INTERACTIONS

OF

ANTICONVULSANT DRUGS

WITH

HAEM BIOSYNTHESIS

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SUMMARY

The porphyrias are a group of genetically inherited diseases, each characterized by a deficiency of a particular enzyme in the haem biosynthetic pathway. The condition may be well tolerated until extra demands are made on the pathway. Under such circumstances, the disease may enter an acute phase or there may be a worsening of chronic symptoms. The problems associated with the porphyrias occur as a result of the excessive production of porphyrins and precursors at these times, due to derepression of 5-aminolaevulinic acid synthase (ALA-S), the first and rate limiting enzyme in the pathway. Porphyria can be accompanied with neurological complications, including epilepsy. Seizure control in these patients is difficult because many drugs stress the pathway and will precipitate an attack in susceptible individuals. Controversy exists as regards which drugs are safe to use for control of seizures. This thesis was aimed at resolving if three contentious drugs were safe. These were carbamazepine (CBZ), sodium valproate (VPA) and phenytoin (DPH). Of further interest was a detailed investigation of the effects of CBZ following reports that CBZ treatment resulted in a biochemistry typical of acute intermittent porphyria in non-porphyric individuals. The nature and possible mechanisms of these effects (derepression of ALA-S, depression of porphobilinogen deaminase (PBG-D) activity and an altered urinary porphyrin excretion profile) were investigated at length in man, rat, cell culture and *in vitro*.

The first chapter of this thesis provides an introduction to epilepsy, the drugs used in its management, the enzymes and intermediates of haem

biosynthesis and the porphyrias. It concludes with objectives for this work. The following chapter describes the various methodologies employed in the next six chapters(three to eight) which contain all the practical results and discuss the significance of these.

The results of an assessment of the safety of CBZ, VPA and DPH in the management of epilepsy in individuals with porphyria are presented in chapter three. This was achieved by examining the effects of these drugs on selected aspects of haem biosynthesis in otherwise healthy epileptic patients on long-term anti-convulsant monotherapy. CBZ and VPA were found to be contra-indicated in light of the fact that both drugs caused derepression of ALA-S. There was some evidence that in the case of both CBZ and VPA, ALA-S was derepressed as a consequence of increased cyt P450 synthesis. VPA also caused increased excretion of porphyrins and precursors. This could be explained by the raised ALA-S activity. CBZ also caused a loss of 5-aminolaevulinic acid dehydratase (ALA-D, the second enzyme in the pathway) activity and a loss of PBG-D activity (third enzyme in the pathway). Both of these effects correlated with plasma CBZ levels. However no significant departures from normal were found following quantitative and qualitative analysis of urinary porphyrins and precursors in individuals on long-term CBZ. This disagrees with the findings mentioned earlier. No conclusive results could be drawn from the group of patients on DPH. Since all the changes found during VPA treatment could be satisfactorily explained, the remaining chapters investigated in detail the effects of CBZ treatment on selected aspects of the pathway.

Chapter four constituted a more detailed investigation into the effects of CBZ treatment on ALA-D, PBG-D and the urinary porphyrin excretion profile

in man. CBZ treatment produced a slow, irreversible loss of ALA-D activity which was related to both plasma "total" CBZ and low activity was the result of a reduction in the amount of functionally normal enzyme. The effect of CBZ on PBG-D activity noted in chapter three and by earlier workers was shown to be an artefact caused by inadequate levels of ALA-D, (required as a coupling enzyme for the PBG-D assay). CBZ produced temporary disturbances in the urinary porphyrin excretion profile in the initial stages of treatment. These changes, described by other workers, were probably related to the very high levels of ALA-S found at this time.

Chapter five took these studies a step further by investigating the effects of CBZ on haem biosynthesis in rats. These experiments confirmed the findings made in chapter four regarding ALA-D and PBG-D and also showed that derepression of ALA-S was related to de novo synthesis of the microsomal haemoprotein cytochrome P-450. As suggested earlier, this is probably the mechanism by which ALA-S activity was derepressed in man during CBZ treatment.

