growth hormone was shown by Bartlett and Glynn (10) to decrease transaminase activity in the "tibialis anticus" muscle of immature hypophysectomised rats, and the activities of these muscle enzymes were shown to be lower in rapidly growing normal rats than they were in normal adults, or in immature, hypophysectomised animals.

Treatment of normal adult female rats with growth hormone produced no change in the level of either hepatic or renal transaminase activity, but an increase in the activity of hepatic transaminase was observed in hypophysectomised rats treated with growth hormone. (11). Beaton et al (12) demonstrated a higher level of activity of the transaminases in the livers of adult male rats (322-429G) than in those of younger males (107 - 135G).

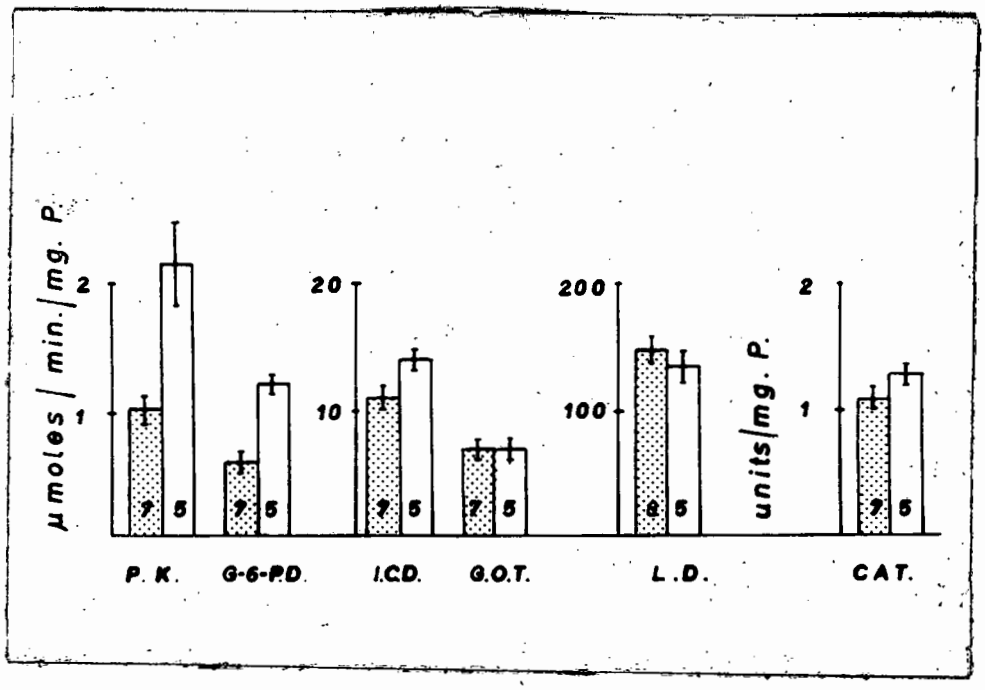


Figure 11. Influence of body weight on the levels of activity of several hepatic enzymes in the rat.

Each pair of columns compares mean levels of activity in groups of heavy and light female animals.

Heavier animal groups are indicated by stippling.

Values one standard error on either side of the mean are shown, as also the number of animals in each group.

Catalase activity was found to be somewhat reduced (27%) in the tissues of older rats of both sexes.

In the majority of 91 measurements, in different tissues, of 41 specific proteins, reviewed by Knox et al (178), no change was found with age in 15 instances, and in only 9 instances were decreases in activity observed.

In my studies two groups of female rats are available for comparison, - one group ranging in weight from 164-198 G, and the other from 110 to 134 G.

Comparison of enzyme activities in these two groups is shown in Figure 11. Detailed data are presented in Tables 1 and 3 (Pyruvate kinase), 8 and 11 (G-6-P-D), 27 and 29 (ICD), 54 and 56 (Catalase), 34 and 36 (GOT) and 20 and 22 (L.D.).

As regards the majority of the enzymes, the level of activity was found to be higher in the smaller animals. Pyruvate kinase activity was 118% higher ($p = < 0.001$), G-6-P-D activity 114% higher ($p = < 0.001$) and I.C.D. activity 34% (not significant) higher. Catalase activity was 22% higher. G-O.T. and L.D. activities showed only negligible differences between the two groups.

All the animals were fasted for 18 hours before being killed, but the larger animals were investigated during the period 10th June to 5th July, 1963, while the smaller ones were investigated between the 14th of October and the 3rd of December, 1963.

All the animals weighed more than 100 G. which is the weight at which Freedland et al (102) found hepatic phenylalanine hydroxylase activity to become constant in female rats. In these animals a steady increase in the activity of this enzyme was observed until the rats were 40 days old and weighed 100 G. Activity then remained at a constant level, and even decreased slightly. In male rats the level of activity continued to increase until they were 77 days old and weighed 316 G.

Age and the porphyrias.

Stich and Decker (306) emphasised the importance of the age of the animal in experimental work on porphyria. They were unable to induce porphyria with Sedormid in rabbits if the animals were more than 5 months old. Rimington and Goldberg (242) agreed with this observation.

Schmid and Schwartz (267) found that they obtained their best results when the rabbits they were studying weighed less than 2 Kg.

De Matteis and Rimington (69) found young animals to react more promptly to griseofulvin administration than did older ones.

Rose et al (248) were unable to induce porphobilinogenuria with AIA in rats weighing more than 200 G.

The Turkish epidemic involved mainly the younger age groups (41, 45, 48, 62) and the rare erythropoietic forms of porphyria have been found to manifest in the very young. (107, 199, 269a).

By contrast, the various forms of hepatic porphyria in man

have usually manifested in somewhat older age groups.

Acute intermittent porphyria has been found to be rare before puberty, and has usually presented for the first time in the 3rd and 4th decades. (107, 152, 326).

Variegate porphyria, while fairly common in the 2nd decade, has been found to have a peak onset in the 3rd decade.(87, 107).

Acquired porphyria has usually appeared for the first time in even older age groups, having a peak onset in the 5th decade. (87, 107, 152, 306).

(v). Seasonal and climatic conditions.

Glock and Mclean (118) noted that hepatic G-6-P-D activity in rats was significantly higher in summer than it was in winter.

Figure 12 compares mean levels of enzyme activity obtaining in 3 groups of animals, one of which was investigated in the winter, and the other two in the spring.

The open columns show the mean levels of activity obtaining in female animals investigated over the period 10th June to 5th July,1963, and ranging in weight from 164 to 198 G. The shaded columns show the mean levels of activity in two groups of animals studied over the period 14th October to 3rd December, 1963, the middle column being comprised of female animals weighing from 110 to 134 G, and the other column of male rats varying in weight between 162 and 218 G.

All the animals were starved for 18 hours before being killed. Details are recorded in Tables 1 - 3 (Pyruvate kinase), 8 - 10 (G-6-P-D), 20 -22 (Lactic dehydrogenase), 27 - 29 (ICD), 34 - 36 (GOT) and 54 - 56 (Catalase).

Levels of pyruvate kinase activity were similar in the groups which were studied over the same period despite differences in sex and weight range; and were much greater in these two groups studied in the spring than they were in a group studied in the winter - a group which might otherwise have been comparable as regards either sex or weight range.

It seems probable, therefore, that the major influence on the level of activity of this enzyme was a seasonal one and that differences apparently occurring as a result of variations in weight range or sex are largely to be accounted for on such a basis.

Levels of hepatic G-6-P-D activity were highest in smaller females studied in spring, and were low in larger females studied in winter, and in males studied in spring. Interpretation is difficult, but these findings are in accord with Glock and Mclean's (118) observations of higher G-6-P-D activity in the summer and in females, but contrast with their observations of greater activity in the adult, as opposed to the young, female.

Differences in catalase activity seem fairly definitely to have been related to variations in sex rather than weight or

seasonal influences. Its greater level of activity in the male seems fairly conclusive. A slight seasonal influence is suggested if differences in weight are accepted as unimportant in the two female groups.

Glutamic-oxaloacetic transaminase activity and lactic dehydrogenase activity seem to have been largely uninfluenced by seasonal factors.

Isocitric dehydrogenase activity was highest in the group of small females studied in the spring. If interpretation is permitted these findings would suggest a greater activity in the spring and in the female sex, although they may equally indicate a more marked influence of body weight on the activity of this enzyme than of either sex or seasonal factors.

Seasonal and Climatic factors and the porphyrias.

Schwartz et al (280) reported that erythrocyte coproporphyrin values in lead-treated rabbits were much lower in the autumn of 1951 than in the spring. They cited the work of Sumegi and Putnoky who described increased porphyrinuria in lead-poisoned rabbits in the spring, but not in the autumn; and of Seggel who described a similar phenomenon for fluorocytes.

The influence of sunlight and seasons on the course of the various porphyrias is well-known, controversial, and outside the scope of this thesis.

Urinary Ascorbic Acid excretion.

The rate of excretion of ascorbic acid in the urine is the one parameter which has been measured which seems to reflect directly *in vivo* metabolic processes in the animals studied.

Musulin *et al* (212) showed a striking influence of the nature of the diet, and of inanition upon the urinary excretion of ascorbic acid by albino rats. There was a considerable variation in the excretion rates of individual animals, which depended in part on their diets prior to the experimental circumstances. Inanition caused a rapid and marked decrease in the ascorbic acid excretion rate.

As all the animals in the present investigation which were studied as regards urinary ascorbic acid excretion rates were starved for the eighteen hours during which the urine was collected, the influence of their dietary status cannot be considered. However, three groups of normal animals are available for comparative studies, and the findings are summarised in Figure 13. In this figure the first column shows the mean excretory rate of ascorbic acid in the urine of a group of female rats ranging in weight from 164 to 204 G and investigated over the period 10th June to 5th July, 1963. The middle column shows that of a group of female rats weighing 85 - 117 G, and the third column that of male rats weighing 151 - 241 G. The latter groups were investigated over the period 14th October to 3rd December, 1963.

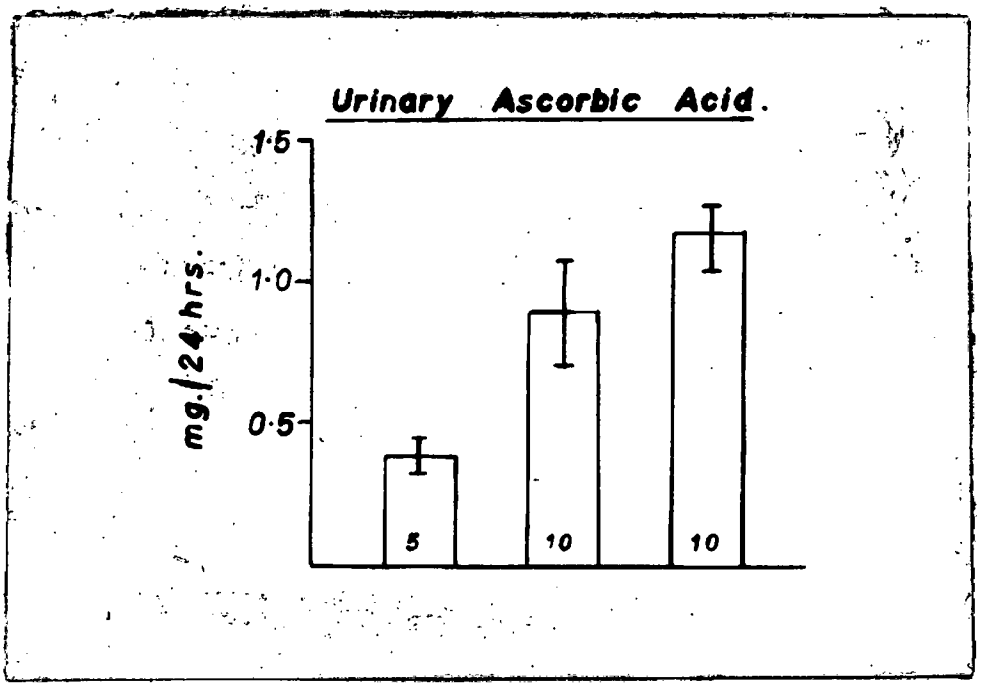


Figure 13.

It is evident that the two groups of animals studied in the warmer months excreted more ascorbic acid per 24 hours than did the group studied in winter. This finding lessens the significance of the alternate observations (i), that heavier females excreted less ascorbic acid in their urine than did the smaller animals or (ii), that females excreted less ascorbic acid than did males of an equivalent weight range.

Even in the intact animal, therefore, a seasonal influence upon a manifestation of enzyme activity has been observed.

ALA synthetase, ALA dehydrase, AA synthetase, and oxygen utilisation.

The effects of nutritional status, sex, body weight and seasonal influences on hepatic ALA synthetase, ALA dehydrase, and AA synthetase activities, and on oxygen utilisation by mitochondria cannot readily be assessed as these parameters have not been studied in at all comparable groups. The groups comprised:- (i), normal male animals weighing between 198 and 209 G, starved for 18 hours before being killed, and investigated during the period 29th, October 1963 to 11th December, 1963. (ii), normal female rats weighing between 160 - 209 G, allowed free access to food to the time of death, and investigated during the period 6th August, 1963 to 23rd August, 1963, and (iii), a group of normal female rats ranging in weight from 110 to 145 G, starved for 18 hours before death, and investigated over the

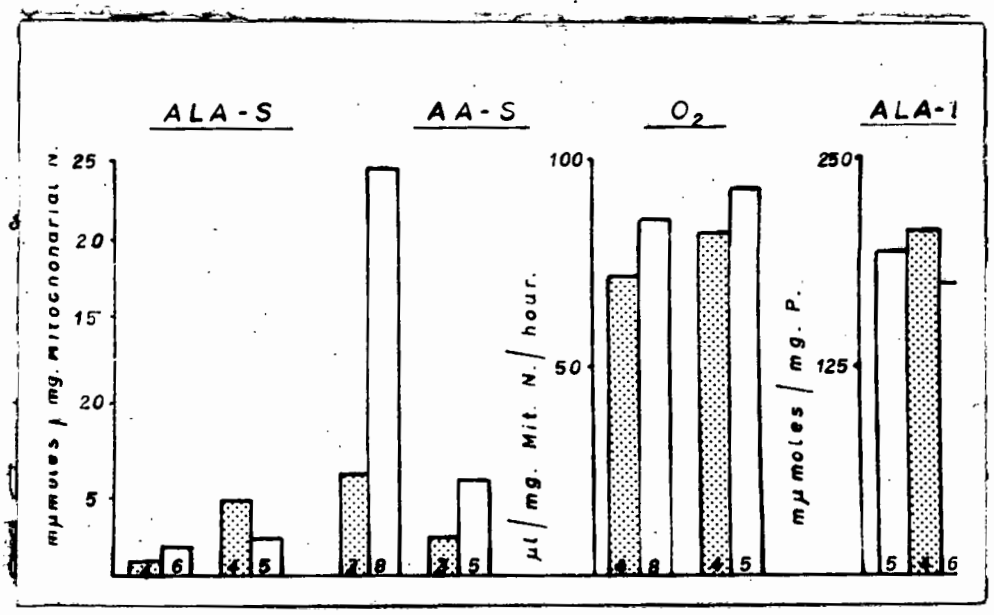


Figure 14. The first pair of columns in each of the first three graphs shows findings when pyruvate served as substrate.

Male groups are indicated by stippling.

period 16th October to 3rd December, 1963 and in which only ALA dehydrase activity was measured.

The results obtaining are shown in Figure 14.

ALA synthetase activity (Tables 41, 43) was minimal - approaching the limits of the experimental technique, and no conclusions at all may be drawn as to the influence of these various factors.

AA synthetase activity (Tables 61, 63) was much greater in the female animals, but the variables of season and nutritional status may have been equally or more important. Very few animals were studied.

No significant differences are apparent between the abilities of mitochondria from male and female livers, respiring on either pyruvate or citrate, to utilise oxygen (Tables 65, 66). This similarity occurred despite differences in the dietary status of the groups compared, and in the season in which they were investigated. Kiessling and Tilander (174) could detect no difference between the rat sexes as regards the ability of normal liver mitochondria to oxidise either pyruvate or succinate.

None of these factors was found to influence ALA dehydrase activity, either, and it was maintained at much the same level in all 3 groups compared. (Tables 45, 46, 47).

Davidheiser and Troyer (61) commented upon variations in the ability of tissue

homogenates from various species to convert delta-aminolaevulic acid to porphyrins as a result of the influences of age and hormonal factors.

They found that the level of such activity in the liver of the hibernating bat was much lower than it was in the livers of non-hibernating rodents.

3. PHARMACOLOGICAL AGENTS AND DISEASE STATES.

A large number of drugs and many diseases have been shown to influence levels of tissue enzyme activity. This subject was reviewed by Knox et al (178) in 1956.

The influence of alcohol on liver enzyme activities is possibly pertinent to the subject of acquired porphyria. Of some interest is the work of Wartburg and Röthlisberger (op cit 165) who demonstrated that chronic administration of alcohol resulted in a marked increase in liver catalase activity.

Figueroa and Klotz (97) showed that in cirrhotic patients with heavy chronic alcoholic histories, isocitric dehydrogenase activity was reduced, while glutamic-oxaloacetic transaminase activity was unchanged.

Alcohol treatment was also shown to cause a 30% reduction in the ability of rat liver mitochondria to oxidise pyruvate. (174). The impairment which such treatment caused in the ability to oxidise succinate was only apparent in male animals,

while mitochondria from female livers respired at about the same rate whether from alcohol-treated or control animals.

Isselbacher and Greenberger (165) recently reviewed the literature relating to the metabolic effects of alcohol.

Hepatic catalase, lactic dehydrogenase and glutamic-oxaloacetic transaminase activities were found to be moderately decreased (348) in acute hepatitis and Dale (58) found catalase activity to be decreased in infectious hepatitis. Schmidt and Schmidt (272) found significant reductions in the activities of several enzymes in acute hepatitis, but showed that significant increase occurred in G-6-P D activity in this disease.

Yoshida (348) reported an increased liver catalase activity in patients with chronic inactive hepatitis.

A large number of drugs has been shown to be capable of increasing urinary ascorbic acid excretion in many animals. (37, 38, 39, 90, 196, 213). These drugs are apparently structurally unrelated.

The major theme of this thesis concerns the influence of drugs known to be capable of inducing porphyria on various liver enzyme activities in the rat. These findings are presented separately in Section 6.

Many drugs and chemicals have been associated with the development of coproporphyrinuria, and also porphyria, in man. These are briefly considered in Section 3.

Kalow (170) has discussed the relationship of such drugs to the porphyrias, and in particular the controversial topics of whether such agents are capable (i), of producing the equivalent of an hepatic porphyria in the absence of a specific genetic defect, (ii), of precipitating an attack of porphyria during a state of remission or latency, or (iii), of aggravating a manifest acute attack.

A variety of disease states, notably diseases of the liver and the haemopoietic system, have been associated with the development of a coproporphyrinuria (op cit 129, 332), and Tio et al (318a) have described the occurrence of a porphyrin-producing benign hepatic adenoma.

Goldberg and Rimington (129) described the precipitation or aggravation of attacks of acute intermittent porphyria in the course of intercurrent infections.

SECTION 6.

RESULTS.

The major theme of this thesis concerns the influence of DDC, AIA, and HCB on the levels of activity of several hepatic enzymes in the rat. The results of the investigation are presented in part A of this Section.

The influence of chlorbutanol administration on porphyrin and ascorbic acid synthesis is shown in part B. It was administered because of the observed influence of DDC, AIA and HCB on urinary levels of ascorbic acid excretion.

The levels of activity of the enzymes studied obtaining in the livers of a number of patients with porphyria are presented in part C. Clinical details of these patients are presented in appendix 2.

A. COMPARISON OF THE LEVELS OF LIVER ENZYME ACTIVITIES, AND RATES OF URINARY ASCORBIC ACID EXCRETION IN NORMAL RATS, AND IN RATS RENDERED PORPHYRIC WITH DDC, AIA and HCB.

Findings in four groups of drug-treated animals, all with disturbances of porphyrin metabolism, are available for comparison with those in normal control groups matched for sex, weight, dietary status and season. The importance of these factors is discussed in Section 5.

The four groups include:-

1. Normally fed female rats administered DDC by stomach tube in a dose of 250 mg/Kg body weight twice daily.

2. A similar group, but starved for 18 hours before being killed.

3. Male rats administered AIA by stomach tube in a dose of 250 mg/Kg body weight, and starved for 18 hours before being killed.

4. Female rats administered HCB as a 0.2% mixture in their diet, and starved for 18 hours before being killed.

All starved animals were allowed free access to water.

The parameters which have been studied are summarised on page 4 , and in Figure 1.

In this section tables are presented which show the results of the measurements of these parameters in individual animals in each group, the mean for the group $\pm$ the standard error of the mean, and the range of activity in each group. The results of statistical comparisons made between each group and its corresponding control group are indicated.(Students "t" test - see appendix 3). The value for P refers to the probability that the two means are drawn from the same population.

The weight range and sex of the animals, the dietary status and the period over which the determinations were made are shown with each table.

The comparisons are demonstrated graphically in figures which show the mean values for the groups indicated $\pm$ the standard error of the mean. The number of animals in each group

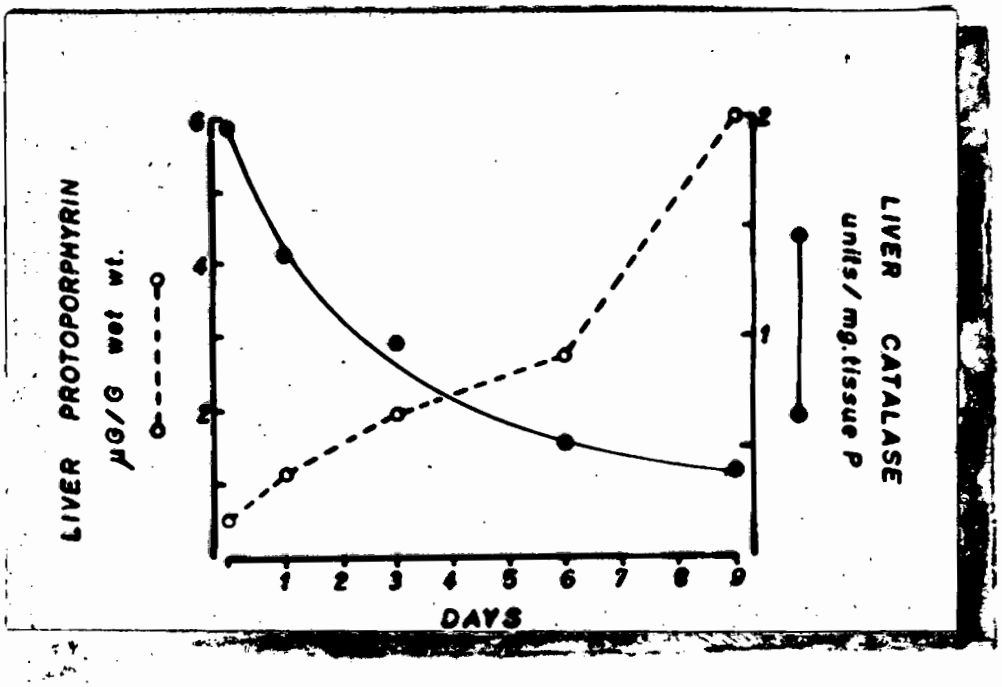

LIVER PROTOPORPHYRIN
µG/G wet wt.
LIVER CATALASE
units/mg.tissue P
1 2 3 4 5 6 7 8 9
DAYS

Figure 15.

is indicated at the foot of the various columns.

Tables and graphs are presented showing the levels of enzyme activity and the rates of urinary ascorbic acid excretion after varying periods of drug treatment.

As can be seen from the preliminary experiments summarised in Figure 15, liver catalase activity had fallen to low levels after 6 days of DDC administration; and liver protoporphyrin concentration, although rising steeply between the 6th and 9th days of administration of this drug was already significantly increased after 6 days too. Investigations for statistical purposes were made at this stage.

Rats treated with AIA showed similar patterns of change of their liver catalase activities and porphyrin concentrations, and, in the main, the various parameters were compared in animals treated for 6 days in this group too.

HCB administration had previously been shown to induce porphyria after between 2 and 8 weeks of its administration as a 0.2% mixture in the diet. (226). In the present investigation the various parameters were studied at weekly intervals, and statistical comparisons are shown between normal animals and animals receiving the drug for between 14 and 57 days.

The effects of feeding DDC as a 0.2% mixture in the diet for 49 days, and of HCB administration in a dosage of 250 mg/Kg body weight twice daily over a period of 6 days are also indicated.

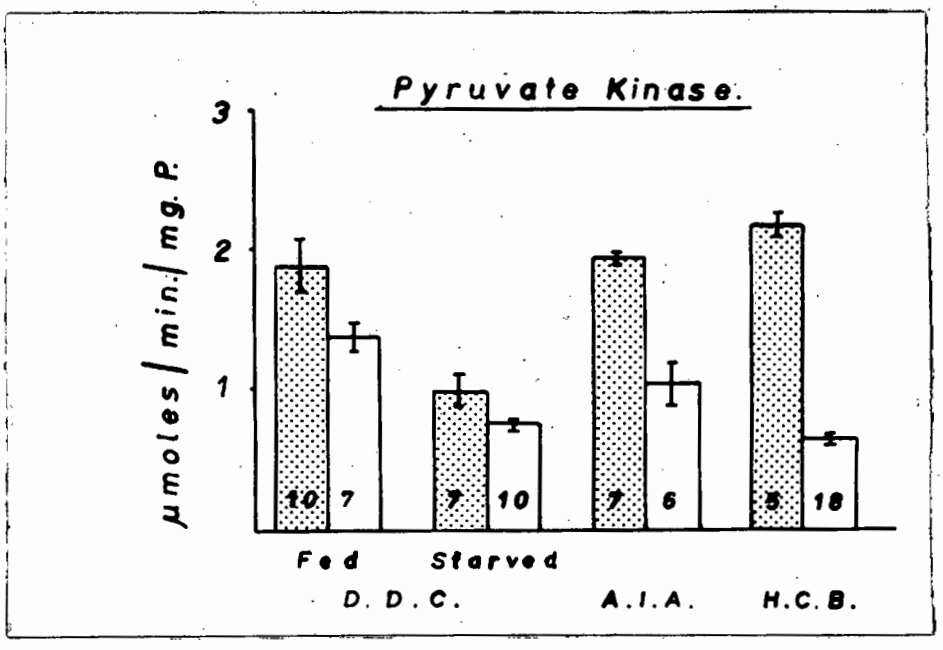


Figure 16. In this, and subsequent graphs, where comparisons between normal and porphyric groups are shown, normal groups are indicated by stippling of the columns.

PYRUVATE - KINASE ACTIVITY.

DDC.

Animals administered DDC by stomach tube for 6 days showed a reduction in their levels of hepatic pyruvate kinase activity (measured *in vitro*) as compared with those in corresponding control groups. (Table 1).

The mean level of activity in the livers of control animals in the "fed" group was 1.88 ± 0.19 µmoles/min/mg. total tissue phosphorus. In the drug-treated animals this was 1.39 ± 0.10 µmoles/min/mgP. - a reduction to 74% the normal level.

The control group which was starved for 18 hours before death showed a mean level of activity of 1.00 ± 0.10 µmoles/min/mgP., while the corresponding DDC-treated group had a mean level of activity of 0.76 ± 0.06 µmoles/min/mgP., which represents a reduction in activity to 76% of the control level. While a period of starvation in itself resulted in a lowering of the level of activity of this enzyme, DDC-administration resulted in a further reduction in activity proportionately equal to that which occurred in the normally fed animals.

This reduction in activity is statistically significant in both fed and starved groups of rats. ($p = < 0.05$).

AIA.

Animals administered AIA for 6 days (Table 2) showed a reduction in the level of activity of this enzyme to 53% the

normal level. In control animals the mean was 1.94 ± 0.37 μmoles/min/mgP., and in the treated animals it was 1.03 ± 0.15. ($p = < 0.05$).

HCB.

A group of animals fed HCB as a 0.2% mixture in the diet for periods varying between 16 and 57 days (Table 3) showed a mean level of activity of hepatic pyruvate kinase of 0.66 ± 0.05 μmoles/min/mgP. In the control group the mean was 2.18 ± 0.37 μmoles/min/mgP. The reduction in activity was thus to 30% the normal level. ($p = < 0.001$).

These results are summarised graphically in Figure 16.

It is evident that with each of the drugs used there was a significant reduction in hepatic pyruvate kinase activity. This was most marked during HCB administration.

Time studies.

The influence of the duration of drug administration in large doses by stomach tube upon the development of changes in hepatic pyruvate kinase activity is shown in Tables 4 - 6., and these findings are summarised graphically in Figure 17.

In both DDC- and AIA-treated rats there was an initial rise in the activity of the enzyme. In the DDC-treated rats there was then a marked fall in enzyme activity after 3 days of drug administration after which the level remained constant until the end of the experiment. In the AIA-treated animals there was

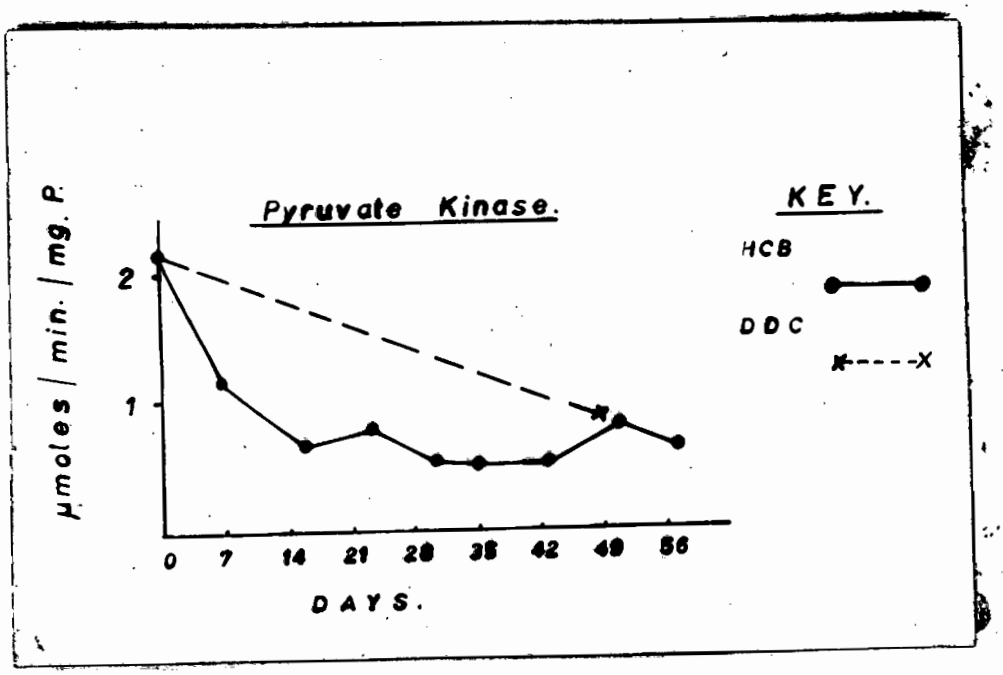


Figure 17.