Chapter six attempted to elucidate the mechanism whereby ALA-D was affected during CBZ treatment. This was done by observing the effects of CBZ and metabolites on erythrocyte ALA-D, rat liver ALA-D and purified bovine liver ALA-D *in vitro*. Activity was not affected under any of the conditions employed, possibly because all of these systems are metabolically inert. Consequently the effects of CBZ and CBZ-E on ALA-D in cultured human lymphocytes were studied in the next chapter (Chapter seven) to try and answer the same question as chapter six. Both compounds successfully reduced ALA-D activity, both dose-dependently and irreversibly. CBZ-E

however was much more effective than CBZ. The effects of these compounds on the levels of ALA-D activity were not due to cessation of enzyme synthesis. Chapter seven also examined the effects of CBZ and CBZ-E on PBG-D activity and on the synthesis of porphyrin from added ALA. There was no effect on the former but in the case of the latter there was a quantitative (but not qualitative) effect: the amount of porphyrin produced was related to the ALA-D activity remaining. This was taken as further evidence that the altered urinary porphyrin excretion profile noted previously was due to derepression of ALA-S.

The experiments presented in Chapter eight attempted to metabolize or activate CBZ to investigate if the effect of CBZ treatment on ALA-D activity could be reproduced *in vitro*. This was achieved by incubating the drug with hepatic drug metabolizing systems in the presence of purified ALA-D. Metabolism of either CBZ or CBZ-E was found to produce a selective loss of ALA-D activity. The relevance of these findings on the *in vivo* situation is uncertain.

Chapter nine is the final chapter in this thesis. It draws together the conclusions of the work and discusses the significance of the findings. The extent to which the aims set out in Chapter one were met is also discussed.

ABBREVIATIONS

AIP	Acute intermittent porphyria
ALA	5-Aminolaevulinic acid
ALA-D	5-Aminolaevulinic acid dehydratase
ALA-S	5-Aminolaevulinic acid synthase
b.d.	twice daily
BSA	Bovine serum albumin
CBZ	Carbamazepine
CBZ-E	Carbamazepine-10,11-epoxide
COPRO	Coproporphyrin
COPRO-O	Coproporphyrinogen oxidase
CYTP-450	Cytochrome P-450
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DHA	4,6-dioxoheptanoic acid
DMAB	p-dimethylaminobenzaldehyde
DMSO	Dimethyl sulphoxide
DPH	Diphenylhydantoin (phenytoin)
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylenediaminotetra-acetic acid
EP	Congenital erythropoietic porphyria
EPP	Erythropoietic protoporphyria
FCS	Foetal calf serum
FERRO-C	Ferrochelatase
GSH	Glutathione (reduced form)
GSSG	Glutathione (oxidized form)
HBSS	Hanks balanced salts solution
HC	Hereditary coproprophyria
H_2CBZ	10,11-dihydrocarbamazepine
HPLC	High performance liquid chromatography
m.f.o.	mixed function oxidase
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NTB	2-nitro-5-thiobenzoic acid
PB	Phenobarbitone
PBG	Porphobilinogen
PBG-D	Porphobilinogen deaminase
PCT	Porphyria cutanea tarda (cutaneous hepatic porphyria)
PROTO	Protoporphyrin
PROTO-O	Protoporphyrinogen oxidase
q.i.d.	four times daily
RBC	Red blood cells
RIA	Radioimmunoassay
S.D.	Standard deviation
t.d.	three times daily
TRANS-CBZ-DIOL	Trans-10,11-dihydrodihydroxycarbamazepine
URO	Uroporphyrin
URO-D	Uroporphyrinogen decarboxylase
VP	Variegate porphyria
VPA	Valproic acid or its sodium salt
6β-OHC	6β-hydroxy cortisol

CHAPTER 1

1. INTRODUCTION

This introductory chapter is divided into four sections. The first is devoted to epilepsy and is comprised of a description of each of the various forms of the condition. This is followed by a section covering each of the anticonvulsant drugs studied in this thesis. The third part is concerned with haem biosynthesis and includes a review of the knowledge accumulated on each of the enzymes in the pathway, a sub-section on the regulation of the pathway and a sub-section devoted to disorders of the pathway. The fourth and final part of the chapter discusses the objectives of this thesis.