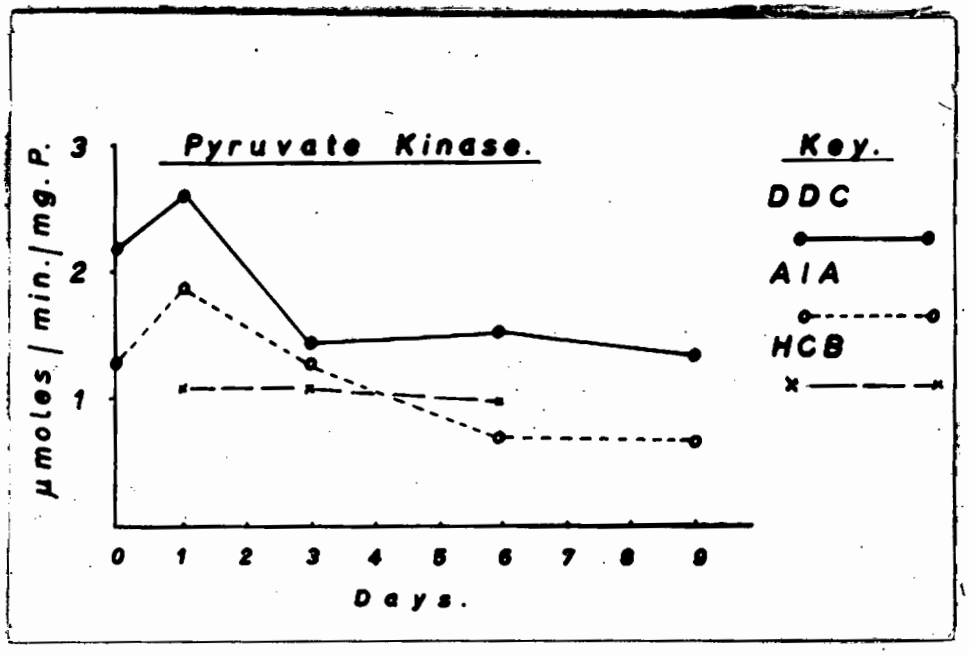


Figure 18.

similarly a fall in activity after 3 days of treatment. It was more marked after 6 days and then also remained constant until the end of the experiment.

No exactly corresponding control animals are available for comparison with the HCB-treated animals. As shown in Section 5 (v) despite a difference in sex and weight range, normal male animals studied over the same period had similar levels of pyruvate kinase activity and probably provide an acceptable control group in this study. It is then evident that a slight reduction in the activity of this enzyme occurred after 6 days of drug administration.

Rats fed HCB in the diet showed a marked reduction in the activity of this enzyme after 7 days of treatment. This was more pronounced after 16 days, and then the level remained fairly constant until the end of the experiment. (Table 3).

These results are summarised in Figure 18 as also the effects of feeding DDC as a 0.2% mixture in the diet for a period of 49 days. (Table 7).

Prolonged DDC-administration resulted in a reduction in the level of activity of this enzyme from a mean of 2.18 ± 0.37 to one of 0.87 μmoles/min/mgP. - a reduction even more striking than that obtaining in the animals administered DDC in larger doses over a shorter period. Too few animals have been studied to allow of statistically valid conclusions being drawn.

These time studies, while not suitable for statistical analysis, suffice to indicate a trend and to suggest certain time relationships in the development of experimental porphyria.

They show that changes occurred early - within 3 days of DDC and AIA administration, and somewhat more slowly in HCB-treated animals. Prolonged drug treatment, especially noticeable in the HCB-fed group, did not result in further decreases in enzyme activity despite the progressive accumulation of porphyrins in the liver.

Table 1.

Pyruvate kinase activity in the livers of rats administered DDC by stomach tube for 6 days.

μmoles/min/mgP.

	Normally fed		Starved for 18 hrs.	
	Controls	Treated	Controls	Treated
Level of activity in individual livers	1.53 2.30 2.63 2.92 0.88 1.35 1.46 2.12 1.60 1.98	1.27 1.48 1.90 1.33 1.39 1.09 1.27	0.73 0.79 1.36 0.88 0.98 1.11 1.15	0.46 0.50 0.75 1.02 0.78 0.79 0.63 0.91 0.92 0.86
Number in group	10	7	7	10
Mean ± S.E.	1.88 ± 0.19	1.39 ± 0.10	1.00 ± 0.10	0.76 ± 0.06
Range	0.88 - 2.92	1.09 - 1.90	0.73 - 1.36	0.46 - 1.46
p.	< 0.05		< 0.05	

All female rats. Weight range 164 - 242 G.

Determinations made over period 21st May to 16th July, 1963.

Table 2.

Pyruvate kinase activity in the livers of rats administered AIA by stomach tube for 6 days.

	μmoles/min/mgP.	
	Controls	Treated
Levels of activity in individual livers.	1.37 1.27 1.17 1.84 1.66 2.19 4.06	0.69 0.87 0.57 1.33 1.43 1.30
Number	7	6
Mean ± S.E.	1.94 ± 0.37	1.03 ± 0.15
Range	1.17 - 4.06	0.57 - 1.43
p.	< 0.05	

All male rats.

Weight range 147 - 241 G. (Majority 170 - 210 G).

Determinations made over period 15th October to 15th November, 1963.

All rats starved for 18 hours before being killed.

Table 3.

Pyruvate kinase activity in the livers of rats given HCB as a 0.2 % mixture in the diet over a period of 57 days. Statistical comparisons are shown between control animals and animals fed HCB for 16 - 57 days.

μmoles/min/mgP.

	Controls	7 days	16 days	23 days	31 days	35 days	43 days	51 days	57 days
Level of activity in individual livers	0.97 2.09 1.93 2.96 2.97	1.10 1.45 0.93	0.72 0.63 0.75	0.56 0.66 1.24	0.52 0.60 0.57	0.51 0.54	0.45 0.45 0.75	0.60 1.02	0.65 0.66
Mean	2.18	1.17	0.70	0.82	0.56	0.53	0.55	0.81	0.65
Number	5		18						
Mean ± S.E.	2.18 ± 0.37		0.66 ± 0.05						
Range	0.97 - 2.97		0.45 - 1.24						
p.	< 0.001								

All female rats . Weight range:- 85 - 177 G.(Majority 100 - 150G).

Determinations made over period 14th October to 3rd December,1963.

All rats starved for 18 hours before being killed.

In a similar investigation performed on 30th September,1963, with somewhat heavier animals, the mean level of activity in two rats fed HCB in the diet for 40 days was 0.50 μmoles/min/mgP. The individual levels were 0.21 and 0.79 μmoles/min/mgP, and a control rat studied on the same day had a level of activity of 1.62 μmoles/min/mgP.

Table 4.

Pyruvate kinase activity in the livers of rats administered DDC by stomach tube over a period of 9 days.

	μmoles/min/mgP.				
	Day 0	1 Day	3 Days	6 Days	9 Days
Level of activity in individual livers	1.53 2.30 2.63	1.87 3.00 2.99	1.45 1.65 1.28	1.27 1.48 1.90	1.29 1.69 1.18
Mean	2.15	2.62	1.46	1.55	1.39

All female rats

Weight range:- 198 - 254 G.

Determinations made over period 21st May to 30th May,1963.

All rats normally fed.

Table 5.

Pyruvate kinase activity in the livers of rats administered AIA by stomach tube over a period of 9 days.

	μmoles/min/mgP.				
	Day 0	1 Day	3 Days	6 Days	9 Days
Level of activity in individual livers	1.37 1.27 1.17	1.97 2.13 1.62	1.67 1.13 1.05	0.69 0.87 0.57	0.89 0.50
Mean	1.27	1.90	1.28	0.71	.69

All male rats

Weight range:- 168 - 241 G.

Determinations made over period 15th October to 24th October,1963.

All rats starved for 18 hours before being killed.

Table 6.

Pyruvate kinase activity in the livers of rats administered HCB by stomach tube over a period of 6 days.

	µmoles/min/mgP.		
	1 Day	3 Days	6 Days
Level of activity in individual livers	1.10 1.23 0.97	1.00 1.06 1.19	1.16 0.98 0.73
Mean	1.10	1.08	0.98

All female rats

Weight range:- 166 - 198 G.

Determinations made over period 2nd October to 7th October, 1963.

All rats starved for 18 hours before being killed.

Table 7.

Pyruvate kinase activity in livers of rats fed DDC as a 0.2% mixture in diet for 49 days.

	µmoles/min/mgP.	
	Treated rats	Control rat.
Level of activity in individual livers	0.53 1.37 0.70	1.72
Mean	0.87	2.18 *

All female rats

Weight range 153 - 160 G.

Determinations made on 25th November, 1963.

All rats starved for 18 hours before being killed.

* The mean for a series of normal female rats of equivalent weight range investigated over the period 14th October to 3rd December, 1963 was 2.18 ± 0.37 and the range 0.97 - 2.97 umoles/min/mgP.

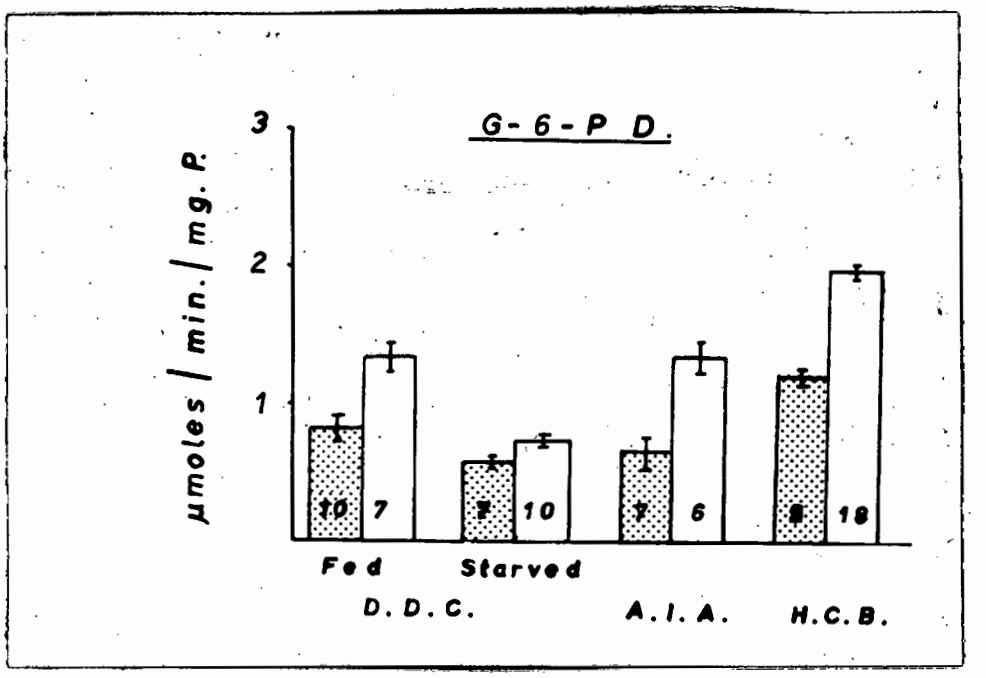


Figure 19.

GLUCOSE - 6 - PHOSPHATE DEHYDROGENASE ACTIVITY.

DDC.

Table 8 shows the effect on the level of liver glucose - 6 - phosphate dehydrogenase activity of administering DDC by stomach tube to rats for 6 days.

Fed controls had a mean level of activity of 0.84 ± 0.10 µmoles/min/mgP. The treated animals had a mean activity of 1.34 ± 0.12 µmoles/min/mgP. - an increase to 159% the control level.

The starved control animals had a mean level of activity of 0.56 ± 0.06 µmoles/min/mgP., while the treated animals showed an increase of activity, proportional to that of the fed group, to 128% the control level - the mean being 0.72 ± 0.05 µmoles/min/mgP.

These changes are not statistically significant ($p \leq 0.1$) but they are important in that (a), the change was opposite to that found for pyruvate kinase activity, (b), it was consistent and (c), there was a wide range of normal activity (also reported by Glock and Maclean (117) with much overlap, which renders the results statistically non-significant, although a real change is suggested.

AIA.

This increased activity which was only suggested in the DDC-fed animals was strikingly significant in rats treated with AIA for 6 days. (Table 9). In these the mean level of activity was more than double that of the control group (210%). In the

control group the mean level of activity was 0.64 ± 0.13 μmoles/min/mgP. Treated animals showed a mean of 1.35 ± 0.13 μmoles/min/mgP. ($p = < 0.01$).

HCB.

In the HCB-treated animals (Table 10) this increased activity was again evident - to 166% the control level. The control animals had a mean level of activity of 1.20 ± 0.21 μmoles/min/mgP. while the animals treated for periods varying between 16 days and 57 days had a mean level of activity of 1.99 ± 0.26. ($p = < 0.05$). A wide range of activity in the various groups was again evident. These results are summarised in Figure 19.

Time studies.

The influence of the duration of treatment on the development of these changes is shown in Tables 10, 12, 13 and 14 and is summarised graphically in Figures 20 and 21.

In the DDC-treated animals there was a fairly steady increase in activity over 6 days. The level dropped slightly after the 9th day of treatment. In the AIA-treated animals there was a definite, marked and regular increase in activity over the first 6 days of treatment after which there was also a slight fall in activity.

Rats treated with HCB by stomach tube over a 6-day period showed a fairly marked increase in activity between the first and the third days of treatment after which, the level remained constant. The significance of this change is doubtful especially

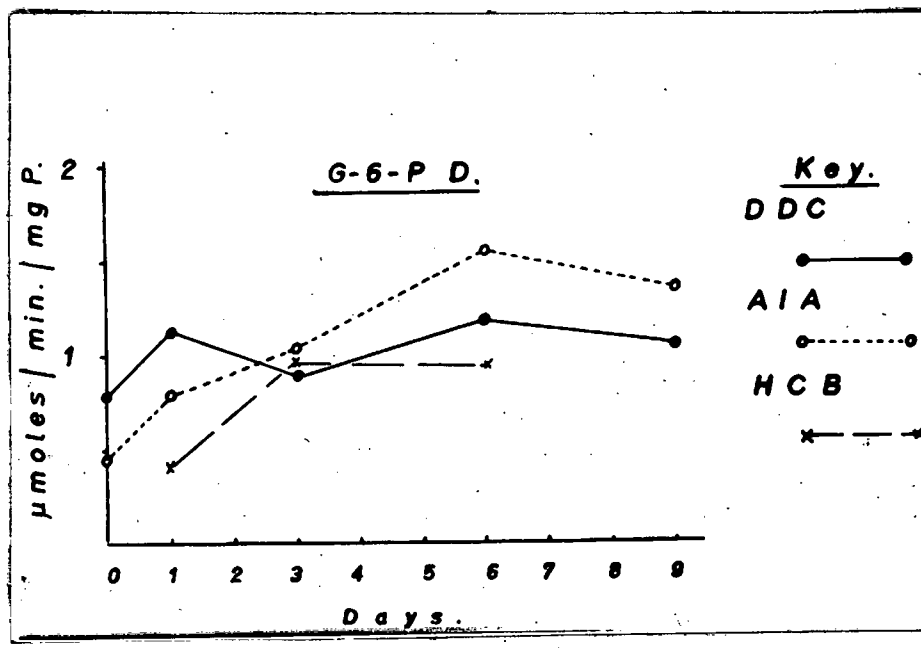


Figure 20.

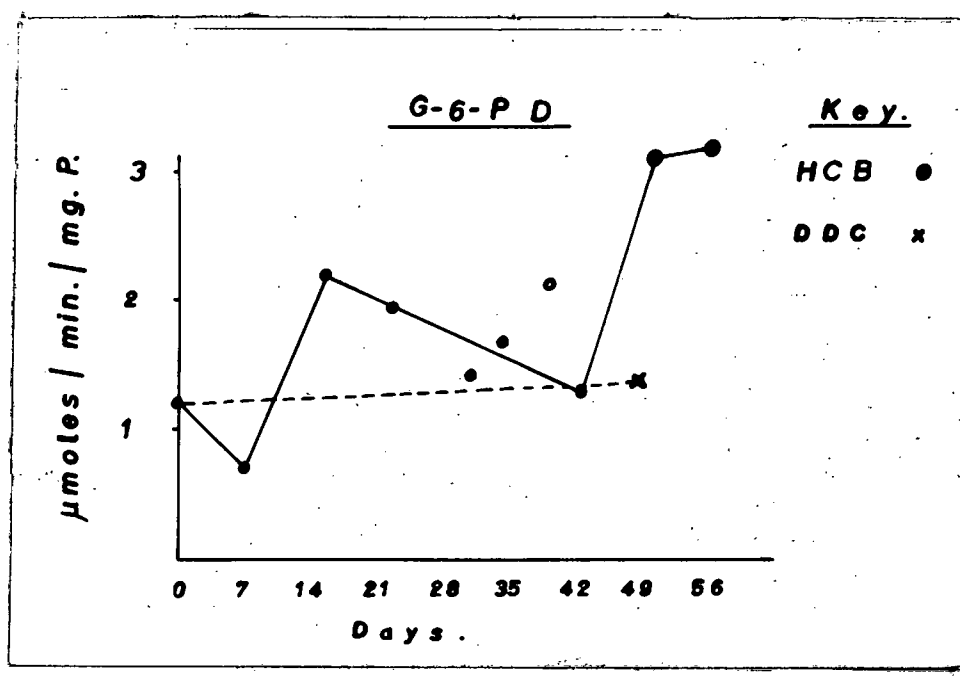


Figure 21.

when considering the small numbers and the wide range of activity of individual rat livers and more especially when considering the changes summarised in Figure 21, which indicate a slight reduction in the activity of this enzyme in rats fed HCB as a 0.2% mixture in the diet for 7 days.

Rats fed HCB in the diet, after an initial fall after 7 days, showed a fairly striking increase in G-6-P D activity after 16 days of treatment. This was followed by a slight but progressive decrease in activity (but remaining above the normal level) until the 42nd day of treatment. After 51 days on the drug there was a further striking increase in activity to 3.10 μmoles/min/mg. tissue P which was maintained until the end of the experiment one week later. (Table 10).

Animals treated with DDC in the diet over a period of 49 days showed very little, or no change in their level of hepatic glucose-6-PO_4 dehydrogenase activity. (Table 14 and Figure 21).

These time studies indicate that a progressive rise in enzyme activity over the first 6 days of treatment with either DDC or AIA occurred.

The results with HCB are slightly contradictory, but it would seem that an initial rise occurred in the 2nd week of treatment, after which the level of activity remained more or less constant for the next few weeks, and that there was a secondary rise in enzyme activity after 7 weeks of drug treatment.

Table 8.

Glucose-6-phosphate dehydrogenase activity in the livers of rats administered DDC by stomach tube for 6 days.

	µmoles/min/mgP.			
	Normally fed		Starved for 18 hrs.	
	Controls	Treated	Controls	Treated
Level of activity in individual livers	0.58 0.96 0.55 1.45 0.58 0.53 0.78 0.66 1.19 1.11	1.00 1.43 1.43 1.35 0.88 0.98 0.77	0.38 0.54 0.84 0.42 0.47 0.57 0.68	0.71 0.67 0.74 0.89 0.67 0.49 0.56 0.87 0.65 0.94
Number in group	10	7	7	10
Mean ± S.E.	0.84 ± 0.10	1.34 ± 0.12	0.56 ± 0.06	0.72 ± 0.05
Range	0.53 - 1.45	0.77 - 1.43	0.38 - 0.84	0.49 - 0.94
p.	< 0.1 (not significant)		< 0.1 (not significant).	

All rats female

Weight range:- 164 - 242 G.

Determinations made over period 21st May to 16th July.1963.

Table 9.

Glucose-6-phosphate dehydrogenase activity in the livers of rats administered AIA by stomach tube for 6 days.

	µmoles/min/mgP.	
	Controls	Treated
Level of activity in individual livers	0.44 0.53 0.42 0.98 0.40 0.66 1.09	1.63 1.35 1.69 1.44 1.23 0.75
Number	7	6
Mean ± S.E.	0.64 ± 0.13	1.35 ± 0.13
Range	0.40 - 1.09	0.75 - 1.69
p.	< 0.01	

All male rats

Weight range:- 147 - 241 G. (Majority 170 - 210 G).

Determinations made over period 15th October to 15th November, 1963.

All rats starved for 18 hours before being killed.

Table 10.

Glucose-6-phosphate dehydrogenase activity in the livers of rats given HCB as a 0.2% mixture in the diet over a period of 57 days. Statistical comparisons are shown between control animals, and animals fed HCB for 16 - 57 days.

	μmoles/min/mgP.								
	Controls	7 days	16 days	23 days	31 days	35 days	43 days	51 days	52 days
Level of activity in individual livers	0.52 1.57 1.18 1.77 0.99	0.53 0.85 0.71	2.18 2.03 2.40	1.18 1.06 3.58	1.06 1.35 1.81	2.00 1.37	1.77 1.08 0.84	1.14 4.90	2.64 3.44
Mean	1.20	0.70	2.20	1.94	1.41	1.68	1.23	3.10	3.20
Number	5		18						
Mean ± S.E.	1.20 ± 0.21		1.99 ± 0.26						
Range	0.52 - 1.77		0.84 - 4.90						
p.	< 0.05								

All female rats. Weight range:- 85 - 177G.(Majority 100 - 150 G).

Determinations made over period 14th October to 3rd December, 1963.

All rats starved for 18 hours before being killed.

In a similar investigation performed on 30th September, 1963, with somewhat heavier animals, the mean level of activity in 2 rats fed HCB in the diet for 40 days was 2.08 μmoles/min/mgP. The individual levels were 1.75 and 2.42 μmoles/min/mgP. A control animal studied on the same day had a level of activity of 0.86 μmoles/min/mgP.

Table 11.

Glucose-6-phosphate dehydrogenase activity in the livers of rats administered DDC by stomach tube over a period of 9 days.

	μmoles/min/mgP.				
	Day 0	1 Day	3 Days	6 Days	9 Days
level of activity in individual livers	0.58 0.96 0.55	0.76 1.36 0.95	1.10 0.77 0.54	1.00 1.43 1.43	1.21 1.09 0.82
Mean	0.70	1.02	0.80	1.29	1.04

All female rats

Weight range:- 198 - 254 G.

Determinations made over period 21st May to 30th May, 1963.

All rats normally fed.

Table 12.

Glucose-6-phosphate dehydrogenase activity in the livers of rats administered AIA by stomach tube over a period of 9 days.

	μmoles/min/mgP.				
	Day 0	1 Day	3 Days	6 Days	9 Days
Level of activity in individual livers	0.44 0.53 0.42	0.69 0.74 0.66	1.29 1.03 0.88	1.63 1.35 1.69	1.53 1.13
Mean	0.47	0.70	1.05	1.56	1.33

All male rats

Weight range 168 - 241 G.

Determinations made over period 15th October to 24th October, 1963.

All rats starved for 18 hours before being killed.

Table 13.

Glucose-6-phosphate dehydrogenase activity in the livers of rats administered HCB by stomach tube over a period of 6 days.

	μmoles/min/mgP.		
	1 Day	3 Days	6 Days
Level of activity in individual livers	0.53 0.28 0.50	0.93 0.76 0.94	1.20 0.69 0.71
Mean	0.43	0.87	0.87

All female rats.

Weight range:- 166 - 198 G.

Determinations made over period 2nd October to 7th October, 1963.

All rats starved for 18 hours before being killed.

Table 14.

Glucose-6-phosphate dehydrogenase activity in the livers of rats fed DDC as a 0.2% mixture in the diet for a period of 49 days.

	μmoles/min/mgP.	
	Treated rats	Control rat
Level of activity in individual livers	0.75 1.84 1.58	1.95
Mean	1.39	1.20 *

All female rats

Weight range:- 153 - 160 G.

All rats starved for 18 hours before being killed.

* The mean for a series of normal female rats of equivalent weight range investigated over the period 14th October to 3rd December 1963, was 1.20 ± 0.21 and the range 0.52 - 1.77 μmoles/min/mgP.

URINARY ASCORBIC ACID EXCRETION.

DDC.

Rats treated with DDC by stomach tube for 6 days showed an increase in their urinary excretion of ascorbic acid from a mean of 0.38 ± 0.07 mg./24 hrs. in the control group to a mean of 1.49 ± 0.16 mg./24 hrs. This represents an increase of 392% ($p = < 0.001$). (Table 15).

No overlap occurred between treated and control animals, and the range of normal was very narrow being only 0.15 - 0.51 mg./24 hrs.

In rats fed DDC in the diet for 7 weeks, the mean urinary ascorbic acid excretion rate was 1.42 ± 0.42 mg./24 hrs. (Table 16). and that by a corresponding control group was 0.90 ± 0.19 mg./24 hrs. (Table 18).

Although the normal animal studied on the 26th November, 1963 excreted only 0.13 mg./24 hrs. the range of normal for the corresponding control group was 0.27 - 2.04 mg./24 hrs. and four of the five treated animals studied, had a urinary ascorbic acid excretion rate falling well within this wide range. It is, therefore, no more than suggestive that prolonged DDC administration can result in increased urinary ascorbic acid excretion. ($p = < .3 > .2$).

AIA.

A very striking rise in urinary ascorbic acid excretion occurred in animals administered AIA over a period of 9 days. (Table 17).

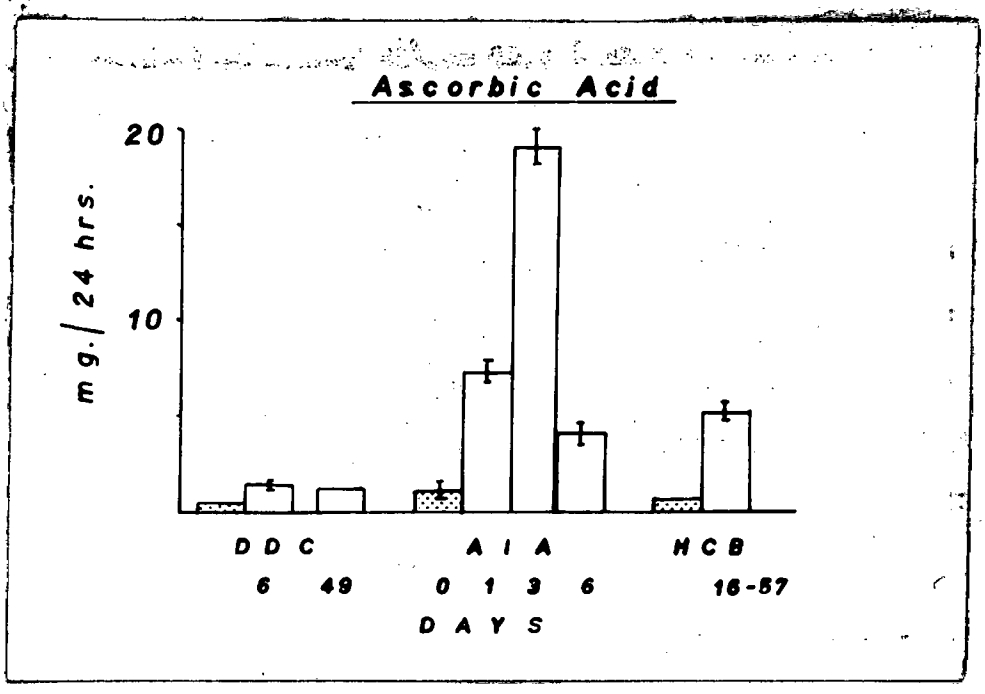


Figure 22.

After the first day of drug administration the excretory rate had risen from a mean of 1.17 ± 0.19 mg./24 hrs. in the control group, to a mean of 8.64 ± 1.00 mg./24 hrs. - an increase of 738%. ($p = < 0.001$). After the 3rd day it had risen to 18.31 ± 1.88 mg./24 hrs. - a rise of 1564%. ($p = < 0.001$). After 6 days of drug administration, the rate of excretion had fallen considerably to only 4.30 ± 0.71 mg./24 hrs., and after the 9th day the mean level was 7.60 mg./24 hrs. Only two animals were studied after this time interval.

HCB.

Animals fed HCB in the diet for periods varying between 16 and 57 days showed a mean urinary ascorbic acid excretion rate of 5.16 ± 0.61 mg./24 hrs. Control animals excreted an average of 0.90 ± 0.19 mg./24 hrs. This represents an increase of 573% in the HCB-fed animals. ($p = < 0.001$). (Table 18).

As may be seen, there was a slight increase in ascorbic acid excretion after one week of drug feeding, and this had risen to a maximum within two weeks, after which it remained more or less constant for the duration of the experiment.

Rats given HCB by stomach tube over 6 days showed an increased excretion of the vitamin after 6 days of drug administration. (Table 19).

The influence of the administration of these drugs on the rate of urinary ascorbic acid excretion is shown graphically in Figure 22.

Table 15.

Urinary ascorbic acid excretion by rats administered DDC for 6 days.

	mg/24 hrs.	
	Normal	Porphyric
Rate of excretion by individual animals	0.50 0.38 0.51 0.15 0.36	2.01 1.52 0.76 2.19 1.44 0.85 1.63 1.30 1.75
Number	5	9
Mean ± S.E.	0.38 ± 0.07	1.49 ± 0.16
Range	0.15 - 0.51	0.76 - 2.19
P.	< .001	

All female rats

Weight range:- 164 - 204 G.

Determinations made over period 10th June to 16th July, 1963.

All rats starved for 18 hours before death during which time the urine was acollected.

Table 16.

Urinary ascorbic acid excretion by rats fed DDC as a 0.2% mixture in the diet for 7 weeks.

	mg/24 hrs.	
	Treated	Control
Excretion by individual animals	1.70 0.17 2.71 1.53 1.00	0.13
Mean ± S.E.	1.42 ± 0.42	0.90 *

All female rats

Weight range:- 143 - 160 G.

Determinations made on 25th and 26th November, 1963.

Rats starved for 18 hours before being killed, during which time the urine was collected.

* The mean for a series of normal female rats of equivalent weight range and investigated over the period 14th October to 3rd December, 1963 was 0.90 ± 0.19, and the range 0.27 - 2.04 mg/24 hrs.

Table 12.

Urinary excretion of ascorbic acid by rats administered AIA for the periods shown.

mg/24 hrs.

	Control	1 Day	3 Days	6 Days	9 Days
Excretion by individual animals	0.92 1.18 1.28 0.44 2.05 0.97 0.74 0.75 1.04 2.37	6.73 4.56 8.15 7.99 8.32 8.21 13.95 11.19	14.67 24.82 19.35 18.22 14.49	1.59 1.16 3.98 2.12 8.33 1.72 3.11 12.42	6.20 9.00
Number	10	8	5	8	2
Mean ± S.E.	1.17 ± 0.19	8.64 ± 1.00	18.31 ± 1.88	4.30 ± 0.71	7.60
p. vs. Control		< 0.001	< 0.001	< 0.001	

All male rats

Weight range:- 151 - 241 G.

Determinations made over period 15th October to 11th December, 1963.

All rats starved for 18 hours before being killed, during which time the urine was collected.

Table 18.

Urinary ascorbic acid excretion by rats administered HCB as a 0.2% mixture in the diet for a period of 57 days. Statistical comparisons are shown between control animals and animals fed HCB for 16 - 57 days.

mg/24 hrs.

	Controls	7 - 9 days	16 - 18 days	23 - 24 days	29 - 31 days	35 - 36 days	43 - 45 days	51 days	57 days
Excretion by individual animals	1.54 0.64 0.59 0.70 1.50 2.04 0.34 0.27 0.44 0.92	3.81 0.33 1.75 2.47 2.94	4.32 1.13 12.41	4.58 5.21 6.44 2.60 3.47	4.87 7.82 2.26 1.56 3.60	3.03 7.12 2.03 4.67	5.47 10.84 10.84 2.97 6.11	7.09 2.58	3.20 4.19
Number	10	5	3	5	5	4	5	2	2
Mean	0.90	2.26	5.95	4.46	4.02	4.21	7.25	4.83	3.70
Number	10	26							
Mean ± S.E.	0.90 ± 0.19	5.16 ± 0.61							
Range	0.27 - 2.04	1.13 - 12.41							
P.	< 0.001								

All female rats. Weight range:- 85 - 177 G.

Determinations made over period 14th October to 3rd December, 1963.

All rats starved for 18 hours before being killed, during which time the urine was collected.

In a similar investigation performed on 30th September, 1963, with somewhat heavier animals, the mean rate of urinary ascorbic acid excretion by 2 rats fed HCB in the diet for 40 days was 4.35 mg/24 hrs. The individual rates were 4.25 and 4.44 mg/24 hrs. A control rat studied on the same day had an excretory rate of 0.97 mg/24 hrs.

Table 19.

Urinary excretion of ascorbic acid by rats administered HCB by stomach tube over a period of 6 days

	mg/24 hrs.		
	1 day	3 days	6 days
Excretion by individual animals	1.24 0.35 0.29	0.58 0.78 1.14	1.04 2.63 2.69
Mean	0.63	0.83	2.12

All female rats

Weight range:- 166 - 198 G.

Determinations made over period 2nd October to 7th October, 1963.

All rats starved for 18 hours before being killed during which time the urine was collected.

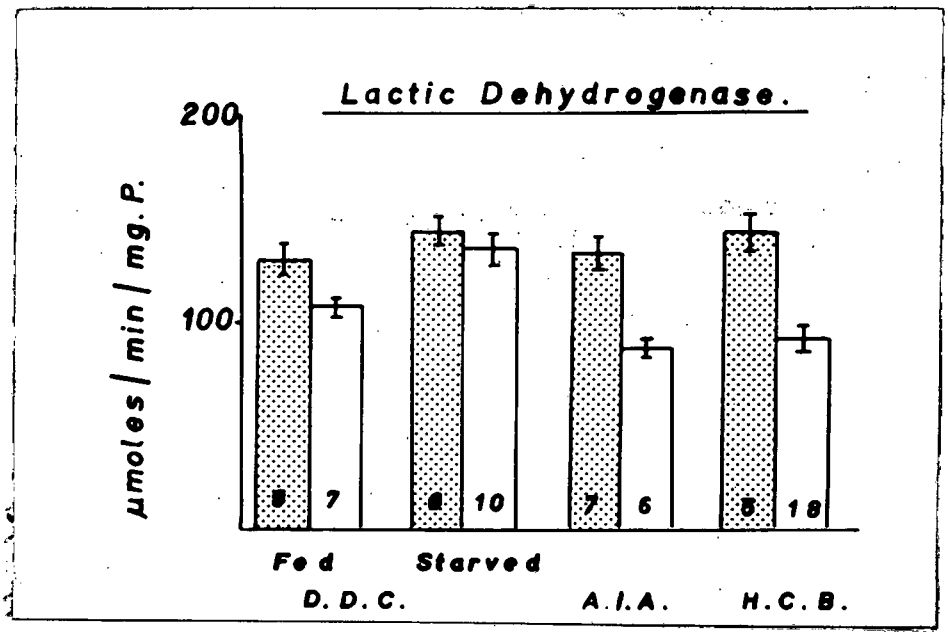

Lactic Dehydrogenase.
200
100
µmoles / min / mg. P.
8
7
8
10
7
6
8
18
Fed
Starved
D. D. C.
A. I. A.
H. C. B.

Figure 23.

LACTIC DEHYDROGENASE ACTIVITY.

DDC.

Animals administered DDC for 6 days (Table 20) showed a reduction in the activity of lactic dehydrogenase to 81% the normal level in the fed groups - from a mean level of 129 ± 10 µmoles/min/mgP. in the control animals to one of 104 ± 5 µmoles/min/mgP. ($p = < 0.05$).

In the starved groups the reduction in activity was minimal and not significant - from a mean of 144 ± 10 µmoles/min/mgP. to a mean of 137 ± 16 µmoles/min/mgP.

In all four groups there was a very wide range of activity in individual livers which limits the value of any comparative studies. It may be merely fortuitous that a significant reduction occurred in the "fed" group of animals and not in the fasted. However, in both groups there is evidence of a reduction in the activity of this enzyme.

AIA.

Rats administered AIA by stomach tube for 6 days (Table 21), had a mean level of activity of the enzyme of 92 ± 6 µmoles/min/mgP., while the mean for the control group was 144 ± 12 µmoles/min/mgP. This represents a reduction in activity to 64% the normal level in the drug-treated animal livers and is highly significant statistically. ($p = < 0.01$).

HCB.

Rats fed HCB in the diet showed a reduction in the activity

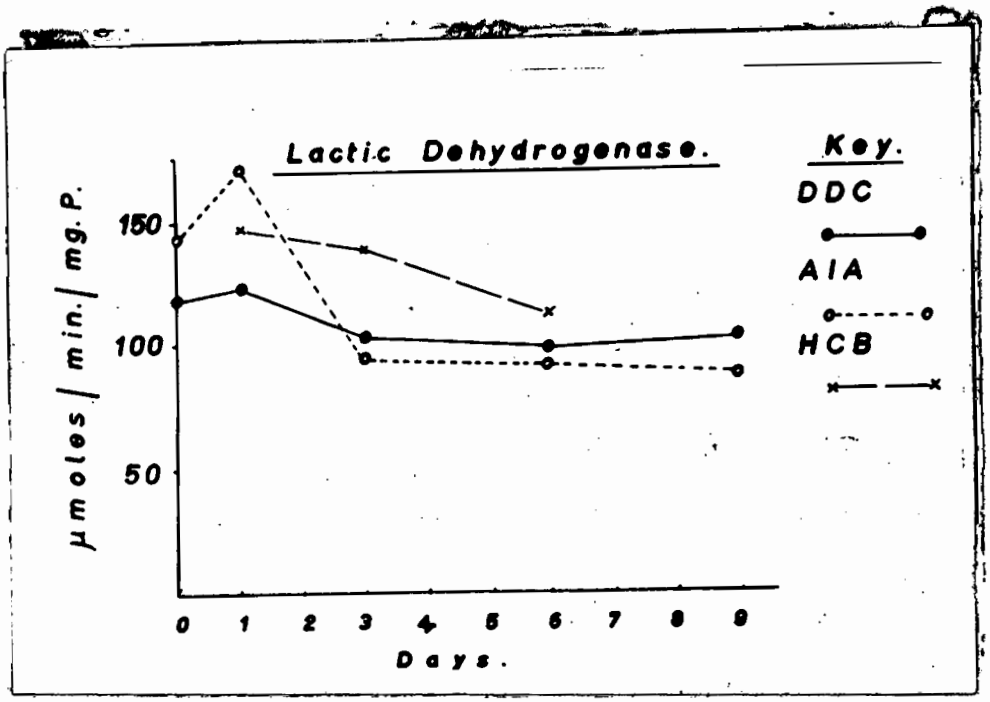


Figure 24.

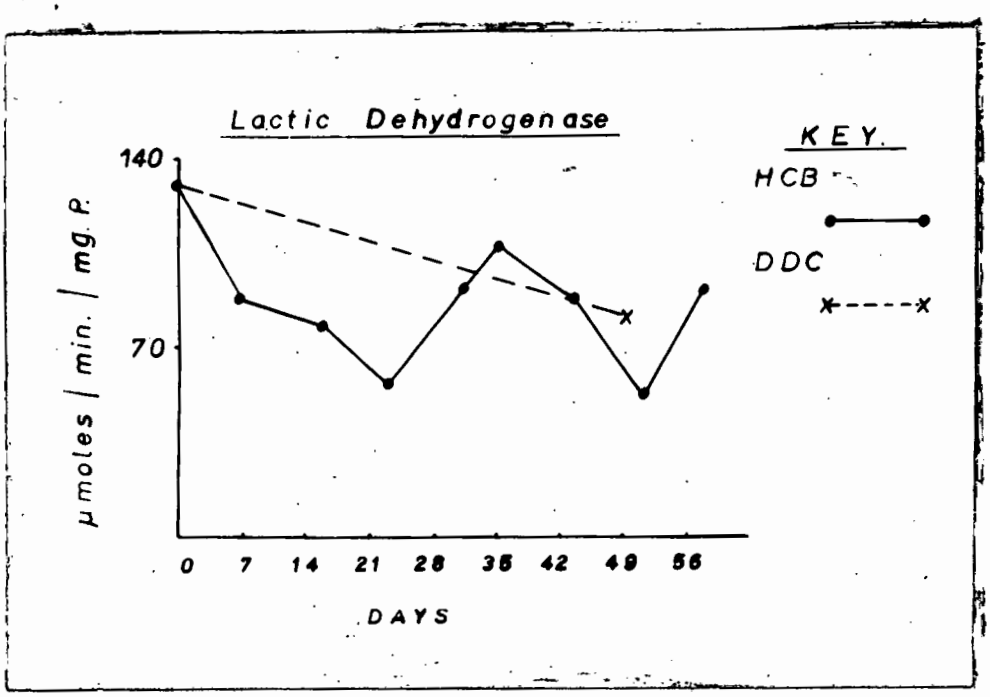


Figure 25.

of this liver enzyme to 60% of normal - from a mean of 133 ± 9 µmoles/min/mgP. in the control group to a mean of 80 ± 5 µmoles/min/mgP. (Table 22).

The results are summarised graphically in Figure 23.

The rate at which the changes occurred is shown in Tables 22, 24, 25 and 26, and is summarised graphically in Figures 24 and 25.

Time studies.

DDC-treated rats showed a reduction in hepatic enzyme activity maximum after 3 days of receiving the drug with a maintenance of this level of activity over the next 6 days.

The AIA-treated animals showed an initial rise in the level of enzyme activity after one day's treatment, but a maximal fall in activity had occurred by the 3rd day, and the level remained steady for the remainder of the experiment.

HCB administered by stomach tube had little effect on the level of activity of this enzyme until it had been administered for 6 days when a moderate reduction became evident.

Prolonged HCB administration caused a fall in the level of enzyme activity, already noticeable 7 days after receiving the drug. The fall was more marked after 16 days and the level then remained more or less constant with variations irregularly above and below the mean level of 80 ± 5 µmoles/min/mgP.

Animals which received DDC as a 0.2% mixture in the diet also showed a fall in the level of activity of lactic dehydrogenase after 7 weeks of treatment - from a mean of 133 ± 9 to one of 78

μmoles/min/mgP. This level may be too low as a result of extraneous factors as the liver of the control animal investigated on the same day had a level of activity of lactic dehydrogenase of only 92 μmoles/min/mgP., whereas the range of activity of the corresponding control group was 102 - 160 μmoles/min/mgP.

Table 20.

Lactic dehydrogenase activity in the livers of rats administered DDC by stomach tube for 6 days.

	µmoles/min/mgP.			
	Normally fed		Starved for 18 hrs.	
	Controls	Treated	Controls	Treated
Level of activity in individual livers	96 128 124 108 166 148 105 180 107	98 116 81 92 121 106 115	165 154 159 160 120 107	185 181 194 151 142 128 113 84 77 110
Number	9	7	6	10
Mean ± S.E.	129 ± 10	104 ± 5	144 ± 10	137 ± 16
Range	96 - 180	81 - 121	107 - 165	77 - 194
p.	<0.05		not significant.	

All female rats

Weight range:- 164 - 242 G.

Determinations made over period 21st May to 16th July, 1963.

Table 21.

Lactic dehydrogenase activity in the livers of rats administered AIA by stomach tube for 6 days.

	µmoles/min/mgP.	
	Controls	Treated
Level of activity in individual livers	183 128 107 131 190 135 134	88 92 90 116 73 98
Number	7	6
Mean ± S.E.	144 ± 12	92 ± 6
Range	107 - 190	73 - 116
P.	< 0.01	

All male rats

Weight range:- 147 - 241 G. (Majority 170 - 210 G).

Determinations made over period 15th October to 15th November, 1963.

All rats starved for 18 hours before being killed.

Table 22.

Lactic dehydrogenase activity in the livers of rats fed HCB as a 0.2% mixture in the diet over a period of 57 days. Statistical comparisons are made between control animals and animals fed HCB for 16 - 57 days.

	μmoles/min/mgP.								
	Controls	7 days	16 days	23 days	31 days	35 days	43 days	51 days	57 days
Level of activity in individual livers	102 119 160 143 142	87 88 90	67 82 77	46 48 64	93 82 106	118 103	77 96 78	54 44	80 100
Mean	133	88	75	53	93	111	84	49	90
Number	5		18						
Mean ± S.E.	133 ± 9		80 ± 5						
Range	102 - 160		44 - 118						
P.	< 0.001								

All female rats

Weight range:- 85 - 117 G.(Majority 100 - 150 G.).

Determinations made over period 14th October to 3rd December, 1963.

All rats starved for 18 hours before being killed.

Table 23.

Lactic dehydrogenase activity in the livers of rats administered DDC by stomach tube over a period of 9 days.

	μmoles/min/mgP.				
	day 0	1 day	3 days	6 days	9 days
Level of activity in individual livers	96 128 124	106 126 125	91 113 103	98 116 81	103 100 102
Mean	116	119	102	98	102

All female rats

Weight range:- 198 - 254 G.

Determinations made over period 21st May to 30th May,1963

All rats normally fed.

Table 24.

Lactic dehydrogenase activity in the livers of rats administered AIA by stomach tube over a period of 9 days.

	μmoles/min/mgP.				
	day 0	1 day	3 days	6 days	9 days
Level of activity in individual livers	183 128 107	199 119 169	76 103 91	88 92 90	99 73
Mean	139	162	90	90	86

All male rats

Weight range:- 168 - 241 G.

Determinations made over period 15th October to 15th November,1963.

All rats starved for 18 hours before being killed.

Table 25.

Lactic dehydrogenase activity in the livers of rats administered HCB by stomach tube over a period of 6 days.

	μmoles/min/mgP.		
	1 day	3 days	6 days
Level of activity in individual livers	148 143 153	157 135 117	104 118 109
Mean	148	137	111

All female rats

Weight range:- 166 - 198 G.

Determinations made over period 2nd October to 7th October, 1963.

All rats starved for 18 hours before being killed.

Table 26.

Lactic dehydrogenase activity in the livers of rats fed DDC as a 0.2% mixture in the diet for a period of 49 days.

	μmoles/min/mgP.	
	Treated rats	Control rat
Level of activity in individual livers	58 93 83	92
Mean	78	133 *

All female rats

Weight range:- 153 - 160 G.

Determinations made on 25th November, 1963.

All rats starved for 18 hours before being killed.

* The mean for a series of normal female rats of equivalent weight range investigated over the period 14th October to 3rd December, 1963 was 133 ± 9 and the range 102 - 160 μmoles/min/mgP.

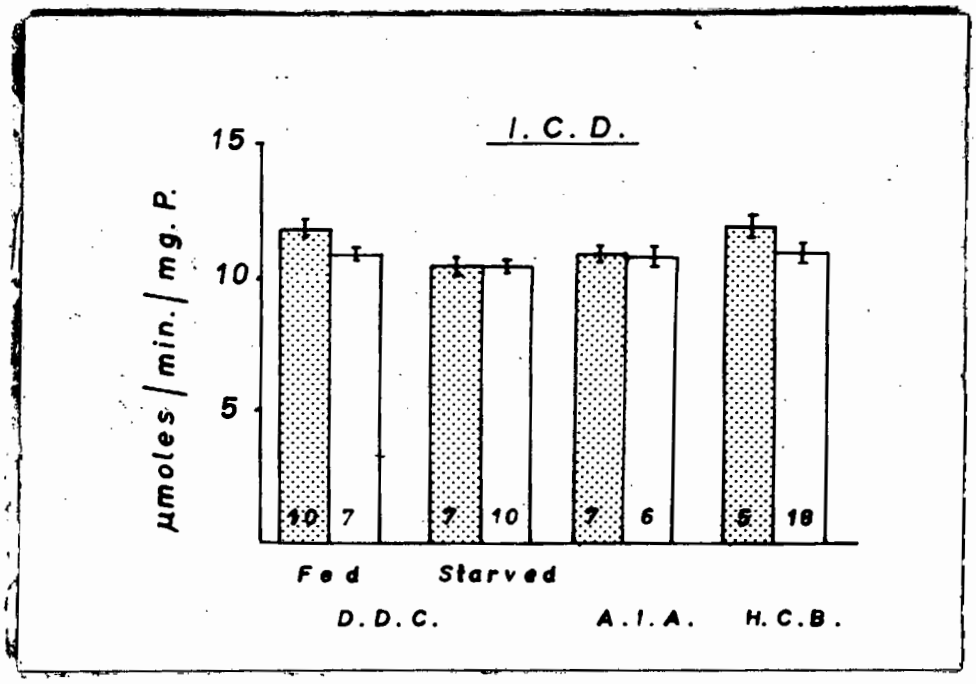

I. C. D.
15
10
5
µmoles / min. / mg. P.
10
7
7
10
7
6
5
18
Fed
Starved
D. D. C.
A. I. A.
H. C. B.

Figure 26.

ISOCITRIC DEHYDROGENASE ACTIVITY.

Observed changes in hepatic isocitric dehydrogenase activity were not as uniform as were those of the other enzyme activities presented thus far, and the final conclusion suggested is that no real change in the activity of this enzyme occurred.

DDC.

The "fed" group of animals treated with DDC (Table 27) showed a reduction in the mean level of activity of this enzyme to 86% the control level from 13.7 ± 0.5 μmoles/min/mgP. in the control group to 11.8 ± 0.3 μmoles/min/mgP. in the treated animals. ($p = < 0.02$). No change was evident in the fasted animals, the mean level of activity in both control and treated animals being 10.4 μmoles/min/mgP.

AIA.

AIA administration for 6 days resulted in a minimal, statistically non-significant, reduction in the activity of the enzyme. (Table 28). The mean level of activity in the control animals was 11.9 ± 0.4 while that in the treated animals was 11.4 ± 0.5 μmoles/min/mgP. This represents a reduction of only 4% and $p = < 0.4, > 0.3$.

HCB.

Rats fed HCB for periods varying between 16 and 57 days had a mean level of activity of 11.9 ± 0.4 μmoles/min/mgP. and the mean level of activity of the control group was 13.9 ± 0.5

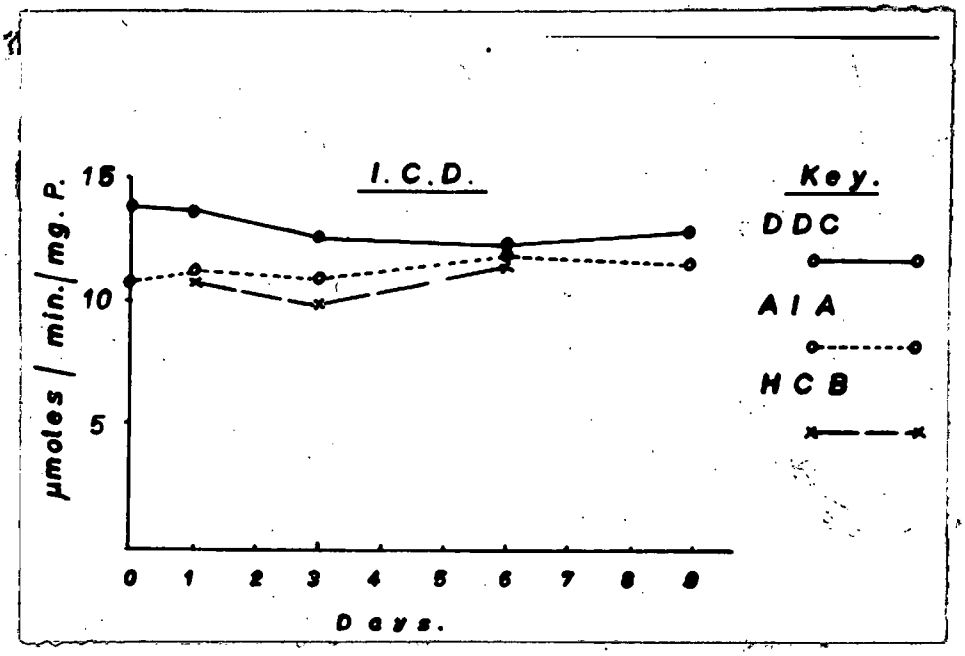


Figure 27.

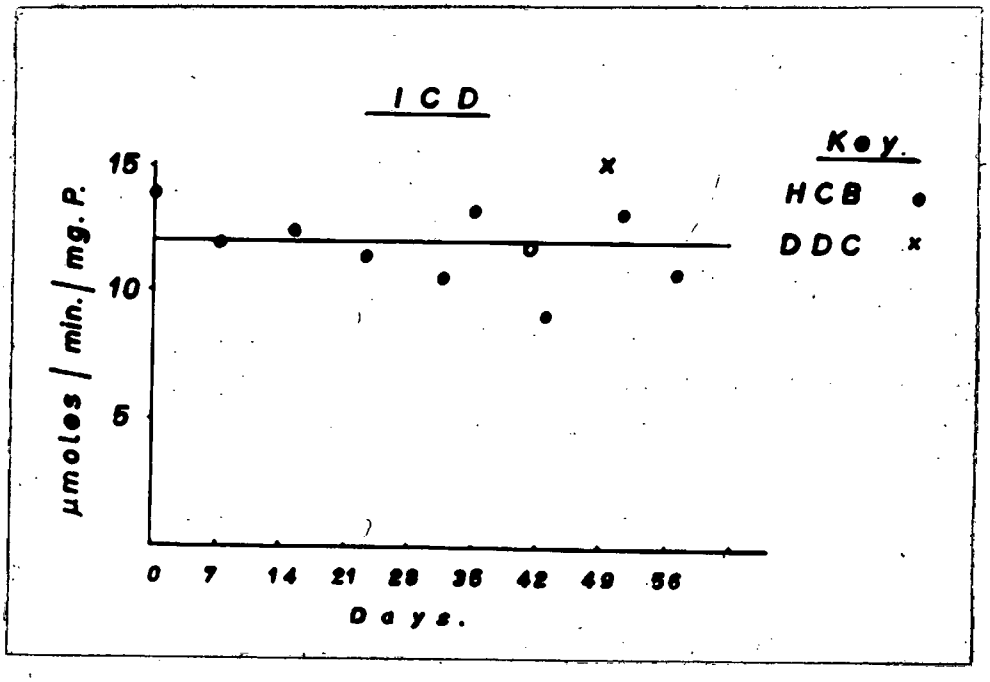


Figure 28.

μmoles/min/mgP. This represents a reduction of 17% in the treated animals and is statistically significant - $p = < 0.01$.

These results are summarised graphically in Figure 26.

Time studies.

From Tables 31, 32 and 33, and from Figure 27 it is evident that the activity of this enzyme was not influenced by 9 days of drug administration by stomach tube while from Table 29 and Figure 28 it is evident that a very slight fall in isocitric dehydrogenase activity occurred after 7 days of feeding HCB in the diet. During the remainder of the experiment the mean level of activity fluctuated fairly widely, and no definite pattern of change in the activity of this enzyme emerged.

Rats fed DDC in the diet for 7 weeks had a mean level of isocitric dehydrogenase activity of 14.4 μmoles/min/mgP. (Table 30), which was very similar to the mean control value of 13.9 ± 0.5 μmoles/min/mgP.

Apart from a slight, although statistically significant, reduction in isocitric dehydrogenase activity in HCB-fed rats and in the fed group of DDC-treated animals, the activity of this enzyme was not influenced by the feeding of these drugs or the development of disturbances of porphyrin metabolism.

Table 27.

Isocitric dehydrogenase activity in the livers of rats administered DDC by stomach tube for 6 days.

µmoles/min/mgP.

	Normally Fed		Starved for 18 hrs.	
	Controls	Treated	Controls	Treated
Level of activity in individual livers	12.4 16.8 12.4 15.5 12.2 13.8 13.5 13.7 14.0 12.7	11.6 12.7 11.6 12.5 11.9 10.2 12.2	9.2 10.2 12.8 11.1 11.0 9.5 8.9	11.4 9.8 11.7 12.5 11.5 9.7 10.0 9.0 9.6 9.3
Number	10	7	7	10
Mean ± S.E.	13.7 ± 0.5	11.8 ± 0.3	10.4 ± 0.5	10.4 ± 0.4
Range	12.2 - 16.8	10.2 - 12.7	8.9 -12.8	9.0 -12.5
P.	< 0.01		not significant	

All female rats

Weight range:- 164 - 242 G.

Determinations made over period 21st May to 16th July, 1963.

Table 28.

Isocitric dehydrogenase activity in the livers of rats administered AIA by stomach tube for 6 days.

	μmoles/min/mgP.	
	Controls	Treated
Level of activity in individual livers	10.8 10.2 10.6 12.3 11.7 13.0 14.7	12.3 12.8 11.6 11.4 11.3 9.3
Number	7	6
Mean ± S.E.	11.9 ± 0.4	11.4 ± 0.5
Range	10.2 - 14.7	9.3 - 12.8
p.	not significant.	

All male rats

Weight range:- 147 - 241 G. (Majority 170 - 210 G.).

Determinations made over period 15th October to 15th November, 1963.

All rats starved for 18 hours before being killed.

Table 29.

Isocitric dehydrogenase activity in the livers of rats given HCB as a 0.2% mixture in the diet over a period of 57 days. Statistical comparisons are shown between control animals and animals fed HCB for 16 - 57 days.

	µmoles/min/mgP.								
	Controls	7 days	16 days	23 days	31 days	35 days	43 days	51 days	57 days
Level of activity in individual livers	13.6 15.1 13.4 15.0 12.6	10.4 12.3 13.2	12.1 12.6 13.1	11.6 10.7 12.5	9.9 10.4 11.9	12.1 14.7	7.9 8.6 11.4	13.4 13.4	10.1 11.7
Mean	13.9	12.0	12.6	11.6	10.7	13.4	9.3	13.4	10.9
Number	5		18						
Mean ± S.E.	13.9 ± 0.5		11.9 ± 0.4						
Range	12.6 - 15.1		7.9 - 4.7						
P.	< 0.01								

All female rats. Weight range:- 85 - 177 G. (Majority 100-150 G.)

Determinations made over period 14th October to 3rd December, 1963.

All rats starved for 18 hours before being killed.

In a similar investigation performed on 30th September, 1963, with somewhat heavier animals, the mean level of activity in two rats fed HCB in the diet for 40 days was 11.8 µmoles/min/mgP. The individual levels were 11.9 and 11.8 µmoles/min/mgP. A control rat studied on the same day had the remarkable level of activity of 21.7 µmoles/min/mgP.

Table 30.

Isocitric dehydrogenase activity in livers of rats administered DDC by stomach tube over a period of 9 days.

	μmoles/min/mgP.				
	Day 0	1 day	3 days	6 days	9 days
Level of activity in individual livers	12.4 16.8 12.4	12.6 14.2 14.3	13.5 12.7 11.7	11.6 12.7 11.6	12.6 12.5 13.2
Mean	13.9	13.7	12.6	12.0	12.8

All female rats

Weight range:- 198 - 254 G.

Determinations made over period 21st May to 30th May, 1963.

All rats normally fed.

Table 31.

Isocitric dehydrogenase activity in the livers of rats administered AIA by stomach tube over a period of 9 days.

	μmoles/min/mgP.				
	Day 0	1 day	3 days	6 days	9 days
Level of activity in individual livers	10.8 10.2 10.6	10.2 11.1 12.2	9.4 11.5 11.3	12.3 12.8 11.6	12.7 10.4
Mean	10.6	11.2	10.8	12.2	11.6

All male rats

Weight range:- 168 - 241 G.

Determinations made over period 15th October to 24th October, 1963.

All rats starved for 18 hours before being killed.

Table 32.

Isocitric dehydrogenase activity in the livers of rats administered HCB by stomach tube over a period of 6 days.

	μmoles/min/mgP.		
	1 day	3 days	6 days
Level of activity in individual livers	9.5 11.7 12.-	9.2 10.1 10.6	10.9 10.3 10.2
Mean	10.7	9.9	10.5

All female rats

Weight range:- 166 - 198 G.

Determinations made over period 2nd October to 7th October, 1963.

All rats starved for 18 hours before being killed.

Table 33.

Isocitric dehydrogenase activity in the livers of rats fed DDC as a 0.2% mixture in the diet for a period of 49 days.

	μmoles/min/mgP.	
	Treated rats	Control rat
Level of activity in individual livers	13.2 15.4 14.6	15.2
Mean	14.4	13.9 *

All female rats

Weight range:- 153 - 160 G.

Determinations made on 25th November, 1963.

All rats starved for 18 hours before being killed.

* The mean for a series of normal female rats of equivalent weight range investigated over the period 14th October to 3rd December, 1963 was 13.9 ± 0.5 and the range 12.6 to 15.1 μmoles/min/mgP.