1.1 EPILEPSY

1.1.1 GENERAL INTRODUCTION TO EPILEPSY

Epilepsy, which affects 5-6 people per 1000 (Pond, Bidwell and Stein, 1960), has been known to man for centuries. As long as 6000 years ago King Hammurabi of Babylon drew up a code which contained laws pertaining to the marriage and employment of epileptics. Through the centuries there have been many diverse treatments for the disease, ranging from hippopotamus testicles and tortoise blood at the time of Hippocrates (400 BC) to castration in the late 19th century. Though Hippocrates suggested that epilepsy was due to a disorder in the brain almost two and a half thousand years ago, it was not until the development of the electroencephalogram in the 1930s that it was demonstrated that seizures were caused by abnormal, uncontrolled, electrical discharges from neurones throughout the brain. Interestingly, such a concept was proposed by Dr.Hughlings Jackson in the latter part of the 19th century. The pathogenesis of the developing fit, however, is still unknown. There is no detectable biochemical or physiological abnormality in the brain cells of epileptic patients. This has made analysis of the mechanism of anticonvulsant drugs, which show great chemical diversity, very difficult. Even today the means by which these drugs control seizures are still not understood and the only way of screening new anticonvulsants is by their ability to prevent experimentally induced seizures in animals.

1.1.2 CLASSIFICATION OF EPILEPTIC SEIZURES

Epilepsy can generally be divided into two categories: generalised and partial epilepsy. Generalised epilepsy symmetrically

involves the whole brain whereas partial epilepsy, as the name suggests involves only a part of the brain. These two very broad categories can be sub-divided into different classes of seizure and the nature of these will be discussed.

All forms of generalised epilepsy are characterized by abnormal electrical discharges throughout the cortex of both cerebral hemispheres causing a sudden loss of consciousness. This can take the form of a grand mal (or tonic-clonic) or a petit mal (or absence) seizure. In a grand mal seizure there is a sudden, complete loss of consciousness followed by contraction of all the muscles. This is known as the tonic phase and may last from about 30 seconds to 1 minute. This is followed by the clonic phase, characterized by violent contraction and relaxation of all the muscles in the body causing a generalised convulsion. These convulsions gradually subside as the brain becomes exhausted and the individual may enter a state of deep unconsciousness which becomes sleep as consciousness returns. On awakening, the individual feels exhausted, is aching all over and often has a headache. During the actual convulsion there may be incontinence and the patient may bite his tongue. Status epilepticus is a condition where one grand mal seizure follows another without any regaining of consciousness between fits. In petit mal epilepsy there is only a transient interruption of consciousness lasting for up to 30 seconds which often goes unnoticed, as the individual does not fall, or is attributed to day-dreaming. The only visible sign may be a rapid fluttering of the eyelids. Whereas grand mal seizures are commonest in adolescents and adults, petit mal seizures occur almost entirely in children.

Partial epilepsy (also known as focal epilepsy) can also be divided into two classes - simple or complex partial epilepsy - depending on the region of the brain where the disturbance originates (the focal point). Simple partial epilepsy is an uncommon form of epilepsy and occurs when the site of abnormal electrical discharge is an area of the brain concerned with simple movements such as bending a finger, or simple sensations such as heat or cold. An attack occurs as a twitch, on one side of the body only, of some part of the body corresponding to the focal point of the seizure. This may spread as more areas of the brain become involved and may eventually become a generalised convulsion with involvement of the entire brain. Alternatively the seizure may remain localized and stop. Complex partial fits originate in the temporal lobes and for this reason are also known as temporal lobe epilepsy. This area of the brain is concerned with complex movements involving groups of muscles, for example walking and complex sensations such as sound, taste, sight and smell. The temporal lobes are closely linked to areas of the brain controlling autonomic function, emotions, memory and consciousness. There is a great variety in the forms complex fits may take, from bizarre complex movements to a sensation of strangeness and often unpleasant tastes and smells. Due to the proximity of the temporal lobes to the aforementioned areas of the brain, these may also become involved leading to disturbances of the heart rate, sensations of choking or uncomfortable feelings in the gut, memory upsets causing feelings of deja vu and emotional involvement leading to feelings of rage or fear. There is generally some impairment of consciouness which leaves the individual feeling confused after the attack. These fits can also develop into generalised convulsions.