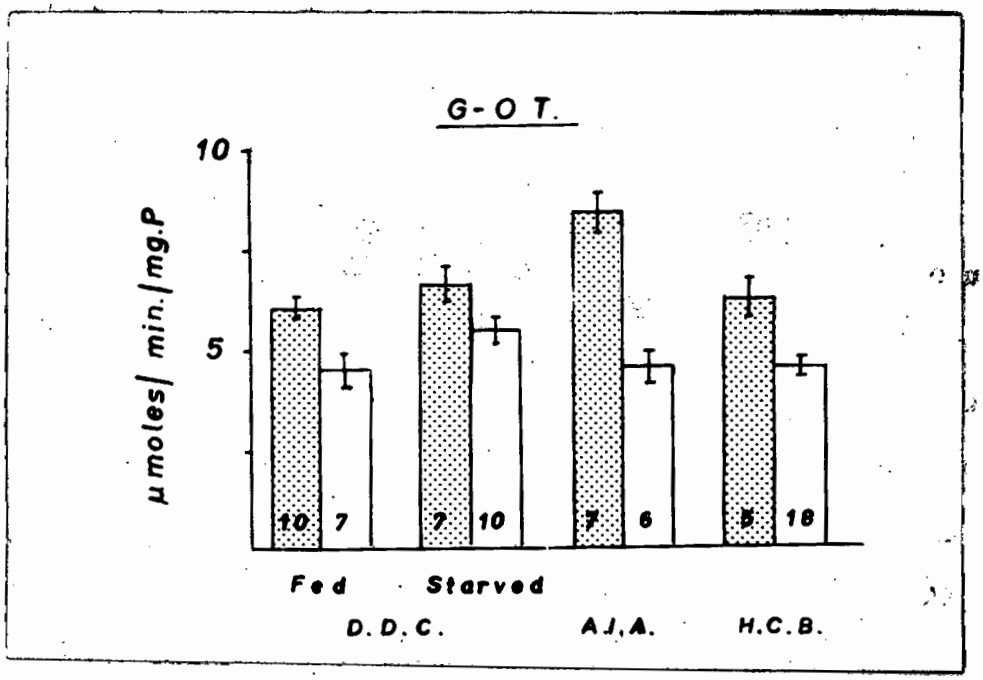


Figure 29.

GLUTAMIC - OXALOACETIC TRANSAMINASE ACTIVITY.

DDC.

Fed animals administered DDC showed a significant reduction in the mean level of activity of hepatic glutamic-oxaloacetic transaminase to 69 % the control value. Control animals had a mean level of activity of 6.65 ± 0.36 μmoles/min/mgP. while the drug-treated animals had a mean level of activity of 4.55 ± 0.31 μmoles/min/mgP. ($p = < 0.001$).

In the starved animals, the control group had a mean level of activity of 6.93 ± 0.53 μmoles/min/mgP. while in the treated animals it was 5.36 ± 0.34 μmoles/min/mgP. - representing a reduction to 78% of the control value. ($p = < 0.05$).(Table 34).

AIA.

Rats administered AIA by stomach tube for 6 days (Table 35) had a mean level of activity of this enzyme of 4.53 ± 0.61 μmoles/min/mgP. In the corresponding control group this value was 8.70 ± 0.63 μmoles/min/mgP. which represents a reduction in activity to 52% of normal in the AIA-treated animal livers. ($p = < 0.001$).

HCB.

HCB-fed rats showed a reduction in the activity of glutamic-oxaloacetic transaminase to 67% of normal from a mean of 6.34 ± 0.70 in the control group to a mean of 4.27 ± 0.27 μmoles/min/mgP.

These results are summarised graphically in Figure 29.

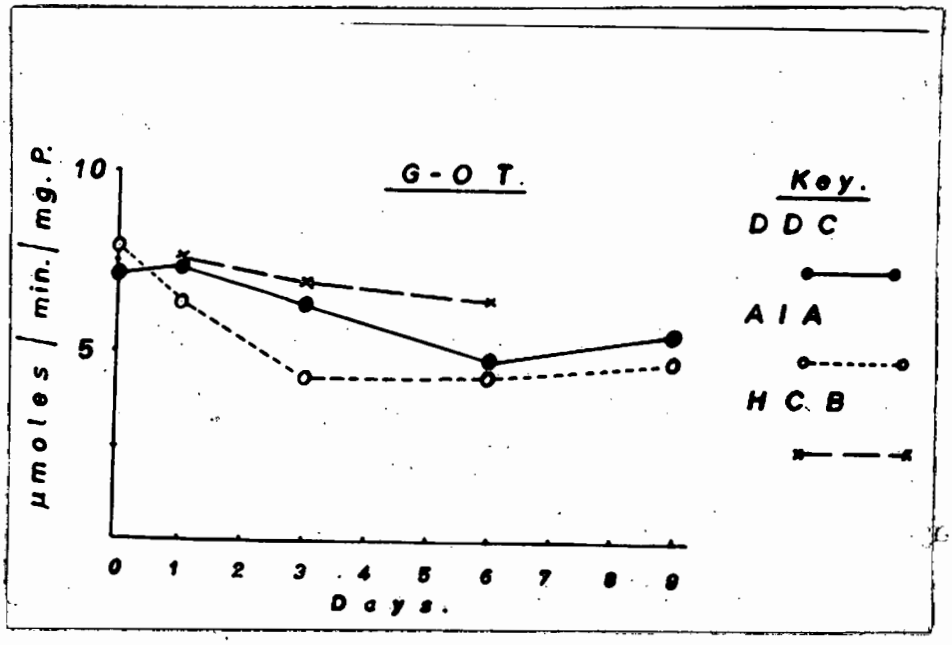


Figure 30.

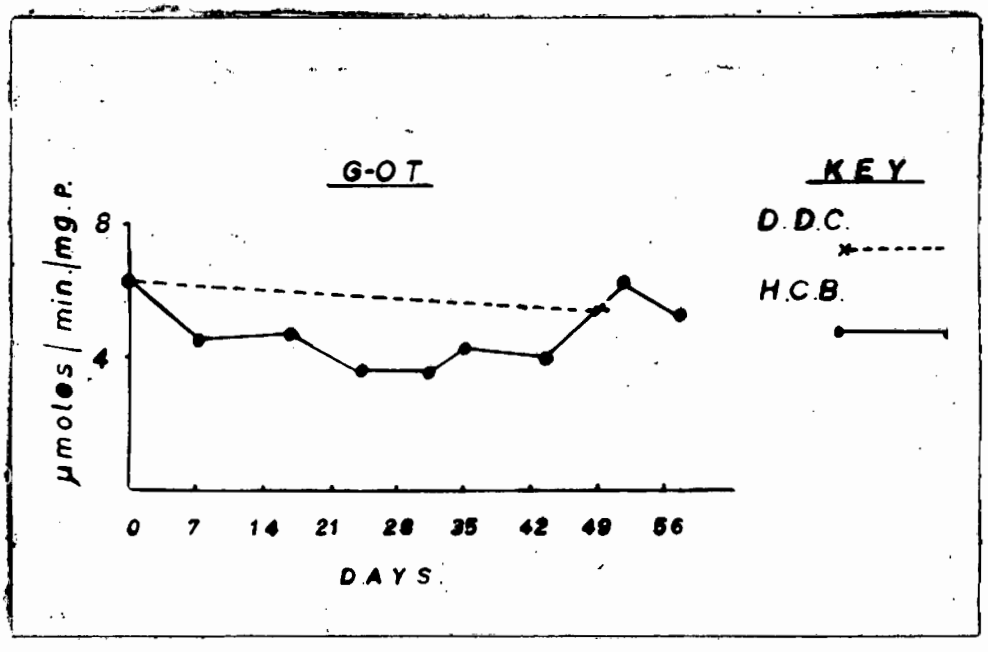


Figure 31.

Time studies.

The influence of the duration of drug treatment upon the development of these changes is shown in Tables 36, 38, 39 and 40 and is summarised in Figures 30 and 31.

Activity in the DDC-treated rats began to fall after the 3rd day of treatment and reached minimum levels after 6 days of treatment. There was a slight increase in activity after the 9 days.

AIA-treated animals showed a steady fall in activity from the onset until the 3rd day after which a relatively constant level was maintained until the end of the experiment.

HCB-treated animals showed a slight fall in activity after 6 days of treatment.

In the animals which were fed HCB in the diet a marked fall in enzyme activity was apparent after a week, after which the level of activity remained more or less constant for the next 5 weeks. Then a return of activity to near normal levels occurred.

The rats fed DDC in the diet for 49 days showed no appreciable change in their level of hepatic glutamic-oxaloacetic transaminase activity.

Table 34.

Glutamic-oxaloacetic transaminase activity in the livers of rats administered DDC by stomach tube for 6 days.

	µmoles/min/mgP.			
	Normally fed		Starved for 18 hrs.	
	Controls	Treated	Controls	Treated
Level of activity in individual livers	7.50 7.14 6.65 6.78 7.50 7.60 4.57 7.79 4.90 5.95	4.84 4.84 4.78 4.29 5.78 3.04 4.37	6.25 5.73 6.71 5.38 9.10 6.70 8.64	6.96 5.02 7.25 4.43 5.32 5.98 3.91 4.99 4.61 5.65
Number	10	7	7	10
Mean $\pm$ S.E.	6.65 $\pm$ 0.36	4.55 $\pm$ 0.31	6.93 $\pm$ 0.53	5.36 $\pm$ 0.34
Range	4.90 - 7.29	3.04 - 5.78	5.38 - 9.10	3.91 - 7.25
P.	<0.001		<0.05	

All female rats

Weight range:- 164 - 242 G.

Determinations made over period 21st May to 16th July.1963.

Table 35.

Glutamic-oxaloacetic transaminase activity in the livers of rats administered AIA by stomach tube for 6 days.

	µmoles/min/mgP.	
	Controls	Treated
Level of activity in individual livers	7.00 8.28 7.75 11.41 8.39 10.63 7.40	4.60 4.70 5.03 3.24 2.72 6.89
Number	7	6
Mean ± S.E.	8.70 ± 0.63	4.53 ± 0.61
Range	7.00 - 11.41	2.72 - 6.89
P.	<0.001	

All male rats

Weight range:- 147 - 241 G. (Majority 170-210 G.).

Determinations made over period 15th October to 15th November, 1963.

All rats starved for 18 hours before being killed.

Table 36.

Glutamic-oxaloacetic transaminase activity in the livers of rats given HCB as a 0.2% mixture in the diet over a period of 57 days. Statistical comparisons are shown between control rats and rats receiving HCB for 16 - 57 days.

	μmoles/min/mgP.								
	Controls	7 days	16 days	23 days	31 days	35 days	43 days	51 days	57 days
Level of activity in individual livers	5.68 5.23 6.32 9.11 5.37	4.41 5.41 3.34	4.70 3.58 5.88	3.41 3.58 3.22	3.48 3.32 3.16	4.57 3.62	5.11 4.03 2.90	5.29 6.91	5.35 4.93
Mean	6.34	4.38	4.75	3.40	3.32	4.10	4.00	6.10	5.14
Number	5		18						
Mean ± S.E.	6.34 ± 0.70		4.27 ± 0.27						
Range	5.23 - 9.11		2.90 - 6.91						
P.	< 0.02								

All female rats. Weight range:- 85 - 177 G. (Majority 100-150 G.).

Determinations made over period 14th October to 3rd December, 1963.

All rats starved for 18 hours before being killed.

In a similar investigation performed on 30th September, 1963, with somewhat heavier animals, the mean level of activity in two rats fed HCB for 40 days was 7.32 μmoles/min/mgP. The individual levels were 5.45 and 9.10 μmoles/min/mgP. A control rat studied on the same day had a level of activity of 10.95 μmoles/min/mgP.

Table 37.

Glutamic-oxaloacetic transaminase activity in the livers of rats administered DDC by stomach tube over a period of 9 days.

	µmoles/min/mgP.				
	Day 0	1 day	3 days	6 days	9 days
Level of activity in individual livers	7.50 7.14 6.65	7.45 7.59 6.00	6.57 5.91 5.60	4.84 4.84 4.78	5.78 5.21 5.10
Mean	7.10	7.01	6.03	4.82	5.36

All female rats

Weight range:- 198 - 254 G.

Determinations made over period 21st May to 30th May,1963.

All rats normally fed.

Table 38.

Glutamic-oxaloacetic transaminase activity in the livers of rats administered AIA by stomach tube over a period of 9 days.

	µmoles/min/mgP.				
	Day 0	1 day	3 days	6 days	9 days
Level of activity in individual livers	7.00 8.28 7.75	6.93 7.09 4.38	4.00 3.69 4.31	4.60 4.70 5.03	4.03 5.40
Mean	7.67	6.14	4.00	4.77	4.72

All male rats

Weight range:- 168 - 241 G.

Determinations made over period 15th October to 24th October,1963.

All rats starved for 18 hours before being killed.

Table 39.

Glutamic-oxaloacetic transaminase activity in the livers of rats administered HCB by stomach tube over a period of 6 days.

	μmoles/min/mgP.		
	1 day	3 days	6 days
Level of activity in individual livers	7.74 7.75 6.90	7.15 6.05 7.05	7.75 3.54 7.60
Mean	7.47	6.75	6.30

All female rats

Weight range:- 166 - 198 G.

Determinations made over period 2nd October to 7th October, 1963.

All rats starved for 18 hours before being killed.

Table 40.

Glutamic-oxaloacetic transaminase activity in the livers of rats fed DDC as a 0.2% mixture in the diet for a period of 49 days.

	μmoles/min/mgP.	
	Treated rats	Control rat
Level of activity in individual livers	6.17 6.66 5.00	7.50
Mean	5.94	6.34 *

All female rats

Weight range:- 153 - 160 G.

Determinations made on 25th November, 1963.

All rats starved for 18 hours before being killed.

* The mean for a series of normal female rats of equivalent weight range investigated over the period 14th October, 1963 to 3rd December, 1963 was 6.34 ± 0.70 and the range 5.23 - 9.11 μmoles/min/mgP.

ALA SYNTHETASE ACTIVITY.

Controls.

The level of activity of this enzyme in normal rat livers was very low as measured in vitro - approaching in fact the limits of accuracy of the experimental techniques employed, and it was, therefore, not possible to assess the influence of nutritional status, sex, age and weight, and seasonal and climatic factors upon its activity.

The level of activity recorded in individual control animal livers is shown in Tables 41 and 43. Statistical comparisons have been made between mean levels obtaining in the various drug-treated groups and a mean control value incorporating levels in all the control animals studied.

It is also evident that measurements of enzyme activity in control animal livers were largely independent of the nature of the substrate. The range of activity with pyruvate as substrate varied from 0 - 3.8 mμmoles/ ALA formed / mg. mitochondrial nitrogen/2 hours; and the mean for the entire group of 10 controls was 1.5 ± 0.4 mμmoles.

The range of activity with citrate as substrate was 0 - 9.2, with eight of the nine control animals falling in the range 0 - 4.9. The mean for the entire group was 3.5 ± 0.9 mμmoles/mg. mitochondrial N/2 hrs.

In the drug-treated animals IN ALL OF WHICH AN INCREASED LEVEL OF ACTIVITY OF THIS ENZYME WAS APPARENT, there was very

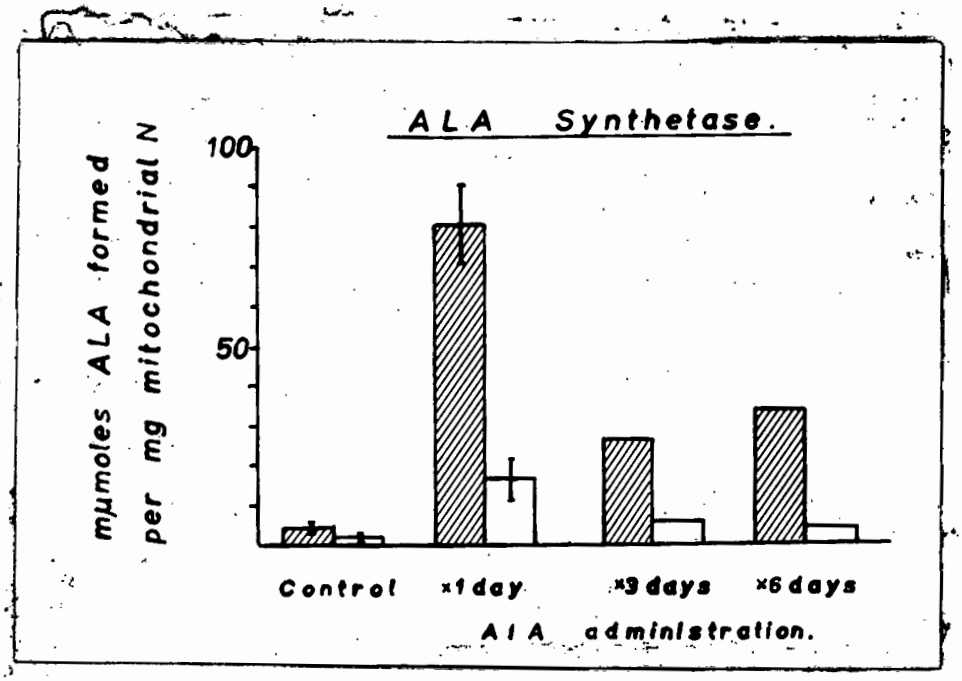

ALA Synthetase.
mμmoles ALA formed per mg mitochondrial N
100
50
Control
×1 day
×3 days
×6 days
ALA administration.

Figure 32.

pronounced evidence of substrate dependence.

DDC.

The liver mitochondria of animals administered DDC by stomach tube for 6 days (Table 41, Figure 34) had a mean level of activity of ALA synthetase of 15.5 ± 3.4 mμmoles/mg. mitochondrial N. when citrate served as substrate and of only 5.0 ± 1.2 with pyruvate as substrate. The citrate-based activity was five times greater than normal ($p = < 0.01$), while the pyruvate-based activity was increased to three times the control level. ($p = < 0.05$).

Statistical analyses are not permitted for the groups treated with DDC for different periods of time, but it is evident from Table 42 that enzyme activity was already increased 24 hours after administering the drug for the first time in one of the two animals studied, and was almost above the normal range in the other. The same increased level of activity was evident in animals studied after 3 days of drug administration, and also in animals fed DDC in the diet for 49 days.

AIA.

The effect of administering AIA by stomach tube for varying periods of time is shown in Table 43 and is summarised in Figure 32.

Maximum activity obtained after the first 24 hours of treatment when a mean of 80 ± 10 mμmoles of ALA were synthesised in 2 hours per mg. of mitochondrial N. with citrate as substrate, and

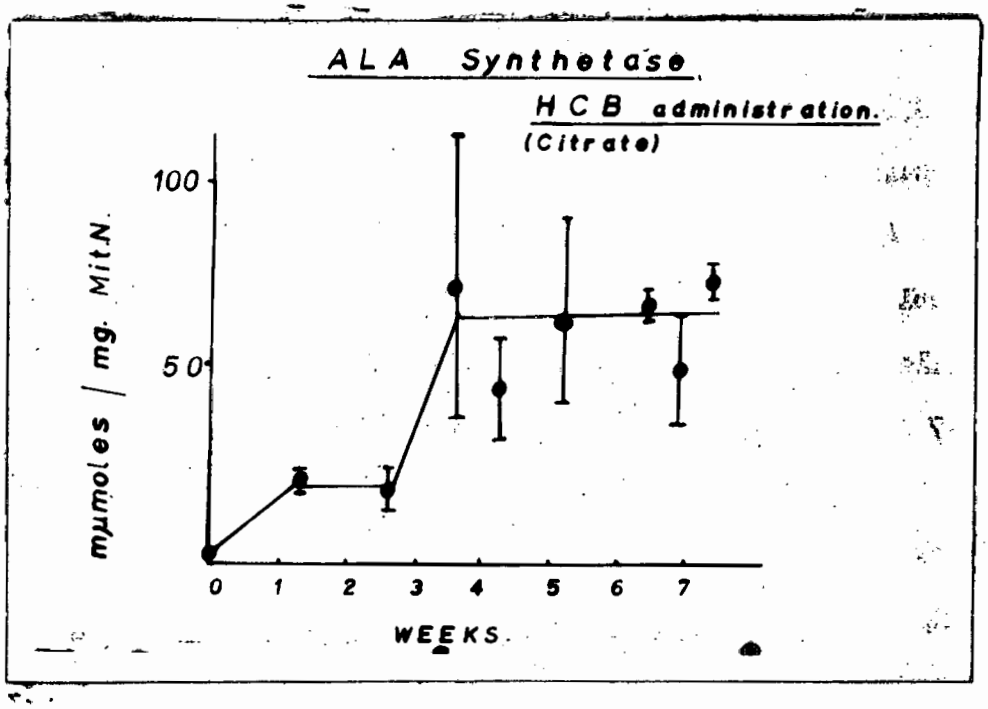

ALA Synthetase
HCB administration.
(Citrate)
100
50
mμmoles / mg. Mit.N.
0
1
2
3
4
5
6
7
WEEKS.

Figure 33.

a mean of 16.5 ± 5 mμmoles with pyruvate as substrate. This represents a fortyfold increase in activity as compared with levels of activity in control animals in the case of citrate, and a tenfold increase in the case of pyruvate. ($p = < 0.001$ in both instances).

After 3 days of drug administration a mean of only 25 mumoles was synthesised from citrate and of 4.7 from pyruvate, while after 6 days, 33 mμmoles were synthesised from citrate, and 2.7 from pyruvate - per mg. mitochondrial N/2 hrs.

AIA administration, therefore, resulted in a very rapid and pronounced increase in hepatic ALA synthetase activity which then quickly fell to lower, but nevertheless much greater than normal, levels.

HCB.

Control animals of equivalent sex and weight range were not studied concurrently with the HCB-treated animals as regards measurement of ALA synthetase activity. However, control male animals were studied throughout the period of this investigation (although not on the same days). As discussed previously, all the control animals studied, irrespective of sex and weight, had very low levels of activity of this enzyme, and the same corporate control group has been utilised for comparative analyses.

Feeding of HCB in the diet resulted in a moderate increase in ALA synthetase activity after 9 days, which remained constant

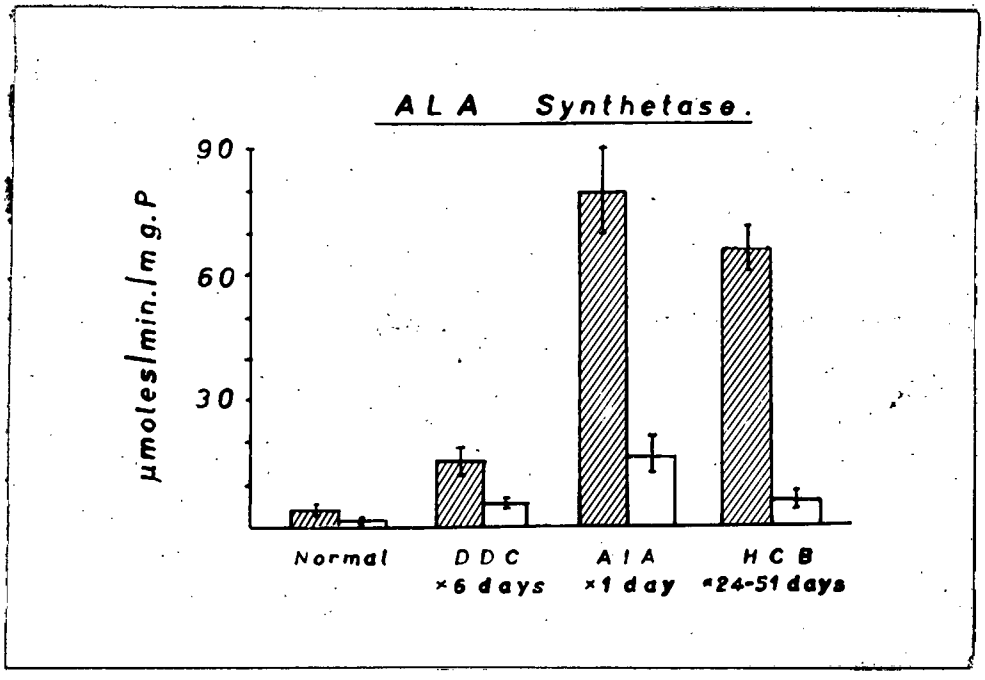

ALA Synthetase.
μmoles/min./mg.P
90
60
30
Normal
DDC
×6 days
AIA
×1 day
HCB
×24-51 days

Figure 34.

to the 18th day of drug administration and which then increased rapidly after 24 days again to remain constant, but at a greatly elevated level. (Table 44 and Figure 33).

The mean levels of activity found in the livers of twelve animals studied between the 24th and the 51st days of drug feeding were 66.5 ± 7.0 mμmoles/mg. mitochondrial N/2 hrs (citrate) and 5.3 ± 1.8 (pyruvate). The increase with citrate as substrate was highly significant, ($p = < 0.001$) and it is very interesting that in this group there should have been no increased activity with pyruvate as substrate. Moreover, oxidation of pyruvate by these liver mitochondria was shown to be impaired. (Table 67).

The changes occurring in levels of ALA synthetase activity with drug-feeding are summarised graphically in Figure 34.

ALA synthetase activity was increased to more or less the same extent in both ALA- and HCB-fed rats. In the DDC-fed rats this increase was very much less. This difference may have been due purely to intrinsic differences in the modes of action of the drugs, but it should be noted that the DDC-treated rats were normally fed until the time of being killed, while rats in each of the other two groups were starved for 18 hours. Through oversight, animals studied after 18 days of HCB-administration were not fasted, and this may have contributed towards the perhaps unexpectedly low levels of activity in these rats.

Table 41.

ALA synthetase activity in the livers of rats administered DDC by stomach tube for 6 days.

	mumoles/mg mitochondrial N/2hrs.			
	Pyruvate as substrate		Citrate as substrate	
	Normal	Treated	Normal	Treated
Level of activity in individual livers	0.8 2.6 0.0 3.8 3.2 1.6	9.1 4.9 1.8 5.6 3.4	0.0 0.5 3.8 4.9 4.2	17.3 31.2 14.2 6.4 15.4 8.2
Number	6	5	5	6
Mean $\pm$ S.E.	2.0 $\pm$ 0.6	5.0 $\pm$ 1.2	2.7 $\pm$ 1	15.5 $\pm$ 3.4
Range	0 - 3.8	1.8 - 9.1	0 - 4.9	6.4 - 31.2
* p.	not significant		0.01	

All female rats

Weight range:- 177 - 207 G.

Determinations made over period 8th August to 23rd August, 1963.

All rats normally fed

* Statistical comparisons are shown between drug-treated animals and the mean value in all the control animals. (See text).

Table 42.

ALA synthetase activity in the livers of rats administered DDC over varying periods of time.

mμmoles/mg. mitochondrial N/2 hrs.

	Pyruvate as substrate					Citrate as substrate				
	Day 0	1 day	3 days	6 days	49 days	Day 0	1 day	3 days	6 days	49 days
Level of activity in individual livers	Table 41	4.6 1.8	5.1 7.4	Table 41	2.8 4.5	Table 41	33.0 5.7	8.2 12.8 18.5*	Table 41	24.2 14.4
Number	6	2	2	5	2	5	2	4	6	2
Mean	2.0	3.2	6.3	5.0	3.5	2.7	19.4	14.0	15.5	19.3
Weight range:-	172 - 255 G.				143 - 150G.					
Period	8/8 - 23/8	1/10/63 - 11/10/63		8/8 - 23/8	26/11/63					
All female rats All rats normally fed					Starved for 18 hrs.					

* Level of activity in pooled mitochondria from 2 rat livers - treated as two separate results when calculating mean.

Table 43.

ALA synthetase activity in the livers of rats administered AIA by stomach tube over a period of 6 days.

mumoles/mg mitochondrial N/2 hrs.

	Pyruvate as substrate				Citrate as substrate			
	Day 0	1 day	3 days	6 days	Day 0	1 day	3 days	6 days
Level of activity in individual livers	2.1 0.5 0.7 0.0	31.2 2.7 9.4 19.8 *	7.9 3.3 0.9 6.7	2.5 3.1 2.6	9.2 2.6 2.3 4.1	72 119 40 76 86*	44 21 12 25	31 32 34
Number	4	5	4	3	4	6	4	3
Mean ± S.E.	0.8	16.5 ± 5	4.7	2.7	4.5	80 ± 10	25	33
p. **	.001				.001			

All male rats

Weight range:- 151 - 209 G.

Determinations made over period 29th October to 11th December, 1963.

All animals starved for 18 hours before being killed.

* Level of activity of pooled mitochondria from 2 rat livers - treated as two separate results in statistical analyses.

** Statistical comparisons are shown between the mean for all control animals (see text) and animals administered AIA for one day.

Table 44.

ALA synthetase activity in the livers of rats fed HCB in the diet for periods varying up to 51 days.

mumoles/mg mitochondrial N/2 hrs.

	Pyruvate as substrate							
	9 days	18 days	24 days	29 days	36 days	45 days	48 days	51 days
Level of activity in individual livers	2.2 2.3	5.9 2.4	1.6 14.4	5.5 20.2	2.3 4.7	0.7 0.0	* 2.3 2.6	5.3 4.2
Mean	2.3	4.2	8.0	12.9	3.5	0.4	2.5	4.8
Number			12					
Mean ± S.E.			5.3 ± 1.8					
Range			0.0 - 20.2					

			Citrate as substrate					
	9 days	18 days	24 days	29 days	36 days	45 days	48 days	51 days
Level of activity in individual livers	17.1 15.4	17.1 13.9	39.6 116.0	36.6 55.0	95.5 47.8	75.0 71.4	* 38 71	77.5 74.5
Mean	16.3	12.5	77.8	45.8	71.7	73.2	54	76
Number			12					
Mean ± S.E.			66.5 ± 7.0					
Range			36.6 - 116					
** p.			< .001					

All female rats. Weight range:- 110 - 145 G.

All starved for 18 hours before being killed - except 18 day group.

Determinations made over period 16th October to 27th November, 1963.

* Separate experiments - performed on 8th October, 1963.

** Statistical comparisons are shown between the mean for all the control animals, and the mean for animals fed HCB in the diet for 24 - 51 days (see text).

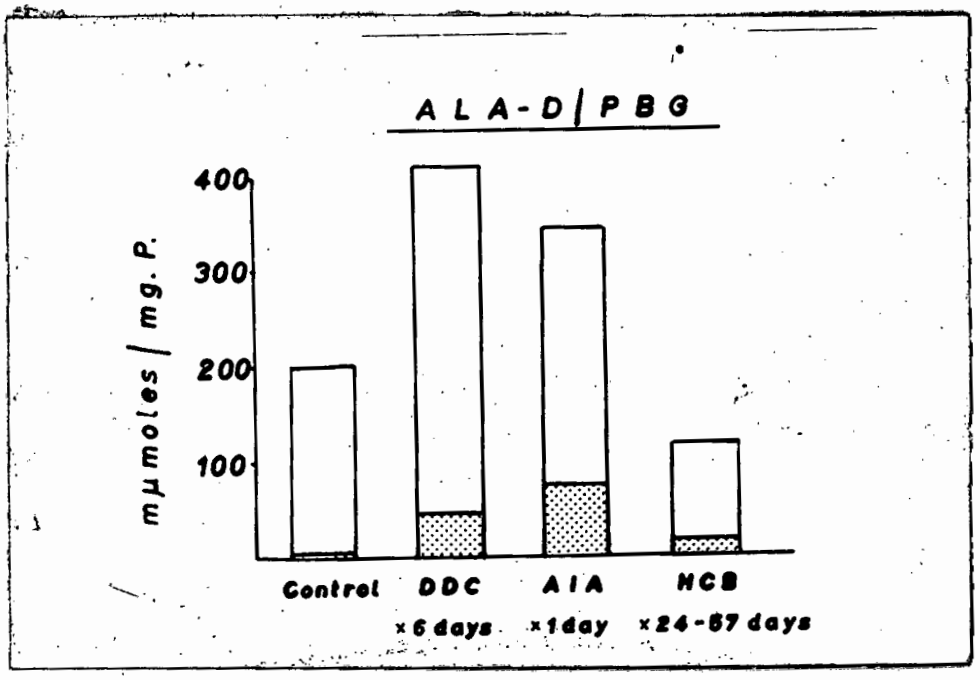

ALA-D/PBG
mμmoles/mg.P.
400
300
200
100
Control
DDC
×6 days
AIA
×1day
HCB
×24-67 days

Figure 35.

HEPATIC ALA DEHYDRASE ACTIVITY AND PORPHOBILINOGEN CONCENTRATION.

Controls.

The normal level of hepatic ALA dehydrase activity was fairly constant despite variations in the nutritional status, sex and weight range of the animals studied; and seasonal factors seem to have been unimportant.

These results are shown in Tables 45 - 47 and are summarised in Figure 14.

The mean levels of activity in 3 groups of normal rat livers were 206 ± 15, 215 ± 18 and 186 ± 5 mμmoles PBG formed/hour/mg. tissue P. The range of activity for the entire group of controls was 163 - 257 mμmoles/hour/mgP, and the mean 200.

Changes in the level of activity of this enzyme occurred with each of the three drugs administered, but these changes were very different according to the drug used.

DDC.

Administration of DDC (Table 45, Figure 35) resulted in a twofold INCREASE in activity. The mean level of activity in the livers of rats administered DDC for 6 days was 374 ± 43 mμmoles/hour/mgP. ($p = < 0.01$).

After one day there was but a slight and insignificant increase in activity in the liver of only one of the two animals studied; and after 49 days of drug-feeding the activities in the livers of the two animals studied were within the normal range.

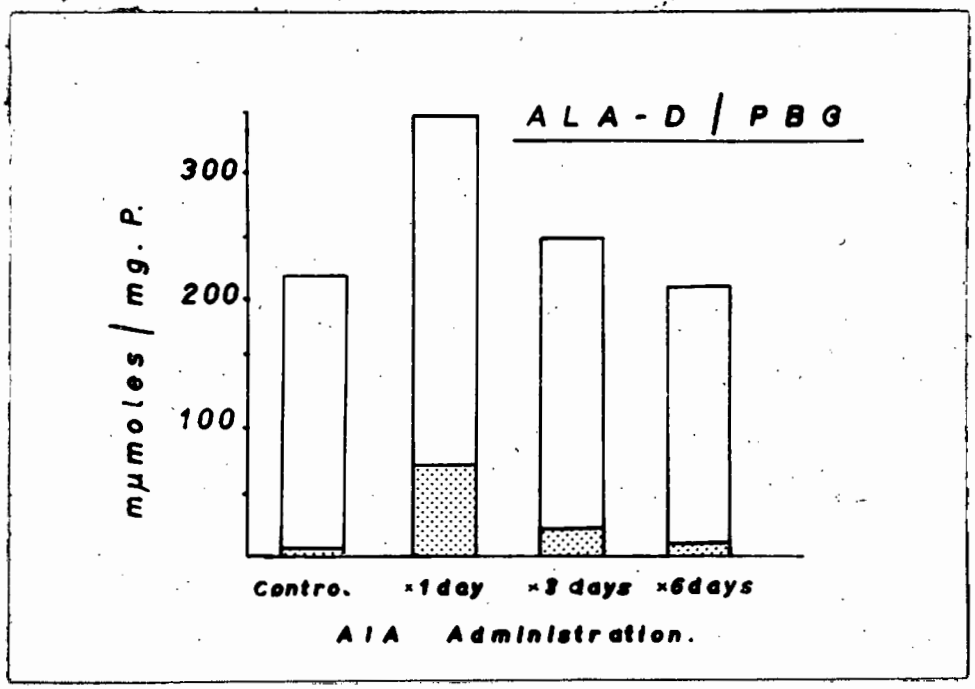


Figure 36.

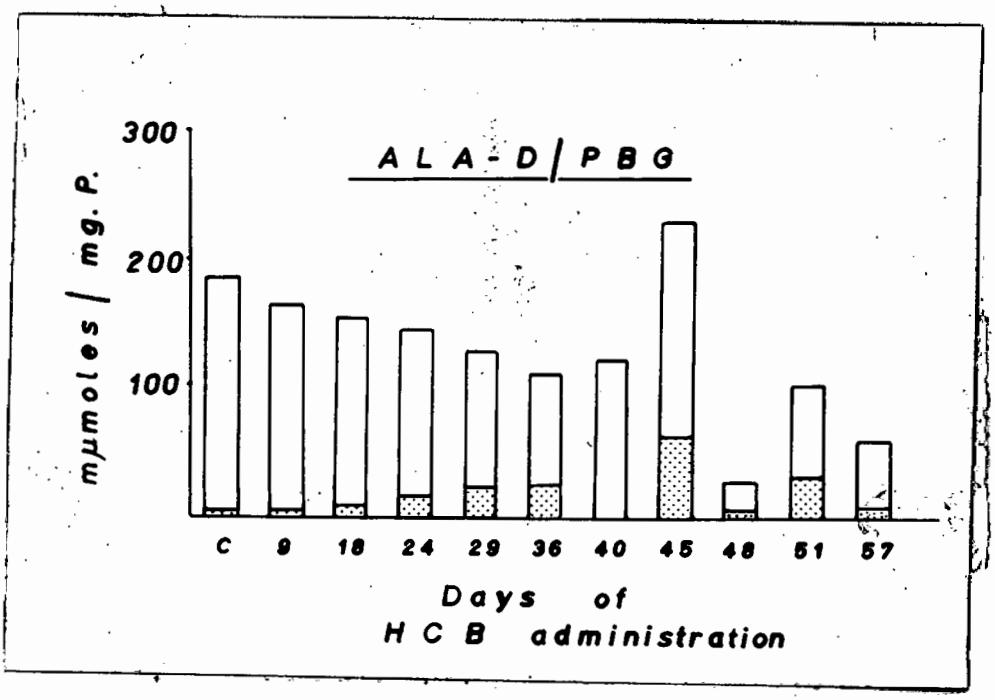


Figure 37.

Technical error precludes the presentation of results after 3 days of drug administration.

AIA.

Administration of AIA for one day resulted in a slight increase in the mean level of activity of this enzyme to 273 ± 23 mμmoles/hour/mgP. (Not significant).

After 3 days the activity had returned to normal levels.

These results are shown in Table 46, and are summarised graphically in Figure 36.

HCB.

HCB-fed rats (Table 47) showed a striking and progressive DECREASE in the activity of this enzyme almost directly proportional to the duration of feeding the drug. This very striking finding is summarised in Figure 37.

After 45 days a sudden sharp increase to well above normal levels occurred in the activity of the enzyme. I can offer no explanation for this finding besides invoking that of technical error, but apart from this single exception, a steady decrease was evident.

The mean level of activity of the enzyme in animals fed HCB for between 24 and 57 days was 100 ± 15 mμmoles/hour/mgP. This is significantly lower than that found in the corresponding control group. ($p = < 0.001$).

Porphobilinogen content of the liver.

Normal animal livers contained negligible amounts of porphobilinogen. The livers of DDC-treated animals contained a moderate amount of PBG after the drug had been administered for 6 days. (Table 45, Figure 35), and there was a very slight increase after 1 day in one of the two animals studied. After 49 days of drug feeding the content of PBG was again very small.

The AIA-treated rats showed a very marked increase in liver PBG content 24 hours after the drug was administered (mean 73 mμmoles/mgP.; range 22 - 121). After 3 days the PBG content had fallen to a mean of 19 mμmoles/mgP. and after 6 days had almost returned to normal levels, being only 7 mμmoles/mgP. (Table 46, Figure 36).

HCB-administration resulted in a moderate increase in liver PBG content from about the 24th to the 51st days of feeding the drug. The occurrence of PBG in the livers of these animals was very variable as may be seen from Table 47 and Figure 37. The mean liver PBG content of the entire group was only 9, and the range 0 - 68 mμmoles/mgP.

Table 45.

ALA dehydrase activity in, and porphobilinogen content of, the livers of rats administered DDC by stomach tube for 6 days.

mumoles/mgP - formed per hour.

	Normal		Treated 1 day		Treated 6 days		Treated 49 days	
Porphobilinogen	Formed	Present	Formed	Present	Formed	Present	Formed	Present
Level	163 197 250 193 228	0 0 1 2 0	266 185	8 0	163 408 441 356 445 433	0 87 24 26 65 32	172 184	1 2
Mean ± S.E.	206 ± 15	0.6 ± 0.4	226	4	374 ± 43	39 ± 13	178	2
P		-	-	-	< 0.01	< 0.02	-	-
Weight range	160 - 214 G.						143 - 150 G.	
Period of exp.	15/8/63 - 28/8/63		1/10/63		15/8/63-28/8/63		26/11/63	
Nutritional status	Fed						Starved	

All female rats

Table 46.

ALA dehydrase activity in, and porphobilinogen content of, the livers of rats administered AIA by stomach tube over a period of 6 days.

μmoles/mgP - formed per hour.

	Normal		Treated 1 day		Treated 3 days		Treated 6 days	
	Formed	Present	Formed	Present	Formed	Present	Formed	Present
	169	1	198	38	269	15	236	7
	257	3	253	121	293	1	215	13
	214	2	302	78	57	46	147	2
	220	2	387	35	299	14		
			248	22				
			238	110				
			283	107				
Mean ± S.E.	215 ± 18	2 ± 0.4	273 ± 23	73 ± 15	229	19	199	7
P.			N.S.	<0.001	not significant		not significant	

All male rats

Weight range:- 151 - 209 G.

Determinations made over period 29th October to 11th December, 1963.

All rats were starved for 18 hours before being killed.

Table 47.

ALA dehydrase activity in, and porphobilinogen content of, the livers of rats fed HCB as a 0.2% mixture in the diet over a period of 57 days.

mumoles/mgP. - formed per hour

	Day 0		9 days		18 days		24 days		29 days		36 days		** 40 days		45 days		** 48 days		51 days		57 days	
	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P	F	P
	191	1	165	2	151	3	124	4	66	5	99	25	114	0	214	68	20	3	118	39	53	3
	171	3	163	3	155	4	146	18	149	33	78	17	126	0	203	89	20	2	22	22	53	3
	176	2																				
	196	4																				
	178	1																				
	203	1																				
Mean	186	2	164	2	153	3	135	11	108	19	89	21	120	0	208	58	20	2	70	30	53	3
Mean ± S.E.	186 ± 5	2					Formed 100 - 15								Present 9							
P.							< 0.001															

All female rats

Weight range:- 110 - 145 G.

Determinations made over period 16th October to 3rd December, 1963.

** Separate experiments performed on 30th September, and 8th October, 1963. Rats weighed 155 and 195 G., and 189 and 180 G.

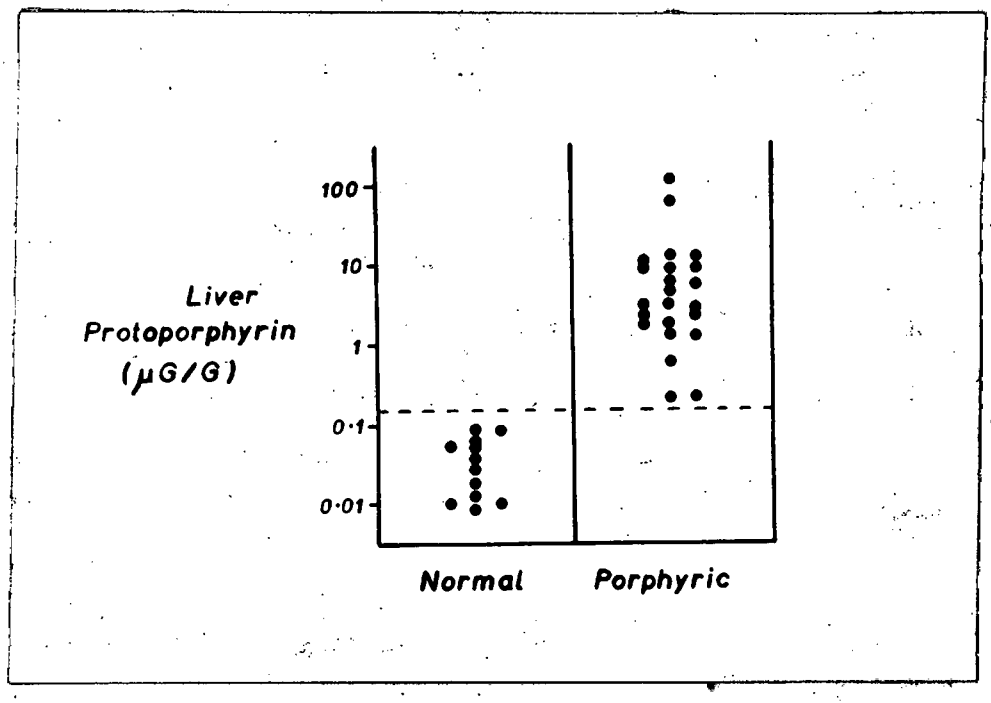


Figure 38.

LIVER PORPHYRINS.

Protoporphyrin concentration only was measured in the livers of the majority of rats treated with DDC.

As may be seen from Table 48, the protoporphyrin content of the livers of rats administered DDC for 6 days was greatly in excess of the minute levels obtaining in normal animal livers.

In the livers of the fed control animals, the protoporphyrin concentration ranged from 0 - 0.06 µg/G wet weight, and in the starved controls the range was 0 - 0.09 µg/G. In the DDC-treated group, the range in the fed animal livers was from 1.70 - 10.90 µg/G, and in the starved animal livers it was from 0.17 - 111.67 µg/G. Although it would seem that the protoporphyrin concentration was greater in the livers of the starved animals, this observation is not supported by statistical analysis ($p = < 0.3 > 0.2$). This lack of statistical significance may possibly be the result of the very wide range of protoporphyrin concentration obtaining in the individual livers - so much so, that Figure 38, comparing protoporphyrin concentrations in normal and porphyric animal livers, has had to be drawn to a logarithmic scale.

As may be seen from Table 51 and Figure 15, the protoporphyrin content of rat livers increased early - within 24 hours - and progressively, with DDC administration.

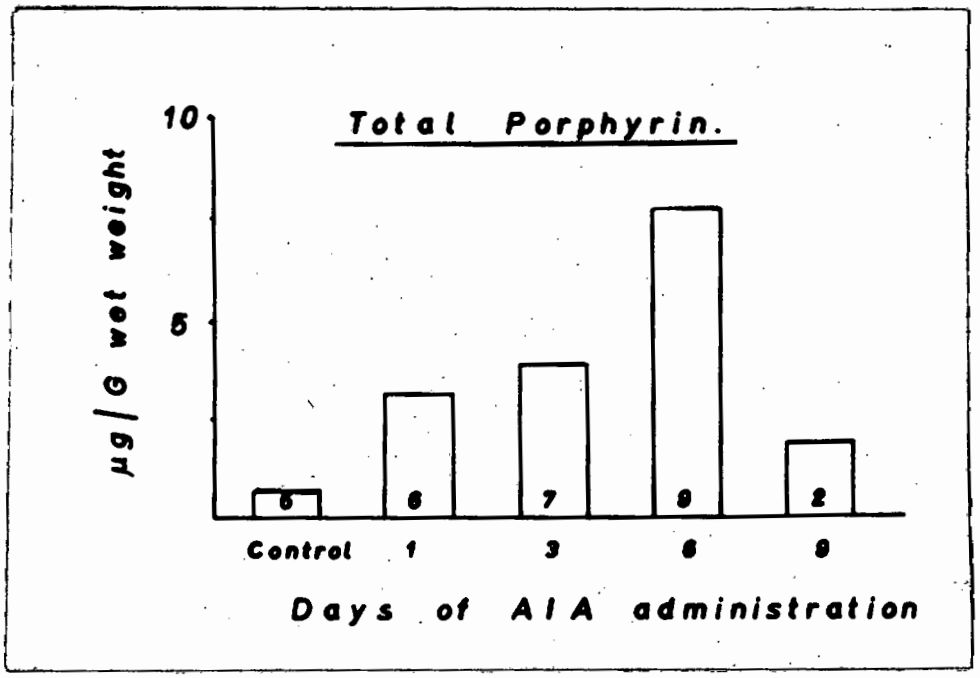


Figure 39.

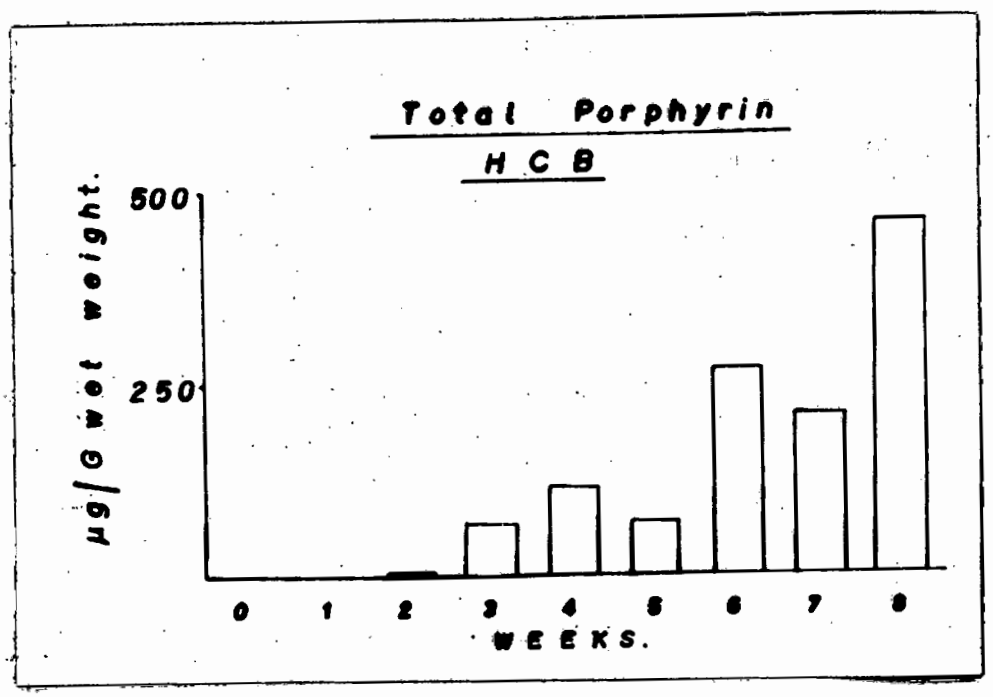


Figure 40.

Total liver porphyrin concentration was measured in AIA- and HCB- treated rats, and in rats receiving DDC for 49 days.

AIA.

Normal male rats had a mean hepatic total porphyrin concentration of 0.59 ± 0.09 µg/G and rats receiving AIA for 6 days had a mean total porphyrin concentration in their livers of 7.02 ± 2.4 µg/G. (Table 49).

An increased hepatic total porphyrin concentration was apparent within 24 hours of AIA administration, and increased progressively over 6 days. After 9 days of drug administration, in the two animals studied the level seemed to be returning towards normal values. (Table 52, Figure 39).

HCB.

Normal female rats had a mean hepatic total porphyrin concentration of 1.56 ± 0.61 µg/G wet weight.

HCB administration resulted in a moderate increase in the total hepatic porphyrin concentration only after 16 days. In animals studied after 9 days the porphyrin concentration was normal.

Between the 3rd and 5th weeks of HCB administration, hepatic total porphyrin concentration was high, and after the 6th week concentrations rose to very high levels. (> 400 µg/G wet weight). These results are shown in Table 50 and summarised in Figure 40.

DDC administration for 7 weeks resulted in a very slight and nonsignificant increase in hepatic total porphyrin concentration.

Table 48.

Protoporphyrin concentration in the livers of rats administered DDC by stomach tube for 6 days.

µg/G wet weight

	Normally fed		Starved for 18 hours	
	Controls	Treated	Controls	Treated
Concentration in individual livers	0.06 0.05 0.04 - 0.01 0.00 0.02 0.02 0.00	1.70 2.76 3.73 10.90 2.15 7.48 1.80	0.09 0.08 0.08 0.00 0.01 0.03	3.12 12.91 2.85 0.17 1.45 1.49 53.11 8.05 111.67 8.69
Numbers	8	7	6	10
Mean ± S.E.	0.03 ± 0.01	4.36 ± 1.34	0.05 ± .02	20.35 ± 11
Range	0.00 - 0.06	1.70 -10.90	0.00 - 0.09	0.17 -111.67
p.	< .001		< .001	

All female rats

Weight range:- 164 - 242 G.

Determinations made over period 21st May to 16th July, 1963.

Table 49.

Total porphyrin concentration in the livers of rats administered AIA by stomach tube for 6 days.

	µg/G wet weight	
	Controls	Treated
Concentration in individual livers	0.41 0.79 0.81 0.76 0.29 0.51	3.14 1.15 2.05 4.92 6.14 22.90 14.50 6.96 1.42
Number	6	9
Mean ± S.E.	0.59 ± 0.09	7.02 ± 2.4
Range	0.29 - 0.81	1.15 - 22.90
p.	<0.02	

All male rats

Weight range:- 147 - 241 G.

Determinations made over period 15th October to 11th December, 1963.

All rats starved for 18 hours before being killed.

Table 50.

Total porphyrin concentration in the livers of rats fed HCB in the diet over a period of 57 days. Statistical comparisons are shown between control animals, and animals fed HCB for 23 - 57 days.

µg/G wet weight

	Day 0	9 days	16 - 18 days	23 - 24 days	29 - 31 days	35 - 36 days	43 - 45 days	51 days	57 days
	1.07	1.85	3.17	42.66	225.51	48.73	465.76	106.26	523.40
	0.54	1.90	6.02	37.92	77.32	49.86	112.82	294.26	402.30
	1.90		2.91	24.73					
	6.23				136.30	68.64	210.30		
	0.78		4.20	81.74	51.44	23.46			
	0.73		1.59		93.54				
	0.22								
	0.95								
	1.66								
Mean	1.56	1.88	3.58	46.76	116.82	47.67	262.96	200.26	462.85
Number	9			20					
Mean ± S.E.	1.56 ± 0.61			153.84 ± 35					
p.	< 0.001								

All female rats. Weight range:- 85 - 177 G.

Determinations made on livers of animals killed during the period 14th October to 3rd December, 1963.

All animals starved for 18 hours before being killed, apart from those studied after 18 days of drug administration.

Table 51.

Total porphyrin concentration in the livers of rats administered DDC by stomach tube over a period of 9 days.

	ug/G wet weight				
	Day 0	1 day	3 days	6 days	9 days
Concentration in individual livers	0.06 0.05 0.04	0.40 1.86 1.18	1.62 2.28 2.02	1.70 2.76 3.73	4.89 7.02 6.18
Mean	0.05	1.14	1.97	2.73	6.03

All female rats. Weight range:- 198 - 254 G.

Determinations made over period 21st May to 30th May, 1963.

All rats normally fed.

Table 52.

Total porphyrin concentration in the livers of rats administered AIA by stomach tube over a period of 9 days.

	ug/G wet weight				
	Day 0	1 day	3 days	6 days	9 days
Concentration in individual livers	0.41 0.79 0.81 0.76 0.29 0.51	1.80 3.22 1.60 3.65 3.66 3.23	6.65 4.75 6.04 2.84 2.06 2.76 1.22	3.14 1.15 2.05 4.92 6.14 22.90 14.50 6.96 1.42	0.57 3.20
Number	6	6	7	9	2
Mean	0.59	2.86	3.76	7.02	1.89

All male rats. Weight range:- 147 - 241 G.

Determinations made over period 15th October to 11th December, 1963.

All rats starved for 18 hours before being killed.

Table 53.

Total porphyrin concentration in the livers of rats fed DDC as a 0.2% mixture in the diet for a period of 49 days.

	µg/G wet weight	
	Treated rats	Control rat
Concentration in individual livers	5.83 1.97 0.82 1.68 1.21	0.62
Mean	2.30 ± 0.80	1.56 *

All female rats.

Weight range:- 153 - 160 G.

Determinations made on 25th and 26th November, 1963.

All rats starved for 18 hours before being killed.

* The mean for a series of normal female rats of equivalent weight range investigated over the period 14th October to 3rd December, 1963 was 1.56 and the range 0.22 - 6.23 µg/G wet weight.

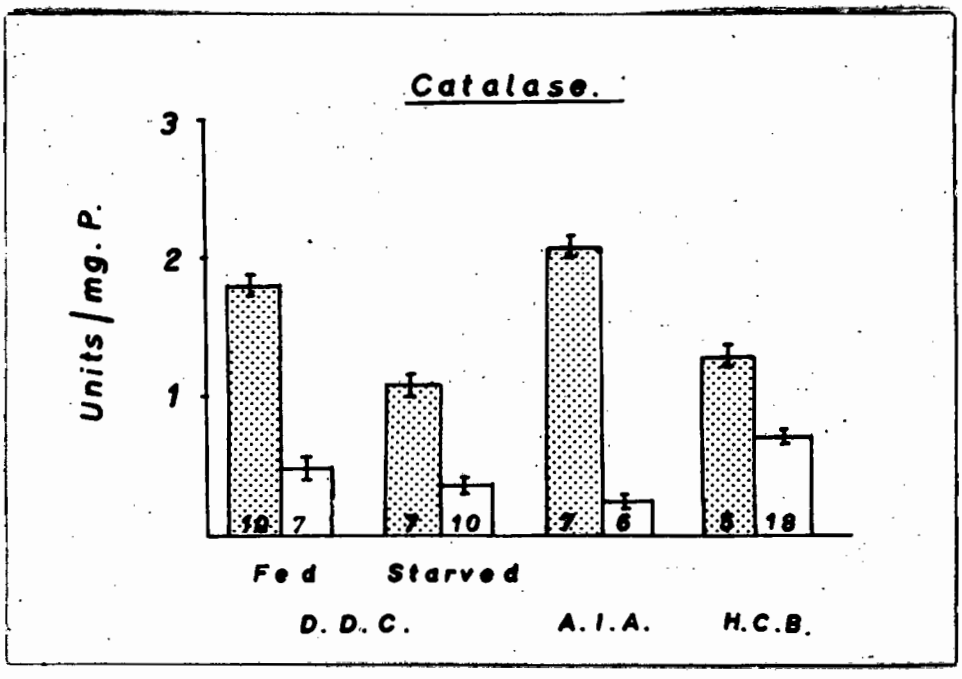


Figure 40.

CATALASE ACTIVITY.

Catalase activity was diminished in all 4 groups of animals investigated.

DDC.

In animals fed DDC for 6 days, the mean level of activity in the fed group was 0.48 ± 0.06 units, and in the starved group it was 0.34 ± .02 units (Table 54).

The corresponding levels of activity in the control animals were 1.80 ± 0.06 and 1.06 ± 0.09 units in the fed and fasted groups respectively. There was thus a reduction of catalase activity to 27% the normal level in the fed group of DDC-treated animals, and to 32% of normal in the fasted group.

AIA.

Treatment with AIA for 6 days (Table 55) resulted in a reduction in the mean level of the activity of this enzyme to 11% of normal. Control animals had a mean level of activity of 2.07 ± 0.05 units, while in the AIA-treated group it was 0.23 ± 0.02.

HCB.

HCB-treated animals, fed the drug in the diet over periods varying between 16 and 57 days (Table 56), had a mean level of hepatic catalase activity of 0.70 ± 0.03 and in the corresponding controls it was 1.29 ± 0.06. This represents a reduction

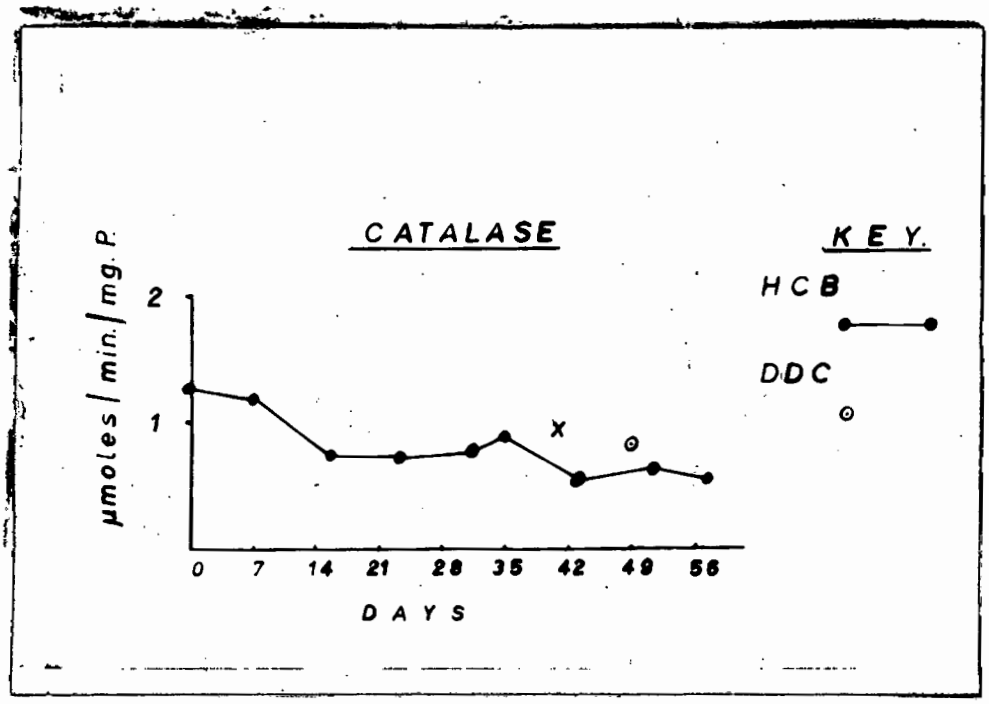


Figure 42.