The causes of epilepsy can either be termed idiopathic where there is no demonstrable brain abnormality, or symptomatic where there is a demonstrable brain lesion. However with greater understanding of how the brain works, fewer and fewer cases are designated idiopathic. The causes of fits may be (a) genetic inheritance of some form of brain abnormality - most idiopathic epilepsies fall into this category, or (b) brain damage. Brain damage is the commonest cause of temporal lobe epilepsy due to the high sensitivity of this part of the brain. Brain damage can occur through trauma at birth, as a result of an infection of the nervous system such as meningitis, as a result of injury to the head, hypoxia, the presence of a tumour or the presence of toxic substances, either due to the accumulation of endogenous toxic metabolites in diseases such as uraemia or ingestion, as in lead poisoning. The commonest precipitating factors of a seizure are stress, fever, flashing lights and lack of sleep.

For a detailed classification of seizures by clinical and electroencephalo-graphical examination as recommended by the International League against Epilepsy, the World Federation of Neurology, the World Federation of Neurosurgical Societies and the International Federation of Societies for Electroencephalography and Clinical Neurophysiology see Gastaut (1969). For further detailed discussion on all aspects of epilepsy see Laidlaw and Richens, (1982).

1.2 DRUGS USED IN THE MANAGEMENT OF EPILEPSY

This section provides an introduction to the four most commonly prescribed anticonvulsants in this country. The four drugs, phenobarbitone, phenytoin, carbamazepine and valproic acid, are presented in chronological order of their introduction into the United Kingdom. Each is discussed in a separate sub-section. Although primidone is now prescribed in preference to phenobarbitone (primidone is metabolized to phenobarbitone and another pharmacologically active compound) no patients receiving primidone therapy were studied in this thesis and therefore this drug is not discussed here. This section discusses the following properties and features of each drug: structure; seizures against which it is effective; therapeutic plasma levels; absorption; transport; metabolism; half-life; excretion; pharmacological interactions; possible mechanisms of action.

1.2.1 PHENOBARBITONE

Phenobarbitone (PB) fig. (1) is a derivative of barbituric acid and was one of the first anticonvulsant drugs used in the treatment of epilepsy over 70 years ago (Hauptmann, 1912). PB is effective in the treatment of generalized tonic-clonic and all forms of partial seizures (So and Penry, 1981).

PB differs from other barbiturates in that it's anticonvulsant properties are manifest without unacceptable sedation. The therapeutic serum levels of PB are 10-40mg/l (43-172μM). The drug is 40-50% bound in serum (Goldbaum and Smith 1954; Waddell and Butler, 1957), principally to albumin. The drug has a relatively long half-life of around 96 hours for a single dose (Viswanathan, Booker and

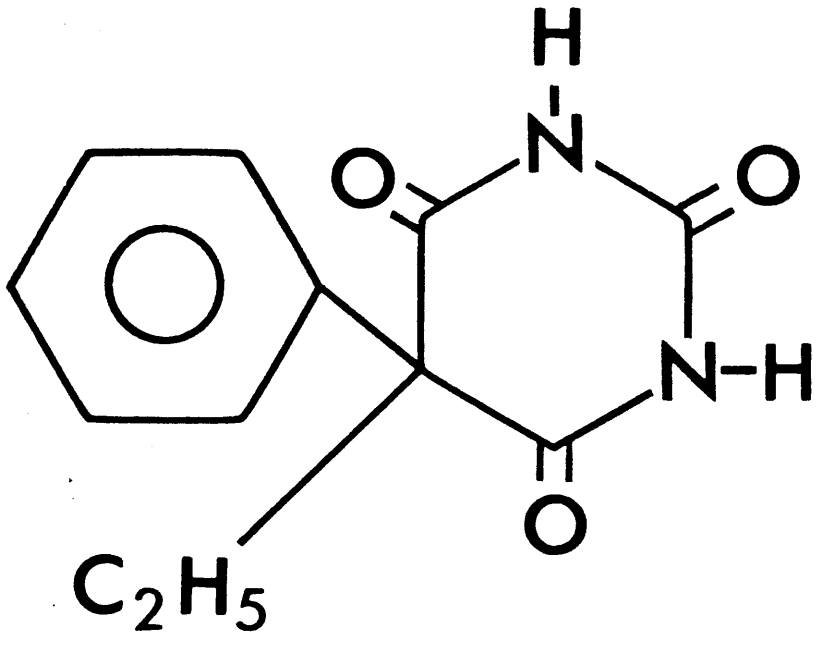


FIG. (1)