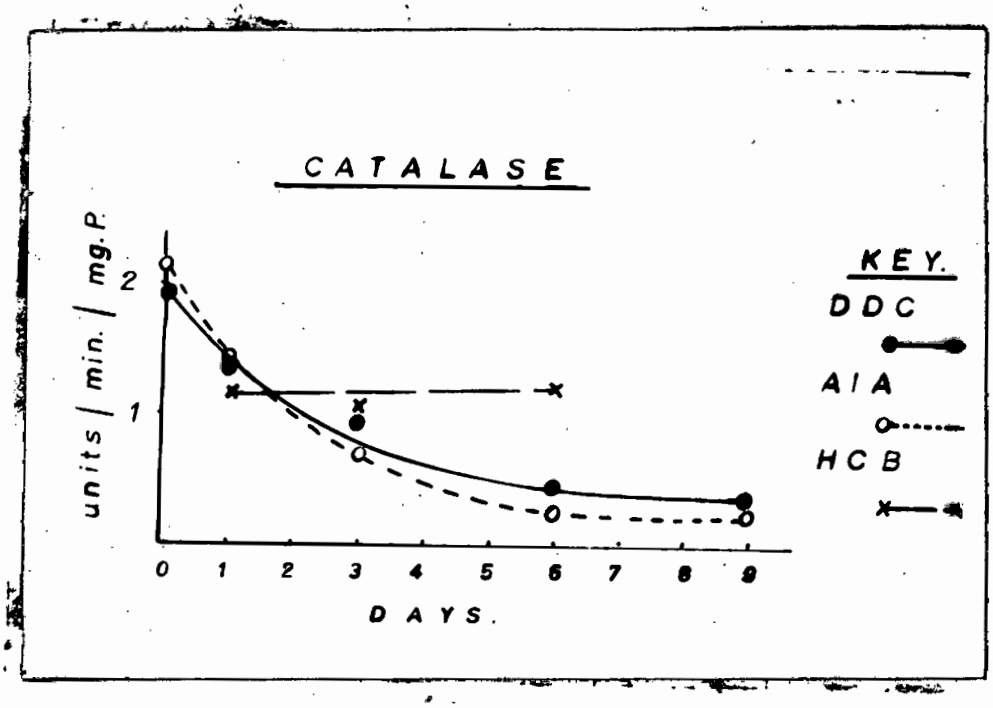


Figure 43.

in activity to 54% the normal level in the HCB-treated animals.

These results are summarised in Figure 41.

Time studies.

Over a period of 9 days DDC-treated animals showed a progressive and rapid reduction in the activity of this enzyme until the 6th day of treatment, after which the level remained fairly constant. (Table 58, Figure 42).

AIA-treated animals showed a very similar pattern of change, although the rate and extent of the reduction was greater. (Table 59, Figure 42).

HCB-treated animals showed no change in the activity of this enzyme over 6 days. (Table 60, Figure 42).

Over a period of 57 days HCB-fed animals maintained their mean level of catalase activity at normal levels for the first 7 days. (Table 56). After 16 days it was reduced to 0.77 units (normal 1.29 $\pm$ 0.06) and this level of activity persisted until the drug had been fed for a total of about 40 days. After 43 days the level of activity was further reduced to 0.57 units, and this new lower level of activity was maintained until the end of the experiment. (Figure 43).

After 49 days of DDC-administration in the diet, activity of this enzyme was reduced from a mean level of 1.29 $\pm$ 0.06 units in a corresponding control group, to one of 0.83 units.

All units are referred to a reference base of 1mg of total tissue phosphorus.

Table 54.

Catalase activity in the livers of rats administered DDC by stomach tube for 6 days.

Units /mgP

	Normally fed		Starved for 18 hours	
	Controls	Treated	Controls	Treated
Level of activity in individual livers	2.23 1.89 1.70 1.89 1.89 1.72 1.70 1.74 1.58 1.71	0.50 0.45 0.43 0.52 0.58 0.41 0.46	1.54 1.01 0.90 0.94 1.11 1.06 0.89	0.39 0.33 0.37 0.42 0.35 0.35 0.26 0.39 0.25 0.33
Number	10	7	7	10
Mean ± S.E.	1.80 ± 0.06	0.48 ± 0.06	1.06 ± 0.09	0.34 ± 0.02
Range	1.58 - 2.23	0.41 - 0.58	0.89 - 1.54	0.25 - 0.42
p.	< 0.001		< 0.001	

All female rats

Weight range:- 164 - 242 G.

Determinations made over period 21st May to 16th July, 1963.

Table 55.

Catalase activity in the livers of rats administered AIA by stomach tube for 6 days.

	Units/mgP	
	Controls	Treated
Level of activity in individual livers	2.16 2.16 2.16 2.19 1.83 2.00 1.97	0.20 0.30 0.23 0.22 0.27 0.17
Number	7	6
Mean ± S.E.	2.07 ± 0.05	0.23 ± 0.02
Range	1.83 - 2.19	0.17 - 0.30
p.	< 0.001	

All male rats

Weight range:- 147 - 241 G. (Majority 170-210 G.).

Determinations made over period 15th October to 15th November, 1963.

All rats starved for 18 hours before being killed.

Table 56.

Catalase activity in the livers of rats fed HCB as a 0.2% mixture in the diet over a period of 57 days. Statistical comparisons are shown between control animals and animals fed HCB for 16 - 57 days.

Units /mgP

	Controls	7 days	16 days	23 days	31 days	35 days	43 days	51 days	57 days
Level of activity in individual livers	1.21 1.18 1.21 1.48 1.35	1.15 1.27 1.18	0.71 0.76 0.83	0.67 0.81 0.68	0.91 0.65 0.76	0.93 0.88	0.54 0.44 0.73	0.73 0.54	0.43 0.64
Mean	1.29	1.20	0.77	0.72	0.78	0.90	0.57	0.63	0.54
Number	5		18						
Mean ± S.E.	1.29 ± 0.06		0.70 ± 0.03						
Range	1.18 - 1.48		0.43 - 0.93						
P.	< 0.001								

All female rats

Weight range:- 85 - 177 G. (Majority 100 - 150 G.).

Determinations made over period 14th October to 3rd December, 1963.

All rats starved for 18 hours before being killed.

In a similar investigation performed on 30th September, 1963, with somewhat heavier animals, the mean level of activity in the livers of two rats fed HCB in the diet for 40 days was 0.96 units. The individual levels were 1.00 and 0.91 units, and in the control animal studied on the same day, the level of activity was 1.01 units.

Table 57.

Catalase activity in the livers of rats administered DDC by stomach tube over a period of 9 days.

	Units/mgP				
	Day 0	1 day	3 days	6 days	9 days
Level of activity in individual livers	2.24 1.89 1.74	1.28 1.46 1.40	1.05 0.95 0.95	0.50 0.45 0.43	0.40 0.39 0.36
Mean	1.96	1.38	0.98	0.46	0.38

All female rats

Weight range:- 198 - 254 G.

Determinations made over period 21st May to 30th May, 1963.

All rats normally fed.

Table 58.

Catalase activity in the livers of rats administered AIA by stomach tube over a period of 9 days.

	Units /mgP				
	Day 0	1 day	3 days	6 days	9 days
Level of activity in individual livers	2.16 2.16 2.16	1.71 1.54 1.22	0.64 0.76 0.72	0.20 0.30 0.23	0.21 0.21
Mean	2.16	1.49	0.71	0.24	0.21

All male rats

Weight range:- 168 - 241 G.

Determinations made over period 15th October to 24th October, 1963.

All rats starved for 18 hours before being killed.

Table 59.

Catalase activity in the livers of rats administered HCB by stomach tube over a period of 6 days.

	Units/mgP.		
	1 day	3 days	6 days
Level of activity in individual livers	1.30 1.13 1.13	0.96 1.10 1.17	1.27 1.20 1.22
Mean	1.19	1.08	1.23

All female rats. Weight range:- 166 - 198 G.

Determinations made over period 2nd October to 7th October, 1963.

All rats starved for 18 hours before being killed.

Table 60.

Catalase activity in the livers of rats administered DDC as a 0.2% mixture in the diet for a period of 49 days.

	Units/mgP.	
	Treated rats	Control rat
Level of activity in individual livers	0.79 0.78 0.91	1.53
Mean	0.83	1.29 *

All female rats. Weight range:- 153 - 160 G.

Determinations made on 25th November, 1963.

All rats starved for 18 hours before being killed.

* The mean for a series of normal female rats of equivalent weight range investigated over the period 14th October to 3rd December, 1963 was 1.29 $\pm$ 0.06 and the range 1.18 - 1.48.

AMINOACETONE SYNTHETASE ACTIVITY.

As opposed to that of ALA synthetase, the level of activity of AA synthetase in normal rat liver mitochondria was fairly high.

It is also evident that pyruvate was superior as a substrate to citrate for its determination.

In normal female rat livers, the mean level of activity with pyruvate as substrate was 24.3± 2.6 mμmoles/2 hours/mg. mitochondrial N. With citrate, only 5.8 ± 0.9 mμmoles of AA were formed in 2 hours per mg. mitochondrial N. In male control animals these figures were 6.4 and 2.9 respectively and the possible significance of this difference between the sexes is discussed in Section 5.

DDC.

DDC-treated animals, after 6 days, had a mean level of activity of this enzyme of 24.0 ± 2.7 (pyruvate) which is almost identical with that of the corresponding control group. With citrate as substrate, the mean was 8.3 ± 1.1 mμmoles/2 hours/mg. mitochondrial N. (Table 61).

Two rats studied after 1 day had an activity of 43 mμmoles/2 hours/mg. N., and after 3 days the level was again 24.7. The significance of this initial rise is doubtful in the face of such small numbers.(Table 62).

After 49 days, the levels of activity in the two rats studied were 3.6 and 6.2 mμmoles/2 hours/mg. mitochondrial N.

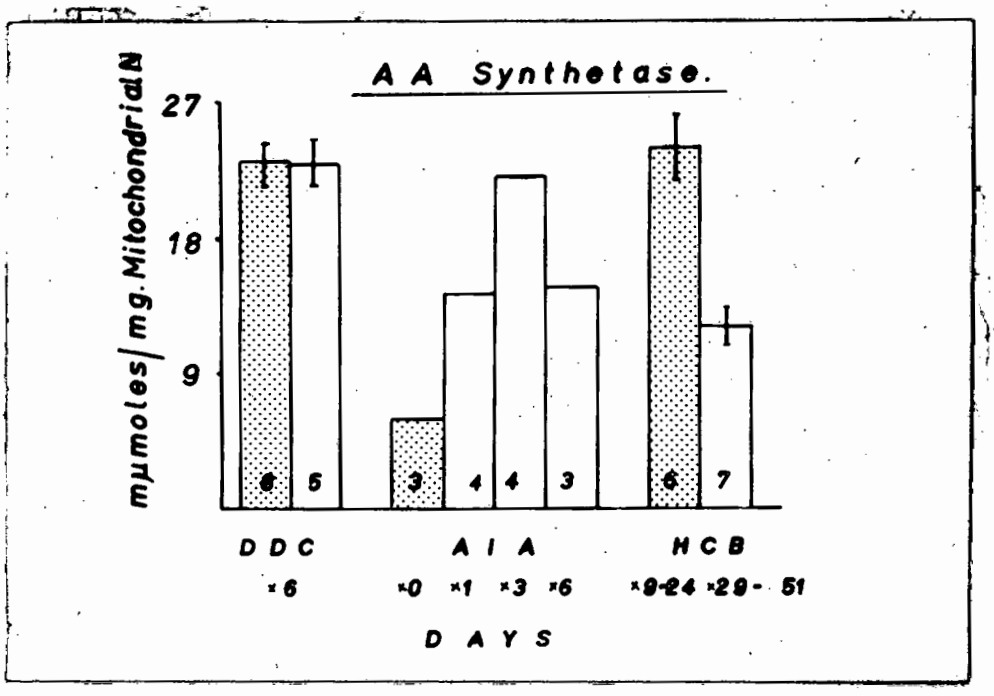

A A Synthetase.
mμmoles/mg.Mitochondrial N
27
18
9
6
5
3
4
4
3
6
7
D D C
×6
A I A
×0 ×1 ×3 ×6
H C B
×9-24 ×29- 51
D A Y S

Figure 44.

It should be stressed that these two rats were starved before being killed while all the other rats in this group were fed until the time of death. (cf. Section 5.).

With citrate as substrate the patterns of change were similar. (Table 62).

AIA.

AIA-administration resulted in a steady increase in activity to the 6th day, and a fall was evident after the 9th day. (Table 63).

HCB.

In the HCB-treated animals, no fully corresponding control group is available for comparison, but it is evident that over the first 24 days of drug treatment there was no real change in the activity of this enzyme, and the mean levels for the six rats studied over this period was 24.6 ± 4.1 mμmoles/2hours/mg.N. (pyruvate), and 7.3 ± 3.4 (citrate) - levels not significantly different from those obtaining in the fed heavier normal group studied in the winter.

Animals fed the drug for between 29 and 51 days showed a significant reduction in the activity of this enzyme, to a level of 12.6 ± 2.6 mμmoles/2 hours/mg.N.(pyruvate) and 5.3 ± 1.3 mμmoles/2 hours/mg. N. (citrate).

These results are summarised graphically in Figure 44.

Table 61.

Aminoacetone synthetase activity in the livers of rats administered DDC by stomach tube for 6 days.

	mμmoles/mg. mitochondrial N/2 hrs.			
	Pyruvate as substrate		Citrate as substrate	
	Normal	Treated	Normal	Treated
Level of activity in individual livers	21.6 26.2 38.2 22.9 15.9 19.5 18.4 31.3	19.7 23.8 24.0 18.4 33.9	5.1 6.0 4.7 4.1 8.9	8.4 9.8 11.6 4.0 6.2 10.0
Number	8	5	5	6
Mean ± S.E.	24.3 ± 2.6	24.0 ± 2.7	5.8 ± 0.9	8.3 ± 1.1
Range	15.9 - 38.2	18.4 - 33.9	4.1 - 8.9	4.0 - 11.6
P.	not significant		not significant	

All female rats

Weight range:- 177 - 209 G.

Determinations made over period 2nd August to 23rd August, 1963.

All rats normally fed.

Table 62.

Aminoacetone synthetase activity in the livers of rats administered DDC for varying periods of time.

mμmoles/mg. mitochondrial N/2 hrs.

	Pyruvate as substrate					Citrate as substrate				
	Day 0	1 day	3 days	6 days	49 days	Day 0	1 day	3 days	6 days	49 days
Level of activity in individual livers	Table 61	43.0 42.0	21.2 28.2	Table 61	3.6 6.2	Table 61	11.4 7.0	6.5 6.1 8.0 *	Table 61	3.8 0.0
Number	8	2	2	5	2	5	2	4	6	2
Mean	24.3	42.5	24.7	24.0	4.9	5.8	9.2	7.2	8.3	1.9
Weight range		177 - 255G.			143 - 150G.					
Period	2/8-23/8	1/10/63 - 11/10/63		8/8/63 23/8/63	26/11/63					
Nutritional status	Normally fed				Starved for 18 hours					

All female rats.

* Level of activity in pooled mitochondria from 2 rat livers - treated as 2 separate results when calculating mean.

Table 63.

AA synthetase activity in the livers of animals administered AIA by stomach tube for the periods shown.

mμmoles/mg mitochondrial N/2 hrs.

	Pyruvate as substrate				Citrate as substrate			
	Day 0	1 day	3 days	6 days	Day 0	1 day	3 days	6 days
level of activity in individual livers	8.9 3.4 6.8	18.1 19.2 9.5 11.0	24.8 19.0 16.3 31.0	11.9 13.5 20.4	4.5 1.2 3.0	7.0 14.0 4.5 9.5	10.2 11.4 4.9 5.8	13.4 1.0
Mean	6.4	15.0	22.8	15.3	2.9	8.5	8.1	7.2

All male rats

Weight range:- 151 - 209 G.

Determinations made over period 24th October to 11th December, 1963.

All animals starved for 18 hours before being killed.

Table 64.

AA synthetase activity in the livers of rats fed HCB in the diet for periods varying up to 51 days.

mumoles/mg. mitochondrial N/2 hrs.

	Pyruvate as substrate						
	9 days	18 days	24 days	29 days	36 days	*48 days	51 days
Level of activity in individual livers	16.4 23.4	38.2 31.8	13.4 27.2	10.9 14.7	- 4.5	6.1 11.2	25.2 15.4
Mean	18.4	35	20.3	12.8	4.5	8.6	20.3
Number	6			7			
Mean ± S.E.	24.6 ± 4.1			12.6 ± 2.6			
**p.	< 0.05						

	Citrate as substrate						
	9 days	18 days	24 days	29 days	36 days	*48 days	51 days
Level of activity in individual livers	3.7 6.1	7.4 7.0	11.8 8.0	8.6 3.5	- 1.2	2.7 -	8.9 6.9
Mean	4.9	7.2	9.9	6.0	1.2	2.7	7.9
Number	6			6			
Mean ± S.E.	7.3 ± 3.4			5.3 ± 1.3			
**p.	<.67>.5						

All female rats. Weight range:- 85 - 177 G.

All rats starved for 18 hours before being killed - except 18 Day group.

Determinations made over period 16th October to 27th November, 1963.

*Separate experiment performed on 8th October, 1963.

** Statistical comparisons are shown between group receiving HCB for 24 days, and those receiving HCB for 29 - 51 days (See text).

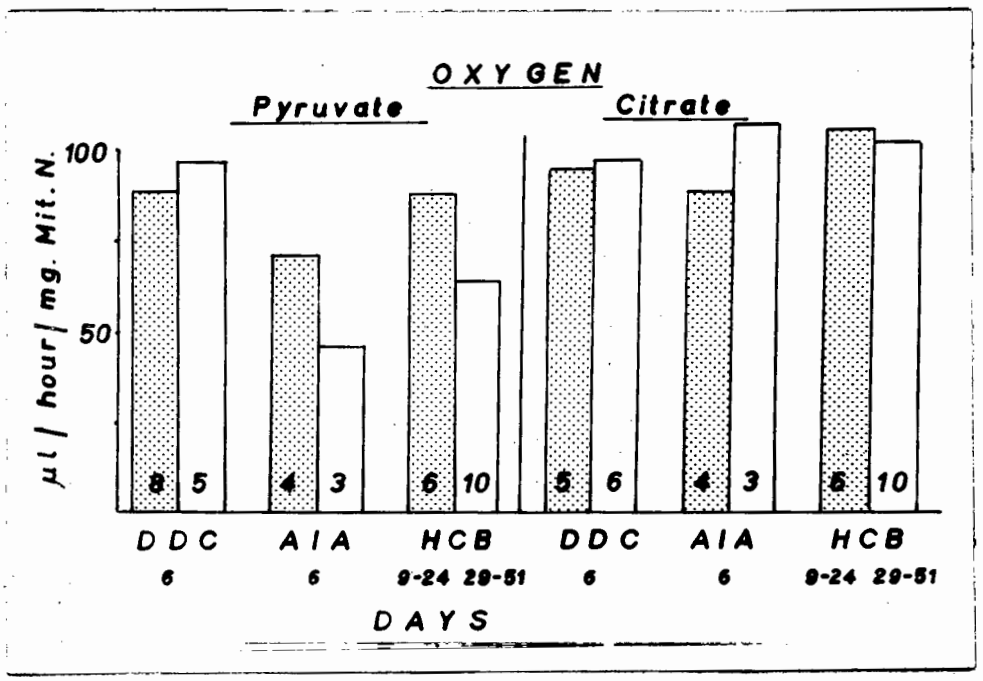

OXYGEN
Pyruvate
Citrate
µl / hour / mg. Mit. N.
100
50
8
5
4
3
6
10
5
6
4
3
5
10
DDC
AIA
HCB
DDC
AIA
HCB
6
6
9-24
29-51
6
6
9-24
29-51
DAYS

Figure 45.

OXYGEN UPTAKE.

Throughout the experiments on the measurement of ALA and AA synthetase activities oxygen consumption was measured as an index of the viability of the mitochondria. In several instances, the rate of oxygen utilisation was noted to be diminished.

When citrate served as substrate, no real change occurred in the rate of oxygen utilisation by any of the rat liver mitochondria. Mitochondria from DDC-treated rat livers showed no alteration in their ability to oxidise pyruvate either. (Table 65). However, AIA administration (Table 66) resulted in a progressive reduction in the ability of the mitochondria to oxidise pyruvate after the 3rd day of drug treatment. Whereas mitochondria from control animal livers utilised oxygen at a mean rate of 70 ul/hour/mg. mitochondrial N. and after one day the mean rate of utilisation of oxygen was 80 umoles/hour, after 3 days this was reduced to 65 ul/hour, and after 6 days it was further reduced to 42 ul/hour. It is also apparent that whereas there was no significant difference in the ability of normal mitochondria to oxidise either pyruvate (70 ul/hour) or citrate (82 ul/hour), after 6 days of AIA administration oxidation of citrate (112 ul/hour) was superior to that of pyruvate (42 ul/hour). It should be noted, however, that there was some overlap amongst these various groups, and

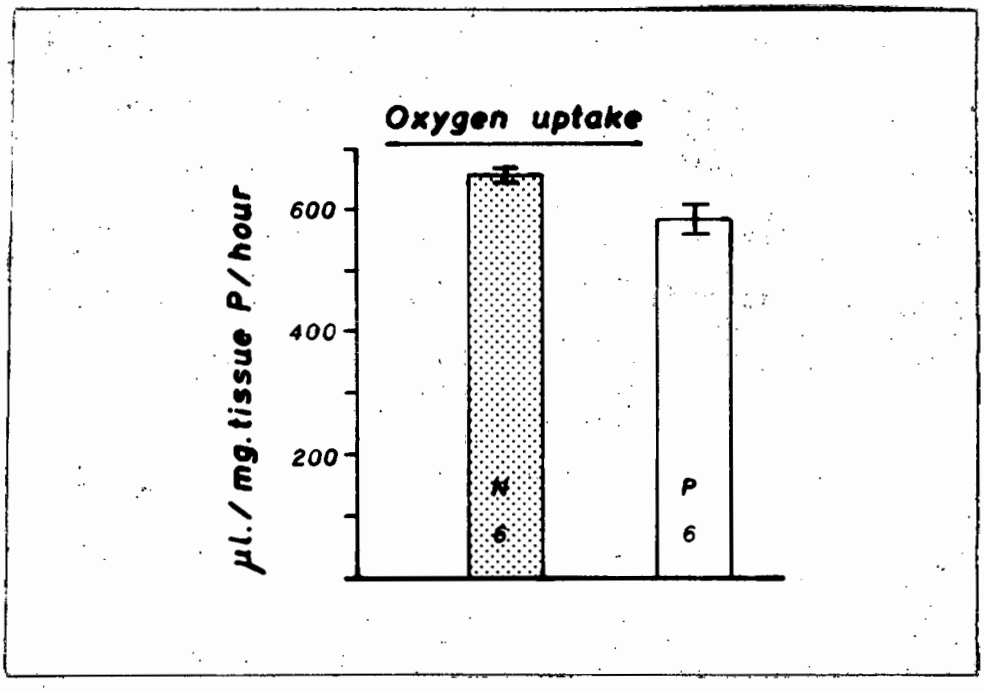


Figure 46.

that the numbers per group are very small.

In the HCB-treated animals, over the first 24 days of drug administration the range of oxygen utilisation by mitochondria from individual livers, with pyruvate as substrate, was 56 - 106, with a mean rate of 85 µl/hour/mg. mitochondrial N.

Between the 29th and 51st days of drug-feeding the range of oxygen utilisation was 36 - 100 µl/hour/mg. mitochondrial N. with a mean rate of 62 $\pm$ 6 µl/hour/mg. N. This rate is significantly less than that over the earlier period. ($p = < 0.05$).

In this group of animals citrate was oxidised at a significantly faster rate (108 $\pm$ 6 µl/hour/mg.N) than was pyruvate over the 9-24 day period of drug-administration ($p = < 0.02$), and over the period 29-51 days, the greater rate of oxidation with citrate as substrate (101 $\pm$ 7 µl/hour/mg.N) was even more marked. ($p = < 0.001$).

These findings suggest that in the drug-induced porphyric state, pyruvate oxidation was impaired.

The findings are summarised in Figure 45.

After 6 days of DDC-administration liver homogenates respiring on endogenous substrate utilised oxygen at a slightly, but significantly, slower rate than did homogenates from normal control animal livers.(Table 68, Figure 46). In these studies, undertaken while measuring rates of glycine oxidation, the homogenates respired in an atmosphere of air, as did the mitochondria in the studies described above.

Table 65.

Oxygen utilisation by mitochondria from the livers of rats administered DDC for varying periods of time.

µl/hour/mg. mitochondrial N.

	Pyruvate as substrate					Citrate as substrate				
	Day 0	1 day	3 days	6 days	49 days	Day 0	1 day	3 days	6 days	49 days
oxygen utilisation by mitochondria from individual livers	91 69 101 92 80 101 74 88	125 94	81 76	70 116 99 97 102	77 98	97 86 86 95 90	117 98	89 93 144*	80 98 131 88 91 95	87 100
Number	8	2	2	5	2	5	2	4	6	2
Mean	87	110	79	97	88	91	108	115	97	94
Mean ± S.E.	87 ± 4.2					91 ± 2.3				
Range	69 - 101			70 - 116		86 - 97			80 - 131	
Period	2/8 - 23/8	1/10-11/10		8/8 - 23/8	26/11					
Nutritional status	Normally fed				Starved for 18 hrs.					

All female animals. Weight range:- 177-255 G.

* Oxygen utilisation by pooled mitochondria from 2 rat livers - treated as two separate results when calculating mean.

Table 66.

Oxygen utilisation by mitochondria from the livers of rats administered AIA for varying periods of time.

	µl/hour/mg. mitochondrial N.							
	Pyruvate				Citrate			
	Normal	1 day	3 days	6 days	Normal	1 day	3 days	6 days
oxygen utilisation by mitochondria from individual livers	87 50 91 51	92 70 65 81 91	71 50 79 61	28 57 40	88 68 95 78	99 105 104 85 97 94 103	84 109 103 61	92 120 123
Number	4	5	4	3	4	7	4	3
Mean	70	80	65	42	82	97	89	112
Mean ± S.E.	70 ± 21.2			42 ± 8.4	82 ± 6.8			112 ± 9.9
Range	50 - 91	65 - 92	50 - 79	28 - 57	68 - 95	85 - 105	61 - 109	92 - 123

All male rats.

Weight range:- 151 - 209 G.

Determinations made over period 29th October to 11th December, 1963.

All animals starved for 18 hours before being killed.

Table 62.

Oxygen utilisation by mitochondria from the livers of rats fed HCB in the diet for varying periods of time.

µl/hour/mg. mitochondrial N.

Pyruvate as substrate

	9 days	18 days	24 days	29 days	36 days	45 days	* 48 days	51 days
Oxygen utilisation of individual livers	77 106	89 88	56 91	70 100	70 49	40 49	36 77	79 53
Mean	92	89	74	85	60	45	57	66
Number	6			10				
Mean ± S.E.	84.5 ± 6.8			62.3 ± 6.4				
Range	56 - 106			36 - 100				
P. **	< 0.05							

Citrate as substrate

	9 days	18 days	24 days	29 days	36 days	45 days	* 48 days	51 days
Oxygen utilisation of individual livers	106 112	97 91	131 110	117 110	141 110	98 100	67 95	99 68
Mean	109	94	120	114	125	99	82	83
Number	6			10				
Mean ± S.E.	108 ± 5.7			101 ± 6.9				
Range	91 - 131			68 - 141				
P. **	< 0.5							

11 female rats. Weight range:- 85 - 177 G.

All rats starved for 18 hours before being killed, except 18-day group.

Determinations made over period 16th October to 27th November, 1963.

* Separate experiment performed on 8th October, 1963.

** Statistical comparisons are shown between group receiving HCB for 24 days, and those receiving HCB for 25 - 51 days - (see text).

Table 68.

Oxygen utilisation by homogenates from the livers of normal rats, and rats administered DDC for 6 days.

	µl/hour/mg.P.	
	Control	Treated
Oxygen utilisation in individual livers.	633 666 645 666 645 690	600 630 645 588 495 525
Number	6	6
Mean ± S.E.	657 ± 8.4	580 ± 2.4
Range	633 - 690	495 - 645
p.	< 0.02	

All female rats.

Weight range:- 160 - 200 G.

Determinations made over period 30th August to 9th September, 1963.

All rats normally fed.

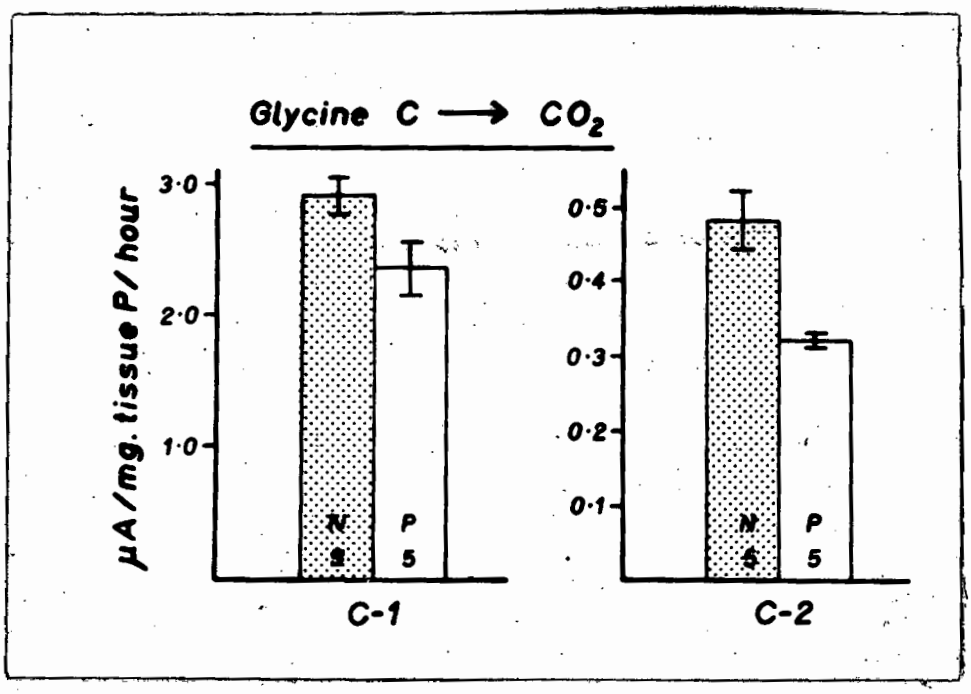

Glycine C ⟶ CO_2
μA/mg. tissue P/ hour
3·0
2·0
1·0
0·5
0·4
0·3
0·2
0·1
N
8
P
5
N
5
P
5
C-1
C-2

Figure 42.

GLYCINE OXIDATION TO CO_2

There was no significant difference in the abilities of normal and porphyric rat liver homogenates to oxidise the carboxyl carbon of glycine to CO_2.