PHENOBARBITONE

Welling, 1979). PB is metabolized by the hepatic mixed function oxidase (m.f.o) system, but a significant fraction (usually around 25%) of administered drug is excreted unchanged. (Whyte and Dekaban, 1977). The major metabolite of the drug is p-hydroxyphenobarbitone (HPB). HPB is found in urine and accounts for, on average, 17% of the excreted compound and exists as both free HPB and the glucuronide conjugate (Whyte and Dekaban, 1977). The production of HPB is important in the detoxification of PB as it is devoid of anticonvulsant activity and is rapidly cleared from the circulation (Butler, 1956). This is supported by the fact that HPB cannot be detected in the plasma of epileptics taking large doses of the drug (Alvin et al, 1975). Other minor metabolites of PB are known (Maynert, 1982).

Drug interactions with phenobarbitone occur at the level of biotransformation rather than binding or absorption and are primarily due to the powerful induction of hepatic m.f.o activity which PB causes. This leads to increased metabolism and consequently shorter half-lives of many drugs including carbamazepine (Christiansen and Dam, 1973; Christiansen and Dam, 1975) and phenytoin (Cucinell et al, 1965). Due to its long half-life, compounds which interfere with PB metabolism and elimination can cause the drug to accumulate to toxic levels. This has been reported to occur with valproate and is thought to be due to an inhibitory action of valproate on the hepatic m.f.o system (Kapetanovic et al, 1980). The pharmacokinetic interactions between PB and other drugs have been catalogued by Kutt and Paris-Kutt, (1982).

In common with the other anticonvulsants to be described, the mechanism by which PB acts to prevent seizures is unknown. The most plausible mechanism, however, may lie in the effects of the drug on synaptic transmission at both pre and post-synaptic levels (Prichard, 1982).

1.2.2 PHENYTOIN

Phenytoin, also known as diphenylhydantoin, fig.(2) was originally identified as having anticonvulsant properties through its ability to prevent experimentally induced seizures in animals (Merrit and Putnam, 1938). In humans it has been shown to be effective against generalized tonic-clonic seizures and all types of partial seizures (So and Penry, 1981).

The drug is principally absorbed by passive diffusion across the lumen of the duodenum (Meinardi *et al*, 1975) rather than the stomach because phenytoin is not soluble at the pH of gastric juice (Dill *et al*, 1956). The therapeutic range of the drug in serum is 10-20mg/l (40-80μM), though departures from this are sometimes necessary depending on the individual (in clinical practice values ranging from 5-50mg/l may be found). Phenytoin in serum is highly protein bound, on average, 90% at 37°C, mostly to albumin (Hooper *et al*, 1973). The half-life of the drug in man is, on average, about 22 hours (Arnold and Gerber, 1970) following an oral dose. Enormous variations are found however because the half-life of the drug is affected by many parameters including dose (Arnold and Gerber, 1970), concurrent drug therapy (see later) and plasma bilirubin (Hooper *et al*, 1973). The drug is extensively metabolized by the mixed function oxidase system in the liver and excreted in the urine with less than 5% of the parent