Homogenates from normal rats converted a mean of 2.90 $\pm$ 0.15 µatoms of carbon to CO_2 in 1 hour, and those from porphyric rats converted a mean of 2.35 $\pm$ 0.21 µatoms of glycine to CO_2 in this time. ($p = < 0.1$).

There was, however, a significant reduction in the rate of glycine-2-C oxidation by porphyric rat liver homogenates - from a mean of 0.48 $\pm$ 0.04 µatoms/mgP/hour in the normal livers, to one of 0.32 $\pm$ 0.02 µatoms/mgP/hour. ($p = < 0.01$).

Glycine oxidation was studied only in rats treated with DDC for 6 days.

The results are summarised in Figure 47 and are shown in detail in Table 69.

Table 69.

C^{14} - Glycine oxidation to $C^{14}O_2$ by liver homogenates from normal animals, and from animals treated with DDC for 6 days.

	µatoms/mgP/hour			
	Normal	Treated	Normal	Treated
oxidation occurring in individual livers	3.36 2.90 3.03 2.71 2.48	3.14 2.08 1.98 2.10 2.43	0.61 0.52 0.45 0.42 0.39	0.36 0.32 0.33 0.25 0.32
Number	5	5	5	5
Mean ± S.E.	2.90 ± 0.15	2.35 ± 0.21	0.48 ± 0.04	0.32 ± 0.02
Range	2.48 - 3.36	1.98 - 3.14	0.39 - 0.61	0.25 - 0.36
p.	Not significant		< 0.01	

All female rats.

Weight range:- 160 - 200 G.

All rats normally fed.

Determinations made over period 30th August to 9th September, 1963.

SUMMARY OF FINDINGS.

(i). DDC-treated animals.

A significant reduction in pyruvate kinase activity occurred in the livers of both the fed and the fasted groups of animals. This was maximum after 3 days of treatment, and was maintained at this level thereafter. A marked reduction in activity was also apparent after 49 days of feeding the drug in the diet.

No change occurred in the ability of mitochondria from the livers of these animals to oxidise pyruvate.

A slight, but statistically non-significant, increase in glucose-6-phosphate dehydrogenase activity occurred after 6 days, which was not evident after 49 days.

A significant increase in the urinary excretion of ascorbic acid was apparent after 6 days of drug treatment. After 49 days there was no real change in ascorbic acid excretion.

Lactic dehydrogenase activity was slightly reduced after 3 days but the change was not marked. After 7 weeks of drug feeding this change was more noticeable.

No consistently significant change occurred in isocitric dehydrogenase activity, although a slight fall was apparent in the fed group.

Glutamic-oxaloacetic transaminase activity was slightly

reduced after 3 days, and maximally reduced after 6 days of drug treatment. No appreciable change was apparent after 49 days.

ALA synthetase activity was probably maximally increased after 24 hours, and was maintained at this level for at least 6 days.

ALA dehydrase activity was unchanged after 24 hours, but was strikingly increased after 6 days. After 1 day there was a very slight increase in hepatic PBG content which was increased to moderate amounts after 6 days.

An increase in hepatic protoporphyrin concentration was apparent after 1 day, which progressed over the 9 days of drug administration. After 49 days there was a slight, but nonsignificant increase in hepatic total porphyrin concentration.

Catalase activity was reduced early, and the reduction occurred progressively over 9 days. After 49 days a slight reduction in catalase activity was apparent.

Aminoacetone synthetase activity was uninfluenced by 6 days of DDC administration.

Glycine oxidation to CO_2 was impaired after 6 days.

(ii). <u>AIA-treated animals.</u>

A significant reduction in pyruvate kinase activity occurred - but only after 6 days of AIA administration. Oxidation of pyruvate by mitochondria from the livers of animals

poisoned with this drug was also impaired at this time.

Glucose-6-PO_4 dehydrogenase activity showed a steady and progressive increase from the 1st day until the 6th day.

Ascorbic acid excretion was very markedly increased, and this increase was noticeable within one day of drug administration. It was most striking after 3 days, after which there was a return towards normal levels.

A significant reduction in lactic dehydrogenase activity occurred which was maximal after 3 days.

No change occurred in isocitric dehydrogenase activity.

Glutamic-oxaloacetic transaminase activity was significantly and maximally reduced after 3 days of drug treatment.

ALA synthetase activity was strikingly increased after 1 day, but was reduced from this very high level after 3 and 6 days.

ALA dehydrase activity was slightly increased after 1 day, and had returned to normal levels after 6 days. PBG concentration was markedly increased after 24 hours, and then fell progressively.

A moderate increase in hepatic total porphyrin concentration was apparent after 1 day. There was a progressive increase to the 6th day, and after 9 days the concentration had fallen.

Catalase activity fell progressively from the first day of AIA administration.

Aminoacetone synthetase activity seemed to increase with continued drug administration, but too few animals were studied to allow of any valid conclusions being drawn.

(iii). HCB-treated animals.

Changes occurring in levels of enzyme activity in the livers of animals treated with HCB took in general much longer to develop than with the two other drugs used.

Pyruvate kinase activity was unaltered after 6 days of HCB administration by stomach tube. When HCB was fed in the diet a reduction in activity was evident after 7 days, but it was only after 16 days that this change was maximal. Oxidation of pyruvate by liver mitochondria from these animals was impaired after 29 days of drug administration.

Glucose-6-Phosphate dehydrogenase activity showed some increase after 3 days of intragastric administration of the drug. When HCB was fed in the diet, there was actually an initial diminution in the activity of this enzyme. Increased activity occurred only after 16 days, and after 7 weeks there was a second, more striking increase.

Ascorbic acid excretion in the urine was slightly increased after 7 days, and maximally increased after 16 days with a persistence at this level for the remainder of the experiment.

Lactic dehydrogenase was significantly reduced after 6 days of intragastric administration and after 7 days of dietary feeding.

A slight lowering of isocitric dehydrogenase activity occurred, and glutamic-oxaloacetic transaminase activity was maximally reduced after 7 days of drug treatment. After 7 weeks of feeding the drug, there was a return to normal levels.

ALA synthetase activity was slightly increased after 9 days, and showed a striking increase after 24 days to a high level which was maintained for the duration of the experiment.

ALA dehydrase activity fell progressively and significantly. Hepatic PBG concentration began to increase after about 24 days, rose progressively to a maximum after 45 days, and then seemed to return to normal levels.

Total porphyrin concentration was unchanged for the first week, had increased slightly after 2 weeks, and then increased strikingly and progressively for the remainder of the experiment.

Catalase activity was normal after one week, had fallen slightly after 16 days, and was further reduced after 43 days of drug administration.

Overall Summary.

Each of the drugs resulted in an increased hepatic porphyrin concentration. ALA synthetase activity was increased by all 3, as were the urinary excretion of ascorbic acid, and G-6-P.D. activity.

Pyruvate kinase lactic dehydrogenase and G-O.T. activities were reduced by all three drugs. No change occurred in isocitric dehydrogenase activity.

The change in ALA dehydrase activity was variable, and the patterns of PBG accumulation in the liver also differed.

AA synthetase activity changes were also variable.

Catalase activity was significantly reduced in the AIA and DDC-treated animals, the reduction in activity being less marked following HCB administration.

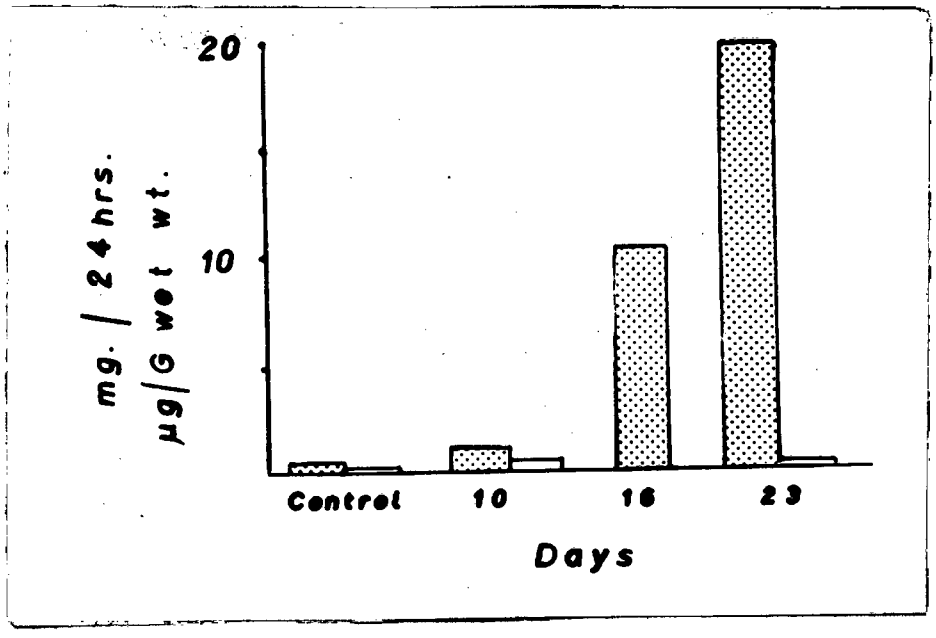


Figure 48.

B. COMPARISON OF URINARY ASCORBIC ACID EXCRETION AND HEPATIC PORPHYRIN CONCENTRATION IN RATS TREATED WITH CHLORBUTANOL.

Because of the striking increase in urinary ascorbic acid excretion by rats with acquired porphyria, the effects on porphyrin metabolism of a drug known to cause an increase in ascorbic acid excretion was investigated.

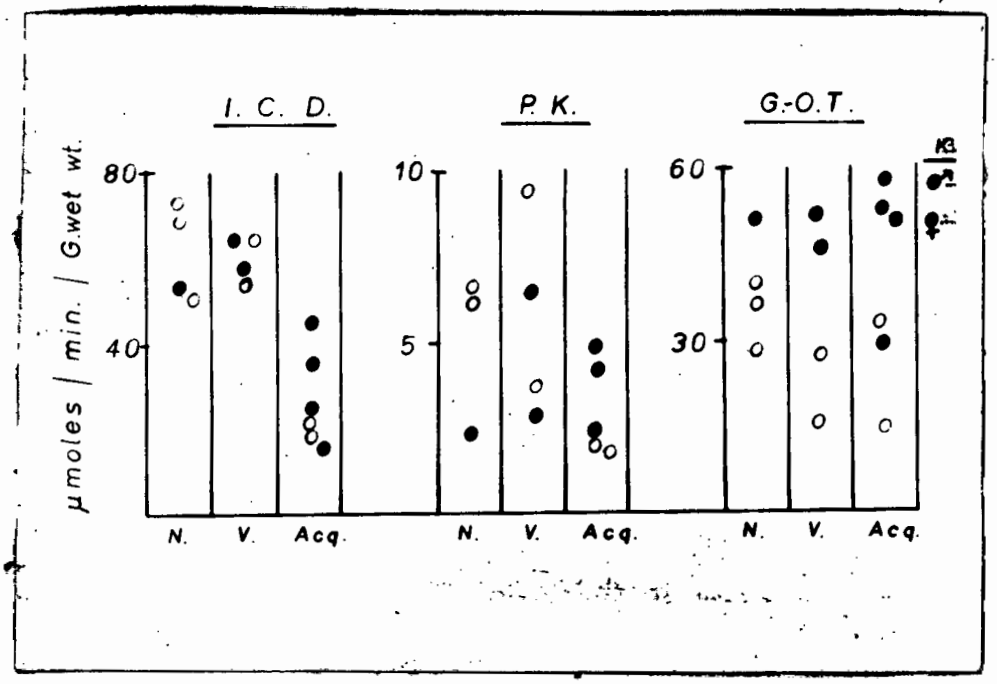
From Table 70 and Figure 48 it is evident that chlorbutanol caused a progressive and marked increase in urinary ascorbic acid excretion, but that no change occurred in the hepatic total porphyrin concentration.

Table 70.

Comparison of urinary ascorbic acid excretion and hepatic porphyrin concentration in rats treated with chlorbutanol.

	Normal		10 days		16 days	23 days	
	* A.A.	** P.	A.A.	P.	A.A.	A.A.	P.
Level in individual animals.	1.16	0.30	1.83	0.55	17.74	19.54	0.34
	0.26	0.46	0.85	0.36	3.34	17.80	0.33
	0.41	-	2.48	0.53	9.42	20.15	0.33
	0.56	-	-	-	-		
Mean	0.60	0.38	1.72	0.48	10.17	19.16	0.33

All female rats.
All rats starved for 18 hours before being killed, during which time the urine was collected.
Weight range:- 128 - 205 G.
Rats administered chlorbutanol over period 25th February to 19th March, 1964.
* A.A. = Ascorbic acid - mg/24 hrs.
** P. = Total porphyrin concentration - µg/G wet weight.


I. C. D.
P. K.
G.-O.T.
µmoles / min. / G.wet wt.
80
40
10
5
60
30
N.
V.
Acq.
KEY

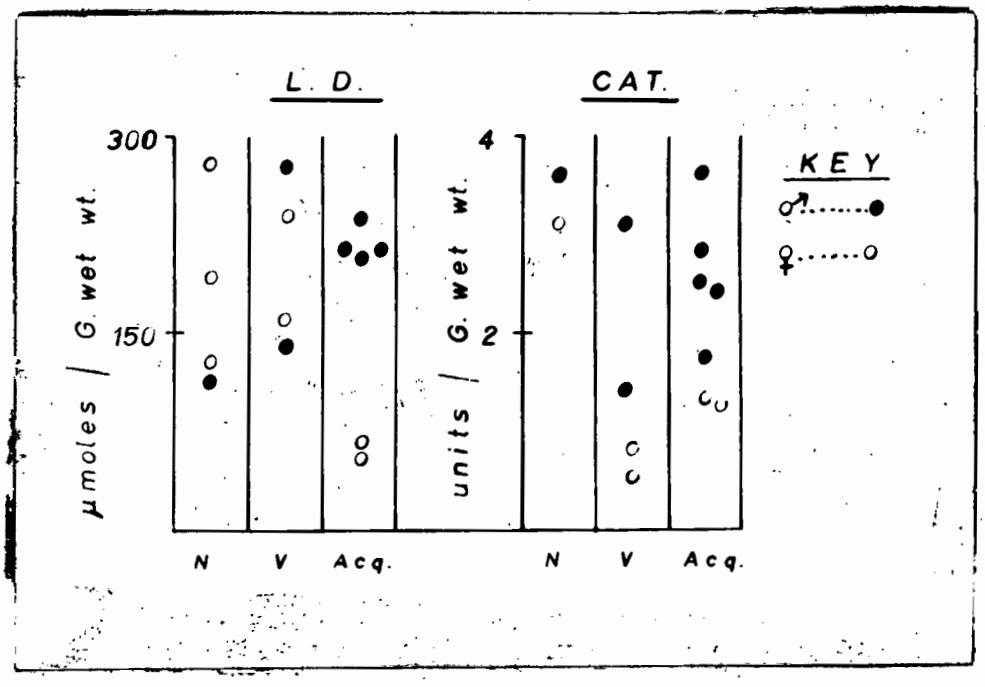

L. D.
CAT.
µmoles / G. wet wt.
units / G. wet wt.
300
150
4
2
N
V
Acq.
KEY

Figure 49.

C. HUMAN MATERIAL.

ICD.

It is evident from Table 71 and from Figure 49 that in persons with acquired porphyria there was a consistent marked reduction in hepatic isocitric dehydrogenase activity.

In the normal group, in the patient with congenital erythropoietic porphyria, and in the patients with variegate porphyria, variation in the level of activity of this enzyme was from 50-73 umoles/min/G wet weight. In the group with acquired porphyria, the range was from 17-45 umoles/min/G wet weight.

Pyruvate kinase.

Levels of pyruvate kinase activity ranged from 2.3 - 9.5 umoles/min/G wet weight in the normal, congenital and variegate groups and from 1.9 - 4.9 in the acquired group - suggestive of a somewhat reduced level of activity in this group.

G-6-P.D.

G-6-P.D. activity was consistently low in all the livers studied.

G-O.T.

G-O.T. activity ranged from 14-58 umoles/min/G wet weight, with both minimum and maximum levels of activity obtaining in the acquired group.

M.S. while recovering from an acute exacerbation of variegate porphyria showed a level of activity of this enzyme

in her liver of only 15 μmoles/min/G wet weight.

Lactic dehydrogenase.

A very wide range of lactic dehydrogenase activity in the livers of these subjects was apparent. There was pronounced overlap amongst the various groups, but it was in 2 of the subjects with acquired porphyria that the lowest levels obtained.

Catalase.

Catalase activity was very low in the two female patients with variegate porphyria, and was lowest in M.S. - perhaps significantly. In the patients with acquired porphyria levels tended towards those obtaining in two normal patients. It was only in the two female patients in this group that the levels were markedly reduced.

Conversely, it was only in the male patient in the variegate group (C.K.) that catalase activity was in the normal range, and he was studied in a presumably quiescent phase at an elective operation for the treatment of a gastric ulcer.

ALA dehydrase.

ALA dehydrase activity in the liver of S.P., a patient with variegate porphyria, was almost twice that in C.P., a control subject. Porphobilinogen was not detected in either liver.

ALA synthetase and oxygen utilisation.

Negligible activity was detected in hepatic mitochondria from S.P., and none in those from C.P. Oxygen utilisation by

these mitochondrial preparations respiring on both pyruvate and citrate was similar.

Levels of enzyme activity in livers from human subjects.

Patient	Age	Sex	Race	μmoles/ G wet weight					Units/ G w.wt	mμmoles /mgP	mμmoles mgN.	μl/hr /mg/N
				ICD	PK	G-6-P.D.	GOT	LD	Cat	ALAD	ALAS	O_2
CONTROLS												
L.S.		F.	C	73	-	-	28	280	-	-	-	-
*C.F.	39	F.	C	50	6.6	1.7	36	189	3.1	428	0	474
J.M.	32	M.	C	52	2.3	1.1	51	110	3.6	-	-	-
CONGENITAL ERYTHROPOIETIC PORPHYRIA.												
M.A.	19	F.	C	67	6.4	1.1	40	124	-	-	-	-
VARIEGATE PORPHYRIA.												
*C.K.	40	M.	C	58	6.5	1.2	52	270	3.1	-	-	-
S.D.	37	M.	E	64	2.9	1.2	46	145	1.4	-	-	-
*S.P.	49	F.	E	54	9.5	1.3	27	238	0.8	826	6.6	442
M.S.	44	F.	E	64	3.6	1.9	15	159	0.5	-	-	-
ACQUIRED PORPHYRIA.												
F.M.	41	M.	B	-	-	-	-	-	2.5	-	-	-
E.N.	44	M.	B	-	-	-	-	-	1.7	-	-	-
G.O.	47	M.	C	25	2.5	0.9	29	121	2.4	-	-	-
R.L.	42	M.	B	17	-	-	51	120	2.8	-	-	-
J.D.	30	F.	C	21	1.9	0.7	33	62	1.2	-	-	-
C.P.	40	M.		45	4.9	-	53	236	3.6	-	-	-
L.M.	57	M.	B	35	4.4	1.2	58	121	2.8	-	-	-
J.J.	52	F.	C	18	2.0	1.1	14	51	1.3	-	-	-

* Specimens obtained at operation.

SECTION 7.

DISCUSSION.

In 1952 Schmid and Schwartz reported the induction of "hepatic porphyria" in rabbits following the administration of the sedative, Sedormid.

Following this report a relatively large literature has accumulated documenting findings in several types of drug-induced porphyria in animals.

These findings have been reviewed in Section 3. The conclusions which may be drawn are that in the experimental porphyrias at least, alterations in carbohydrate, protein, purine and perhaps fat metabolism occur in association with disturbances of porphyrin metabolism. To what extent such changes are interdependent, and how they relate in a cause and effect manner are far from clear.

Perhaps the greatest contribution of the experimental models is to indicate avenues for investigation in human subjects with porphyria in a rational approach to the detection of methods for the prevention and cure of the diseases, and in a search for the explanation of their pathogenesis. They also provide the biochemist with a means whereby porphyrin metabolism may be further investigated and such knowledge must certainly contribute to an understanding of the diseases of porphyrin metabolism. It

is stressed that the utmost caution is essential when attempting to relate data from the experimental animal to the human diseases.

In the present investigation several alterations in levels of hepatic enzyme activity have been documented in rats with drug-induced porphyria. The possible significance of these findings is perhaps increased by the constancy with which the changes occurred despite the chemically unrelated nature of the drugs used to induce the disorder. This suggests a relationship between the observed changes and the disturbance in porphyrin metabolism.

Two major aspects of metabolism have been shown to be altered.

These comprise:-

(i) Alterations in porphyrin metabolism

and, (ii) Alterations in carbohydrate metabolism, and in particular in the activity of the glucuronic acid cycle, and in pyruvate metabolism.

The possible significance of these findings will be considered and I shall speculate on how they correlate with postulated mechanisms of the pathogenesis of the porphyrias.

(1). <u>Alterations in porphyrin metabolism.</u>

<u>Porphyrins.</u>

Various patterns of hepatic porphyrin accumulation have been reported (see pages 68-74) and the present findings are in accord

with previous observations.

Porphobilinogen.

The changes occurring in hepatic porphobilinogen concentration in the drug-induced porphyrias have been described on pages 71-74.

In none of the previous reports has an early accumulation of this compound been noted in AIA-poisoned rats. In contradistinction to an early rise followed by a progressive decrease in hepatic PBG concentration with increasing periods of drug administration (Table 46, Figure 36), both Goldberg and Rimington (128) and Merchante et al (204) could detect PBG in the livers of such animals after several days only, and Rimington and Ziegler (246) noted high PBG concentrations after 6-10 days.

Such differences may be explicable on the same bases as the numerous observed differences in the patterns of PBG excretion in the urine. (See pages 64-66).

ALA synthetase activity.

Increased levels of hepatic ALA synthetase activity have been described in guinea pigs following DDC administration (135, 142) and in rats poisoned with AIA. (208). The present findings show that DDC is capable of causing an elevated level of activity of this enzyme in rats also, confirm the described effects of AIA on rat liver enzyme, and show that HCB is also capable of stimulating ALA synthetase activity in rat livers as measured in vitro.

A uniform finding was that the increase in mitochondrial ALA synthetase activity preceded that in hepatic porphyrin concentration.

In the HCB-treated animals there was initially only a slight increase in enzyme activity - which was associated with a minimal rise in total porphyrin concentration. When a marked increase in enzyme activity occurred this was followed by a progressive rise in the porphyrin content of the liver. It is suggested that at the new level of synthesis, porphyrins were being formed more rapidly than they could be utilised or excreted, with their consequent progressive accumulation.

ALA administration was accompanied by a very sharp increase in ALA synthetase activity, which preceded hepatic porphyrin accumulation. The subsequent reduction in enzyme activity was followed by a fall in porphyrin concentration in the liver.

In the DDC-treated animals there was probably also an immediate increase in enzyme activity with a later progressive increase in porphyrin accumulation.

A cause and effect relationship seems probable.

The increased ALA synthetase activity which has been reported is compatible with the hypothesis that the ability of the mitochondria to make ALA is rate-limiting in porphyrin synthesis in the liver (135), but it is not clear whether the effect of the various drugs was primary and direct, or secondary and indirect, mediated perhaps through interference with a control

mechanism. It is possible that the genetic defect too, is mediated through an increased activity of this enzyme, although the general rule would seem to be for such genetic defects to be associated with a reduction in enzyme activity - at least insofar as the "recessive" diseases of metabolism are concerned.

Part of the inhibitory effects of pyruvate, acetate and certain amino acids (see page 27) might be through the exertion of a controlling influence on ALA synthetase activity.

Burnham (34), and Burnham and Lascelles (35) demonstrated a possible negative feedback control system of porphyrin synthesis via iron-porphyrin complexes. Haemin was shown to repress ALA synthetase activity 3-5 fold when added to a R. spheroides culture. (190). A reduction in the concentration of such compounds consequent, perhaps, on a block in their synthesis, might thus result in increased ALA synthetase activity through the removal of a control mechanism.

Kikuchi et al (176) demonstrated the occurrence of an unidentified inhibitor of ALA synthetase activity that was formed by R. spheroides cells grown aerobically in the dark.

Granick (135) considered that some of the increased activity of this enzyme might be consequent upon activation of an inactive form by the various drugs, but this was not supported experimentally.

ALA dehydrase activity.

Previous reports relating to ALA dehydrase activity are

described on page 88, and it is evident that opposite effects have been observed following the administration of the various drugs to different animals. Moreover, there has been no constant observed effect of a particular drug on a particular species.

The present findings indicate opposite effects of DDC, AIA and HCB in the rat, an increased level of activity following DDC administration, no change occurring after AIA administration, and a progressive decrease following HCB administration.

Although an increased level of activity of this enzyme may be of primary importance in the aetiology of the porphyrias, it would seem more probable that, in those instances where increased levels of activity have occurred, these have been secondary to stimulation by an increased supply of the substrate, ALA.

An observed decrease in the activity of this enzyme is, therefore, of particular interest.

A number of factors may have contributed to this observation:-

(i). It is possible that liver homogenates from such animals were removing PBG faster than they were capable of synthesising it - as opposed to liver tissue from AIA-treated rabbits, and Sedormid-treated rats in which no change in the rate of removal of PBG was observed. (204, 278, 321).

(ii). Some factor may have inhibited the activity of this

enzyme. Protoporphyrin has been shown to be inhibitory (113) as has haemin. (190). It was my impression that those liver homogenates which showed the greatest pink discolouration of their supernates, and the brightest fluorescence in ultraviolet light, showed the lowest levels of ALA dehydrase activity.

(iii). A further consideration is that the development of the colour-compound formed between PBG and Ehrlich's reagent might have been inhibited by some substance in the livers of these animals, a substance which presumably increased progressively in concentration over the period of drug administration. Possibly pertinent is the observation by Granick and Mauzerall (139) of the inhibitory effect of ascorbic acid upon the development of this compound. Such an inhibitory mechanism has also been discussed on page 65 in a consideration of the factors influencing the detection of PBG in the urine.

(iv). It should also be considered, although with great reserve, that in such instances an alternate route of porphyrin synthesis, bypassing PBG as an intermediate, was employed. Kench (173) has suggested the existence of such a pathway in yeasts - organisms in which no ALA dehydrase activity has been detected. (115).

Catalase.

The activity of this enzyme has frequently been shown to be significantly reduced in the drug-induced porphyrias (see page 84).

Activity has not been previously reported to be reduced in HCB-induced porphyria, but this may be because previous authors have not measured activity after a sufficiently long period of drug administration.

A reduction in catalase activity, and perhaps synthesis, may permit of an increased ALA synthetase activity through the removal of a negative feedback control mechanism.

Drabkin (77) considered that the demonstration of reduced catalase activity might be in support of his postulate (76) that the defect in the porphyrias might be as a result of the under-utilisation rather than the overproduction of porphyrins.

Heim et al (158) demonstrated that 3-amino-1:2:4-triazole caused a marked reduction in liver and kidneycatalase activity in the adult female rat, and this compound has not been associated with the development of porphyria - a factor which tends to lessen the significance of the observed reduction in activity.

(2). Alterations in carbohydrate metabolism.

Ascorbic acid.

Perhaps the most important observation of the present investigation was of an increased rate of urinary ascorbic acid excretion (and hence of its synthesis (37, 38, 90, 195a, 196)) in association with a disturbance of porphyrin metabolism.

This was a constant finding in DDC-, AIA-, and HCB-porphyria, and has been confirmed by De Matteis (65).

It may prove of interest to compare levels of urinary glucuronic acid excretion in subjects with porphyria and normal subjects, and also in drug-induced porphyrias in the guinea pig.

Although this increase has thus far always occurred following the administration of porphyria-producing drugs, chlorbutanol administration over 3 weeks was not associated with an increase in hepatic porphyrin concentration. Chlorbutanol is a drug which has previously been shown to stimulate ascorbic acid synthesis (see page 139). However, Granick (136) was able to show an increased synthesis of porphyrins by cells cultured in the presence of this drug and it is possible that the drug was not administered for a sufficiently long period of time.

De Matteis (65) was able to separate the development of increased levels of porphyrin synthesis and ascorbic acid synthesis in rats, and suggested that they were not closely related, but this question is worthy of further investigation.

In the AIA-treated rats in the present investigation levels of ascorbic acid excretion and ALA synthetase activity increased simultaneously to very high levels which does tend to suggest a close correlation. Several possibilities may be postulated as to the nature of this relationship:-

(i) Ascorbic acid has been shown to potentiate the

combination of iron with protoporphyrin to form haem. (122,181). It is possible that consequent upon a primary inhibition of ferro-chelatase activity (as suggested with fairly convincing evidence by Onisawa and Labbe (227)), ascorbic acid synthesis was increased in a compensatory attempt on the part of the liver to enhance this reaction.

An alternative hypothesis is that the primary drug effect, lay in a stimulatory action on ascorbic acid synthesis. Such a mechanism might be expected to reduce porphyrin synthesis via a negative feedback effect of haem compounds on ALA synthetase activity, and to reduce rather than to increase the measured activity of this enzyme. However, when greatly in excess ascorbic acid was found to inhibit ferro-chelatase activity. (122).

It has been considered that the defect in the porphyrias might occur as a result of defects in the redox potential of the cells - permitting of oxidation of porphyrinogen intermediates to porphyrins.

Rimington (240, 241) showed that ascorbic acid or thioglycolate diminished autoxidation of coproporphyrinogen. The increased ascorbic acid synthesis which has been demonstrated may thus have occurred as a compensatory mechanism towards the re-establishment of a milieu favouring reduction in a state where the redox potential was altered in the direction of oxidation.

Should the effect on ascorbic acid synthesis be an important

one, observed alterations in the levels of pyruvate kinase activity might then be explicable on the basis of glucose being deviated away from the Embden-Meyerhof pathway towards ascorbic acid synthesis. A reduced pyruvate kinase activity would reflect a diminished substrate supply, as would the reduced lactic dehydrogenase activity. The increased G-6-P.D. activity might then reflect increased metabolism along the glucuronic acid cycle with an increased supply of endogenous substrate stimulating enzyme activity. (see page 48).

Pyruvate.

Two facts relating to the metabolism of this compound have emerged from the present investigation.

(i) Pyruvate kinase activity was found to be reduced, and (ii) Liver mitochondria from porphyric rats respiring on exogenous pyruvate showed a diminished capacity to utilise pyruvate. If comparisons with findings in tissue culture, in isolated systems, and in human subjects are permitted, this finding is in agreement with the reports (i), of an inhibition, by AIA, of pyruvate oxidation by cells grown in tissue culture (57), (ii), of an inhibition, by drugs capable of inducing porphyria, of beef heart DPNH oxidase activity (56), (iii), of the decreased rate of clearance of exogenous pyruvate from the blood of porphyric Bantu subjects (169), and (iv), of an impaired pyruvate tolerance test in subjects with acute

intermittent porphyria. (129, 161).

It may also be significant in this regard that while liver mitochondria from HCB-poisoned rats showed a marked increase in ALA synthetase activity when citrate was provided as substrate, a similar increase did not occur when pyruvate formed the substrate. (Table 44).

The observations of a diminution in pyruvate kinase activity and in pyruvate oxidation may both have some bearing on the pathogenesis of the porphyrias, although their interpretation is highly speculative.

A reduced activity of pyruvate kinase might be indicative of a diminished synthesis of pyruvate in these animals. Pyruvate has been shown to have an inhibitory effect on porphyrin synthesis in vitro. (see page 27). A primary drug effect might thus have been to inhibit the Embden-Meyerhof pathway.

The findings indicative of impaired pyruvate oxidation in these conditions might then be explicable on the basis of a secondary "atrophy" of the necessary enzymes consequent upon its diminished synthesis.

Increased ascorbic acid synthesis, and increased G-6-P.D. activity might reflect a necessity for alternative routes of glucose metabolism other than through an inhibited central glycolytic pathway.

Such an hypothesis would serve to support the findings of

Tschudy's group (249, 340) and of De Matteis (65) concerning the beneficial effects of carbohydrates upon the porphyric state (see page 94) - carbohydrates might serve to increase the activity of this pathway, and consequently the amounts of pyruvate formed. In the present study it is evident that starvation reduced the activity of this enzyme even further.

An obvious modification and alternative hypothesis is that pyruvate metabolism may be primarily impaired, with a secondary inhibition or "atrophy" of the pathways leading to its synthesis.

Such an hypothesis is supported by the finding of an inhibition of beef heart DPNH oxidase by drugs capable of inducing, or aggravating, the porphyric state (56), and this effect too, may be of aetiological significance.

Contradictory to the suggestion of a block in the central glycolytic pathway are reports of normal, or increased glycolysis in the porphyrias. (57, 320, 321).

Other changes have also been detected in this investigation which may have some bearing on the pathogenesis of the porphyrias.

These include the detection of a diminished rate of oxidation of glycine to CO_2 in DDC-induced porphyria, and of alterations in aminoacetone synthetase activity.

(a). Glycine oxidation.

An impaired oxidation of the alpha carbon of glycine to

CO_2 in several forms of the drug-induced porphyrias has been previously reported. (75, 320, 321).

(b). AA synthetase activity.

Granick and Urata (142) did not detect any alteration in the level of aminoacetone synthetase activity of hepatic mitochondria from DDC-poisoned guinea pigs and in the present investigation no alteration in the activity of this enzyme was noted in mitochondria from DDC-poisoned rat livers. Miyakoshi and Kikuchi (208) reported a similar lack of influence of AIA administration upon the AA synthetase activity of rat liver mitochondria. Tschudy et al (319) could not detect a diminished excretion of aminoacetone in the urine of subjects with acute intermittent porphyria. However, De Matteis and Rimington (68) described such a reduction in the urine of patients with this disorder, and of rats with experimental porphyria. In the HCB-treated rats in the present study a significant reduction in AA synthetase activity is also evident.

Possible role in the pathogenesis of porphyria.

The administration of glycine has been shown to increase porphyrin excretion in both acute intermittent, and drug-induced porphyria (237) and it has been suggested that increased porphyrin biosynthesis might be initiated through an availability of excess glycine.

Excess glycine could result from an impairment in one or

several of its alternate pathways of metabolism. Its conversion to serine has been shown to be impaired in certain forms of porphyria (237), and an impaired conversion to purines in Sedormid-induced porphyria in chick embryos has been described. (183, 315). This may also constitute the significance of the demonstration of an impaired oxidation of glycine to CO_2.

However, it must be considered that these apparent blocks in the alternate pathways of metabolism of this compound may be no more significant than as an indication of increased glycine metabolism along the porphyrin biosynthetic pathway, with perhaps a consequent "atrophy" of its other routes of metabolism.

In subjects with porphyria Dowdle (74) was unable to detect alterations in the rates of conversion of glycine to CO_2 -, or uric acid - but he was able to demonstrate an increased rate of glycine turnover.

Nakada and Weinhouse (214) have suggested that catalase may play a role in the oxidation of the alpha carbon of glycine to CO_2, via formates, and the depressed catalase activity which has been demonstrated in these conditions, may be partially responsible for the observed effects on glycine oxidation. This may even constitute the mode of action of a control mechanism of porphyrin synthesis.

If acetyl-coenzyme A synthesis were impaired in porphyria, as has been suggested (68), less glycine would be metabolised via the aminoacetone cycle, and more would be available for metabolism

via the succinate-glycine cycle, and hence for porphyrin synthesis.

A reduction in AA synthetase activity and in the urinary excretion of aminoacetone may be regarded as supporting such a postulate, but it is evident that these observations have not been consistent.

Some of the inhibitory effect of both pyruvate and acetate upon porphyrin synthesis might be explicable upon their potentiating acetyl-coenzyme A synthesis.

The data obtained in this investigation may also have some bearing on other postulated mechanisms underlying the porphyrias.

Reduction in lactic dehydrogenase, pyruvate kinase, and glutamic-oxaloacetic-transaminase activities might reflect alterations in the DPN:DPNH ratios, while increased Glucose-6-phosphate dehydrogenase and normal isocitric dehydrogenase activities might then be explained on the basis that they are TPN:TPNH dependent enzymes, which ratio must be presumed to be unaffected. Alterations in both these ratios have been described by Rimington and Ziegler (246) in the drug-induced porphyrias. (See page 91). Rimington (240) considered that systems normally reoxidising DPNH and TPNH might be affected during exacerbations in porphyria, and Cowger et al (56) have demonstrated an inhibition of DPNH oxidase by porphyria-producing drugs. Joubert et al (169) postulated a deficiency in the electron transport chain, and suggested that the influence of alcohol as an aetiological factor in porphyria might

be through a decrease in the DPN/DPNH ratio. (See review by Isselbacher and Greenberger (165)). Dowdle(73), however, felt that a decrease in this ratio would favour reduction rather than oxidation.

It is interesting that as early as 1939 alterations in the oxidation-reduction system were invoked to explain the occurrence of methaemoglobinaemia in intoxications with several aromatic amino groups - compounds which were also shown to cause disturbances in porphyrin metabolism. (29).

Sweeney (310) considered that there might be an abnormal permeability of the cell, or parts thereof, to porphyrinogens, and so allow of their escape from the enzymatic sequence and further metabolism, and Sano and Granick (256) considered that the location of certain enzymes of the porphyrin biosynthetic pathway in mitochondria, while others were present in soluble form in the cytoplasm, might provide for a control mechanism of porphyrin biosynthesis through mitochondrial permeability factors.

Dowdle (72) feels that the defect in porphyria might lie in the mitochondrion. Granick and Urata (142) have described an abnormal appearance of the mitochondria of DDC-poisoned guinea-pig livers, and Mauzerall (201) postulated a leak of ALA through a damaged mitochondrial membrane as an aetiological factor.

Alterations in the structure of these particles might have

been responsible for the observed changes in ALA synthetase activity.

It should also be considered that many of the observed alterations in enzyme activity might have been consequent upon the presence of increased concentrations of porphyrins. In this regard it may be significant that Pathak and Fowlks (230) were able to show a fall in skin succinic dehydrogenase activity in animals exposed to light after treatment with the photosensitising agent 8methoxy-psoralen. Bacteria exposed to this agent and light, showed a diminished ability to utilise Kreb's cycle intermediates.

It would be of interest to repeat the present studies on animals housed in the dark.

Finally, the possibility should be considered that some, and perhaps all the observed changes occurred as a nonspecific response to liver damage following drug administration. It would be necessary to investigate the effects of other hepatotoxins on the parameters which have been studied to resolve this consideration. Hoffbauer et al (163) were unable to detect a significant overall influence of choline deficiency, thioacetamide or carbontetrachloride vapour on coproporphyrin excretion by rats, and Schmid and Schwartz (268) could detect no effect of CCl_4 administration on the hepatic catalase activity of rabbits.

However, a significant increase in hepatic G-6-P.D. activity, as reported in the present studies, has previously been associated with liver damage in the form of acute hepatitis in man. (272). Hepatitis has also been associated with a reduction in hepatic catalase, lactic dehydrogenase and G-O.T. activities. (58, 348).

Conclusions.

It is suggested from these studies that in the drug-induced porphyrias increased porphyrin formation is consequent upon an increased level of ALA synthetase activity.

What is not clear is the mechanism responsible for the increased level of activity of this enzyme.

A number of alternatives are suggested by the observed data. These are felt to indicate avenues for further research.

(i). The primary drug effect may have been to stimulate ALA synthetase synthesis or to activate an inactive form of the enzyme.

(ii). The increased levels of ALA synthetase activity, may have been secondary to and dependent upon :-

(a) Increased ascorbic acid synthesis.

(b) An impaired central glycolytic pathway.

(c) An impaired metabolism of pyruvate.

(d) An impaired oxidation of glycine.

(e) Alterations in the redox potential of the cells.

(f) Damage to the mitochondrion,

and (g) Blocks in the porphyrin biosynthetic pathway - especially at the ferro-protoporphyrin chelatase level.

It is also felt that an investigation of human subjects with porphyria, to determine whether any of these factors apply is of importance.

A preliminary study of this sort has already been undertaken and is reported in this thesis. There are serious limitations to this work because of the small number of cases, the variability of their dietary status and sex, and a possible seasonal influence on levels of enzyme activity. Furthermore, while the majority of the liver specimens were obtained by needle biopsy some were obtained at operation, and I have unpublished data to indicate a rapid and marked effect of anaesthesia upon the levels of activity of some enzymes.

Perhaps most significant are the variations in the duration of the disease in the different patients, and more especially the marked variations in the degree of activity of the disease process.

ALA synthetase activity was barely detectable in the liver of the one porphyric subject in whom it was measured but ALA dehydrase activity was doubled.

Pyruvate kinase activity was low even in normal subjects, but a reduced activity in the acquired group is suggested.

A fall in ICD activity in the acquired group is almost in direct contradistinction to the observed findings in the drug-induced porphyrias in the rat. However, chronic alcoholism has previously been shown to be associated with a fall in the activity of this enzyme. (97).

Catalase activity was reduced in the livers of two subjects with variegate porphyria. This is at variance with the reported findings of Gray (144), and Schmid (262) concerning the activities of this enzyme in porphyria. It is possible that these cases differed in the nature, severity and phase of the disturbance in porphyrin metabolism.

Although the majority of the changes which have been described have all been demonstrated in vitro, it is suggested that they may be of significance in the intact animal, and perhaps even in man.

It is hoped that the findings will prove of value in further investigations of the diseases of porphyrin metabolism.

APPENDIX.

1 (a). Composition of reagent mixtures used in measurements of activities of enzymes of intermediary metabolism.

(i). Pyruvate kinase.

Tris buffer pH 7.4 (B.D.H)	-	18.0 µmoles
Phosphoenolpyruvate-tricyclohexylammonium salt (Boehringer)	-	170 mµmoles
DPNH (Sigma; B.D.H.)	-	25.5 mµmoles
ADP (B.D.H.)	-	106 mµmoles
Nicotinamide (B.D.H.)	-	4.25 µmoles
Mg SO_4 (B.D.H)	-	1.70 µmoles
K. Cl (B.D.H)	-	8.49 µmoles
Bovine serum albumin(B.S.A.) (Armour Pharmaceutical Co)	-	0.061%
Lactic dehydrogenase (Sigma) type 3/	-	.165 µ M units

- in a final volume of 210 lambda.

10 lambda of a 1:1 dilution of the original homogenate were added to initiate the reaction.

(ii). G-6-P.D.

Tris buffer pH 7.4	-	15.6 µmoles
Glucose-6-phosphate (B.D.H)	-	777 mµmoles
TPN (B.D.H.)	-	97 mµmoles
Mg Cl_2 (B.D.H.)	-	97 mµmoles

- in a final volume of 200 lambda.

10 lambda of a 1:1 dilution of the original homogenate were added to initiate the reaction.

(iii). I.C.D.

Tris buffer pH 7.4	-	18.1 µmoles
d-Isocitric acid-trisodium salt (Sigma)	-	91 mµmoles
TPN (B.D.H.)	-	91 mµmoles
Nicotinamide	-	4.5 µmoles
MnCl2	-	113 mµmoles
B.S.A.	-	0.068%

- in a final volume of 200 lambda.

10 lambda of a 1:3 dilution of the original homogenate were added to initiate the reaction.

(iv). G-O.T.

Tris buffer pH 7.4	-	17.6 µmoles
Alpha-ketoglutaric acid (B.D.H.)	-	440 mµmoles
L-aspartic acid (B.D.H.)	-	440 mµmoles
DPNH	-	17.6 mµmoles
Nicotinamide	-	4.4 µmoles
B.S.A.	-	0.066%

- in a final volume of 200 lambda.

10 lambda of 1:3 dilution of the original homogenate were added to initiate the reaction. Additional malic dehydrogenase was not provided.

(v). Lactic dehydrogenase.

Tris buffer pH 7.4	-	18.4 µmoles
Sodium pyruvate (Sigma)	-	460 mµmoles
DPNH	-	18.4 mµmoles
Nicotinamide	-	4.61 µmoles
B.S.A.	-	0.046%

- in a final volume of 200 lambda.

10 lambda of a 1:79 dilution of the original homogenate were added to initiate the reaction.

1 (b). Catalase.

The reagent mixture was prepared by diluting 10 ml of 0.1 M phosphate buffer (pH 7.4) and 5 ml of a 1/80 dilution of concentrated H_2O_2 to 100 ml with glass-distilled water.

25 lambda of a 1:5 w/v homogenate were added to 15 cc of this solution, which was constantly agitated by a magnetic stirrer, and at exactly 15-second intervals 1 ml amounts were pipetted into flasks containing a few drops of 2% H_2SO_4 and a trace of $MnCl_2$. These solutions were back titrated against $\pm$ .01 N Potassium permanganate.

The presence of the manganese chloride permitted of the detection of a very precise end point.

1 (c). Preparation of mitochondria.

At lease 5 G of liver were homogenised with 10 volumes of cold 0.25 M sucrose (pH 7.4) in the usual manner.

The homogenate was centrifuged in 10 5 ml amounts in the cold at 500 r.p.m. for 10 minutes in a Beckman Spinco Model L refrigerated ultracentrifuge.

The supernates were decanted into 10 fresh lusteroid tubes and centrifuged at 10,000 r.p.m. for 10 minutes. These supernates were discarded, the pellets were resuspended in 1.5 ml sucrose and the tube contents combined by threes.

Three tubes and the remaining single tube were again centrifuged at 10,000 r.p.m. for 10 minutes and the supernates discarded. The pellets were resuspended in 1.75 ml sucrose containing $Mg\ Cl_2$ in a concentration of 20 m M/L and EDTA in a concentration of 10 m M/L. The smaller pellet in the single tube was resuspended in 0.58 ml.

The suspensions were combined and homogenised in a glass homogeniser.

All procedures were performed in the cold.

1 (d). Preliminary investigations relating to the measurement of ALA.

(i). To determine the molar extinction coefficient of ALA pyrrole + Ehrlich's mercury reagent.

1 ml of a 10^{-4} M solution of ALA hydrochloride was boiled in each of 8 tubes with 16 ml of water, 1 ml acetate buffer pH 4.8, and 0.5 ml acetylacetone for 15 minutes in a water bath.

These solutions were allowed to cool and then passed through 1 x 1 cm Dowex-1-acetate columns. The columns were washed with 5 ml H_2O and with 5 ml 1N acetic acid and the ALA then eluted with 4 ml of a 2:1 Methanol:glacial acetic acid mixture. The eluate was made up to 5 ml with more of this mixture. 5 ml Ehrlich's mercury reagent were added, and the

optical density at 552 mu determined after 15 minutes.

Tubes	1	2	3	4	5	6	7	8
O.D.	.265	.281	.277	.283	.283	.292	.300	.315

Mean .287

The extinction coefficient of this compound in a concentration of 1 M/L at 552 mu in a 1 cm light path was, therefore, calculated as 2.87×10^4.

In subsequent experiments 10^{-3} M solutions of ALA HCl were boiled with 17.5 ml H_2O, 1 ml acetate buffer (pH 4.8) and 0.5 ml acetylacetone. Blank solutions were prepared by replacing ALA with 1 ml H_2O.

(ii). Influence of the duration of boiling.

Tubes were boiled for the periods indicated.

1 ml from each tube was mixed with 4 ml H_2O and 5 ml Ehrlich's reagent. The experiment was performed in duplicate.

Time (mins).		5	10	15	20	25
O.D.	(i)	.229	.241	.235	.240	.235
	(ii)	.225	.239	.230	.243	.235

ALA was, therefore, fully converted to the pyrrole within 10 minutes of boiling.

(iii). Stability of colour compounds.

(a). 1 ml ALA pyrrole was mixed with 4 ml H_2O and 5 ml Ehrlich's reagent. O.D. density was determined at intervals as shown.

Time (mins)		5	10	15	20	25	30	60	120
O.D.	(i)	.229	.229	.225	.222	.221	.221	.215	.212
	(ii)	.239	.241	.239	.237	.238	.238	.239	.225
	(iii)	.227	.227	.225	.221	.220	.220	.215	.212

(b). 1 ml ALA pyrrole was passed through Dowex-1-acetate column (1x1 cm), eluted with 4 ml methanol:glacial acetic acid; made up to 5 ml, and 5 ml Ehrlich's reagent added.
O.D. density was determined at intervals.

Time (mins)		5	10	15	20	25	30	60
O.D.	(i)	.136	.142	.147	.150	.154	.150	.185
	(ii)	.130	.149	.156	.160	.163	.166	.172
	(iii)	.132	.150	.157	.162	.165	.168	.194

(iv). Influence of aging of Ehrlich's reagent.

1 ml ALA was mixed with 4 ml H_2O and 5 ml Ehrlich's mercury reagent - (a) Fresh (b) 1 month old.

O.D.	Fresh	Old
(i)	.247	.297
(ii)	.235	.295

The blank reading was also higher with the old solution.

O.D. Blank - (set to zero on fresh) - .025.

(v). Incomplete elution from column.

(a) 1 ml ALA pyrrole was passed through 1x1 cm Dowex-1-acetate column, eluted with 4 ml methanol:glacial acetic acid and made up to 5 ml with H_2O.

(b) 1 ml ALA pyrrole solution was made up to 5 ml with methanol:glacial acetic acid.

5 ml Ehrlich's reagent added to each solution, and O.D. compared after 15 minutes.

Solution		a	b.
O.D.	(i)	.116	.168
	(ii)	.140	.185

(vi). No loss of ALA when washing columns with water or IN acetic acid.

1 ml ALA pyrrole was passed through column, washed with 5 ml IN acetic acid. Washings were collected separately and 5 ml Ehrlich's reagent added to each. O.D. was determined after 15 mins.

		Water	Acetic acid
O.D.	(i)	.006	.008
	(ii)	.000	.009

(vii). Relation of O.D. to concentration.

ALA solution in amounts of 1 ml, 0.5 ml and 0.25 ml were converted to pyrrole and O.D. of colour:compound with Ehrlich's reagent was determined.

Amounts		1 ml	0.5 ml	0.25 ml
O.D.	(i)*	.225	.123	.055
	(ii)**	.345	.174	.085

* Passed through column

** Not passed through column.

In this experiment the molarity of the ALA was different from those described when measuring the extinction coefficients, and was not accurately measured.

1(e). Method for determination of porphyrin concentrations of liver tissue.

The method of extraction has been dexcribed by Sweeney*.

Total porphyrins.

Water was added to the acetone/HCP extract which was then repeatedly extracted with n-Butanol/ethyl acetate (1:1) in a separating funnel.

The extracts were pooled and washed with water, and petrol ether (40/60°) added.

Porphyrin was extracted with 1.5N HCl, and the concentration was measured spectrophotometrically.

Protoporphyrin.

Two volumes of distilled water were added to the acidified acetone extract, and the pH brought to 3.5 by the addition of saturated aqueous sodium acetate. The porphyrins were repeatedly extracted into ether, and the extracts were pooled and washed with water.

Protoporphyrin was extracted into 1.5N HCl and its concentration measured spectrophotometrically. Completeness of porphyrin extraction was judged under ultraviolet-light control.

* Sweeney, G.D. (1963) : Ph.D. Thesis, page 88. (University of Cape Town).

2. Clinical details of human subjects investigated.

Urinary and faecal porphyrin and porphyrin precursor concentrations, and in some instances hepatic porphyrin concentrations, are shown in Table a. These measurements were made at the time when the liver biopsies were obtained for the purposes of the investigations described in this thesis. The results of the "liver function" tests are shown in Table b.

Control subjects.

L.S. Coloured female aged ± 45.

This subject underwent a cholecystectomy for gall stones.

There were no features to suggest porphyria, and her alcohol intake was not in excess.

The histology of her liver biopsy was not determined.

C.F. Coloured female aged 39.

This patient was also operated on for cholelithiasis. There were no features of porphyria and no history of an excessive alcoholic intake.

The histology of the liver is not known.

J.M. Coloured male aged 32.

This patient was admitted with lobar pneumonia.

There was no evidence of porphyria.

He was known to have drunk alcohol in excess over several years.

The only feature of note in the liver biopsy, was the presence of large amounts of lipofuscin.

Subject with congenital erythropoietic porphyria.

M.A. Coloured female aged 19.

This patient had noticed blisters on her face, hands and feet since the age of 4. She had passed a dark red urine intermittently throughout her life, and had had excessive hair on her face for many years.

Examination revealed scarring and areas of pigmentation and depigmentation on the face, hands and feet. Hypertrichosis was a marked feature and the digits showed sclerodactyly.

The liver edge could just be palpated below the right costal margin.

There was no family history of porphyria, and an excessive alcohol consumption was not suspected.

Liver biopsy revealed a normal architectural pattern. Small amounts of haemosiderin were seen.

Her red cells had a coproporphyrin content of 27 ug%, and a protoporphyrin content of 5.0 ug%.

Subjects with variegate porphyria.

C.K. Coloured male aged 31.

C.K. Coloured male aged 31.

This patient was examined when his brother was diagnosed as having porphyria. Scarring of his face and hands was prominent.

For several years he had noticed that his skin readily formed blisters.

He underwent a gastroenterotomy and vagotomy for chronic duodenal ulceration in May, 1959 and at the time of the present investigation was undergoing a further operation because of a stomal ulcer. A Zollinger-Ellison syndrome was suspected.

For several years he had taken alcohol in excess.

Liver biopsy revealed an increase in periportal fibrous tissue. No iron could be demonstrated.

S.D. European male aged 37.

Undue fragility of the skin of the backs of his hands had been noticed since the age of 24 - following the administration of sulphonamides and aspirins.

His mother and one brother had evidence of cutaneous lesions of porphyria.

He drank an occasional beer.

Liver biopsy showed increased amounts of fibrous tissue in the portal tracts. No iron was present, but lipofuscin was prominent in the liver cells.

S.P. European female aged 49.

This patient had an acute attack of porphyria in 1951 with full recovery.

Abnormal skin fragility had been evident for many years.

A cholecystectomy was performed in August, 1963, when a specimen of liver for the purposes of the present investigation was obtained.

There is a strong family history of porphyria.

Liver biopsy revealed evidence of a very slight degree of periportal fibrosis.

M.S. European female aged 44.

This patient was admitted in an acute attack of porphyria. The biopsy of her liver was performed during the early recovery phase.

For the previous 20 years she had noticed an increased sensitivity of her skin to trauma.

As the patient was a foster child, her family history is unknown.

Her 4 children - eldest 22 - showed no evidence of the disease.

She did not drink alcohol.

She was very ill on admission. There was a muddy pigmentation of the skin of her forehead, and increased hair growth on the sides

of her face. Scars and milia were evident on her hands.

Her liver was not enlarged, but she was tender in the upper abdomen.

She showed the features suggestive of an inappropriate secretion of anti-diuretic hormone.

Liver biopsy was essentially normal.

Subjects with acquired porphyria.

F.M. Bantu male aged 41.

When biopsied this patient had a history of having had intermittent attacks of abdominal pain associated with diarrhoea and vomiting over the period of a year. These symptoms resolved following treatment of a worm infestation.

For some months he had noticed an increased sensitivity of his skin to sunlight and trauma.

His alcohol intake consisted of 2 bottles of sweet wine and a gallon of home-brewed beer over weekends.

There was no family history of porphyria.

Examination revealed typical porphyric skin lesions on the face and hands. The liver edge was just palpable.

Liver biopsy revealed evidence of a marked haemosiderosis. There was focal fatty change, and a slight excess of collagen tissue was apparent.

E.N. Bantu male aged 44.

This patient was admitted in cardiac failure, and a diagnosis of cardiomyopathy of unknown origin was made.

He was noticed to be deeply pigmented. Screening tests for porphyrins were negative in the urine and stool, but his liver contained excessive amounts of porphyrins.

There was no family history of porphyria. His alcoholic habits are not known.

Examination revealed a " 3 finger" enlargement of his liver.

A notable degree of haemosiderosis was evident on liver biopsy. There was no evidence of cirrhosis but there was a degree of periportal fibrosis.

G.O. Coloured male aged 47.

This patient was admitted for the investigation of impotence. He was noted to have typical porphyric skin lesions of the hands and face which had been present for approximately 6 months.

He admitted to a heavy intake of wine and spirits over 8 years.

There was no family history suggestive of porphyria.

His liver was palpable 3 fingers breadth below the right costal margin.

Liver biopsy showed cirrhosis with liver cell regeneration and moderate haemosiderosis.

R.L. Bantu male aged 42.

This patient was admitted with nonspecific complaints, and was noticed to be excessively pigmented. He denied any recent increase in pigmentation.

Since adolescence he had drunk 3-4 tots of brandy, and 2-3 cans of home-brewed beer several times a week.

Liver biopsy revealed marked haemosiderosis with evidence of periportal fibrosis and regeneration of liver cells.

J.D. Coloured female aged 30.

For 4 years before the biopsy was performed she had noticed marked photosensitivity of the exposed skin.

For many years before this she had drunk 2 bottles of wine a day.

There was no family history suggestive of porphyria.

Liver biopsy revealed the presence of small amounts of haemosiderin and mild fatty change. The portal tracts showed an increased cellularity.

C.P. Coloured male aged 40.

This patient was admitted to hospital because of an aspiration pneumonia.

Porphyrin studies were undertaken because of an observed increased pigmentation of his skin.

His alcoholic habits are not known.

Examination revealed his liver to be just palpable.

Liver biopsy showed severe siderosis but no evidence of fibrosis.

L.M. Bantu male aged 52.

When investigated this patient had a one month's history of blistering of the skin of his hands following exposure to light and trauma.

For several years he had drunk 1-2 tots of gin daily.

There was no family history of porphyria.

He was noted to be darkly pigmented, and there were areas of scarring on his hands. His liver was not enlarged.

Liver biopsy showed no evidence of cirrhosis. There was very slight periportal fibrosis with a mild degree of fatty change. Portal tracts showed round cell, plasma cell and eosinophil infiltration.

J.J. Coloured female aged 42.

For 3 months before admission she had noticed an increased pigmentation of her face.

She admitted to an occasional social drink.

There was no family history suggestive of porphyria.

Liver biopsy showed no evidence of cirrhosis, but many cells contained a moderate amount of brown pigment, some of which stained positively for iron.

Table a.

Patient	Urine				Stool		Liver
	ALA	PBG	Uro	Copro	Copro	Proto	Total
	mg/day		ug/L		ug/G		ug/G
L.S.	-	-	-	-	-	-	-
C.P.	-	-	-	-	-	-	-
J.M.	0.82	0.18	Neg.	45	51	29	-
M.A.	1.82	2.74	6754	1814	642	301	-
C.K.	7.54	4.85	Neg.	185	120	159	Fluoresced after heating
S.D.	0.08 mg%	0.32 mg%	350	1007	257	223	-
S.P.	3.69	2.58	40	104	1226	864	-
M.S.	21.96	35.18	4178	1359	554	926	-
F.M.	0.05 mg%	0.43 mg%	595	99	136	107	470
E.N.	-	-	-	-	-	-	55
G.O.	3.34	3.89	1187	297	49	31	197
R.L.	3.10	4.87	4132	693	110	39	Fluoresced
J.D.	2.51	0.70	3355	257	107	65	-
C.P.	3.57	0.59	Neg.	63	52	61	Fluoresced
L.M.	5.53	1.16	3782	343	136	49	-
J.J.	1.05	0.72	1234	68	27	63	Fluoresced

Table b.

Patient	G%		mg%		Units		
			Bilirubin				
	Albumin	Globulin	Total	Conj.	Alk. ph.	SGOT	B.S.P.
L.S.	-	-	-	-	-	-	-
C.P.	3.3	3.6	0.4	0.3	5.2	-	-
J.M.	Normal		0.4	-	3.6	-	Normal
M.A.	3.2	3.8	0.4	0.2	10.7	28	Normal
C.K.	-- N O R M A L --						
S.D.	4.0	2.3	0.8	0.4	14.8	14	24% remaining
S.P.	4.0	2.1	0.5	0.3	11	26	Normal
M.S.	Normal		0.5	0.3	11.7	14	-
F.M.	3.5	2.0	0.1	0.0	10.0	23	Normal
E.N.	2.8	3.8	0.2	-	2.7	-	-
G.O.	4	3.1	1.5	0.5	4	35	Normal
R.L.	4.2	2.9	0.5	0.2	-	57	Normal
J.D.	3.3	3.3	0.5	0.2	9.5	-	-
C.P.	3.0	2.8	0.6	-	8.7	27	-
L.M.	4.0	4.2	1.3	0.9	6.2	41	-
J.J.	4.0	2.6	-	-	5.2	-	Normal

3. Statistical methods.

(a). Calculation of (i) mean ($\bar{x}$) and (ii) variance (S^2).

(i) $$\bar{x} = \frac{\Sigma x}{n}$$

where n = number of observations

and x = sum of all the observations in the sample.

(ii) $$S^2 = \frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}$$

(b). Calculation of the standard error (S.E.) of the mean.

$$S.E. = \frac{S}{\sqrt{n}}$$

The results have been expressed as

$\bar{x} \pm$ S.E.

(c). For comparisons of two means the Student's t with $n_1 + n_2 - 2$ degrees of freedom was calculated according to the formula -

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}}$$

The value of P was ascertained from tables.

EXPLANATORY NOTES.

(i). The discussion was revised at a late stage in the preparation of the manuscript. As a result a few references have been listed which do not appear in the text. These have been allowed to remain because of the inordinate amount of work that would have been entailed in their deletion.

(ii). It will be seen that reference 300 does not appear in its logical sequence. This error has also been allowed to remain because of the difficulties that would have arisen from its correction.

(iii). Reference 165a has been omitted from the reference list and should read:-

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