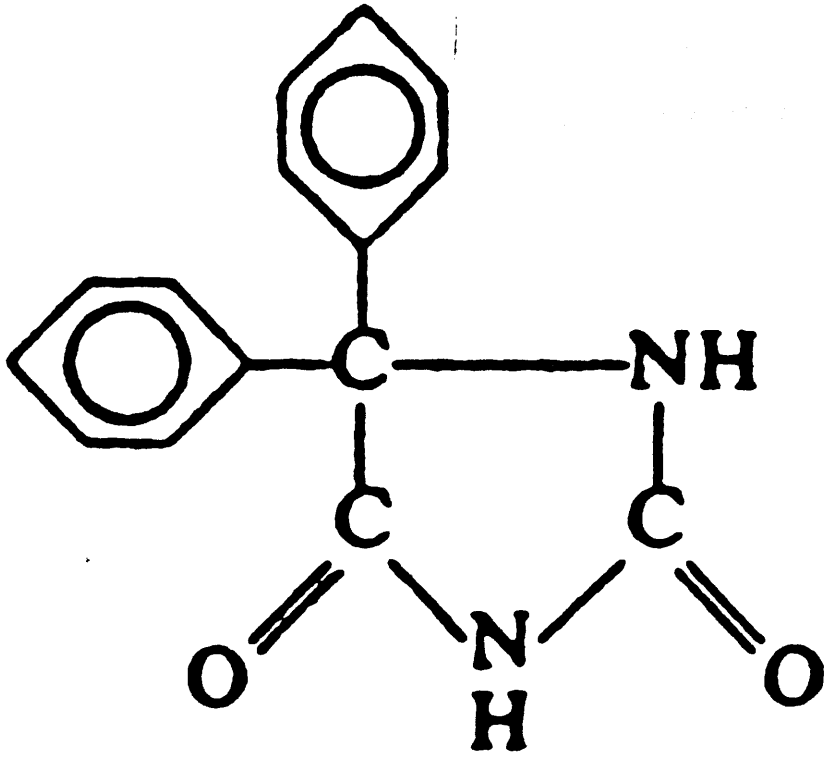


FIG. (2)

PHENYTOIN

drug remaining unchanged (Glazko et al, 1969). The principal metabolite is 5-(4-hydroxyphenyl)-5-phenyl-hydantoin (HPPH) which appears in the urine as a glucuronide conjugate (Maynert, 1960; Thompson et al, 1973). Recent advances in analytical techniques have resulted in the identification of many minor metabolites, (Chang and Glazko, 1982).

The observation made in the previous paragraph that the half-life of the drug varied with the dose given (the half-life increases as the dose increases), is due to the fact that the capacity of the liver to metabolize DPH becomes saturated within the range of plasma concentrations normally used in seizure control, (Arnold and Gerber, 1970). The drug is said to display saturation kinetics.

Phenytoin interacts extensively with many other drugs (Richens, 1977; Kutt, 1982), resulting in alterations of the pharmacokinetics of either itself or the other compound. The most common interactions occur at the level of protein-binding or biotransformation. Drugs which are strongly protein bound, such as salicylates (Fraser et al, 1980) and valproate (Dahlqvist et al, 1977), will displace phenytoin from albumin leading to an increase in the pharmacologically active free fraction of the drug, though this may be compensated by an increased rate of metabolism. Induction of hepatic mixed function oxidase activity may increase the rate of phenytoin metabolism and its rate of clearance. Thus phenobarbitone, a potent inducer of the mixed function oxidase system, can induce phenytoin metabolism (Cucinell et al, 1965) though this example can have additional complicating factors (Patsalos and Lascelles, 1977). Phenytoin

appears to be autoinducing (i.e. to induce its own metabolism) and can also induce the metabolism of other drugs, such as carbamazepine (Eichelbaum et al, 1979).

The mechanism of action of phenytoin is uncertain, but the drug is known to have many effects on events associated with the transmission of impulses through the synapse (Woodbury, 1982). However the most probable mechanism (and the only effect demonstrable using therapeutic free serum levels) is that phenytoin limits the high frequency, repetitive firing of neurons. This has been shown in cell cultures of mouse spinal cord neurons (Macdonald and McLean, 1982). This effect is believed to arise through interference with sodium transport across the neuronal membranes, possibly by temporary blockage of sodium channels after the neuron has fired, increasing the time taken for the action potential to be restored (Matsuki et al, 1981).

1.2.3 CARBAMAZEPINE

Carbamazepine (CBZ) fig.(3) was first synthesized in 1954 (Schindler and Haflinger, 1954). Several years later it was shown to have anticonvulsant properties by its ability to suppress experimentally induced seizures in animals (Theobold and Kunz, 1963). The drug has demonstrated its usefulness in the treatment of partial seizures (particularly of the complex form) and generalised tonic-clonic seizures (Cereghino et al, 1974).

CBZ is highly lipid soluble and therefore is rather slowly absorbed from the gastro-intestinal tract (Morselli, 1975). The therapeutic range of the drug in serum is 4-10mg/l (17-42μM) though some departures from this are occasionally required to achieve satisfactory

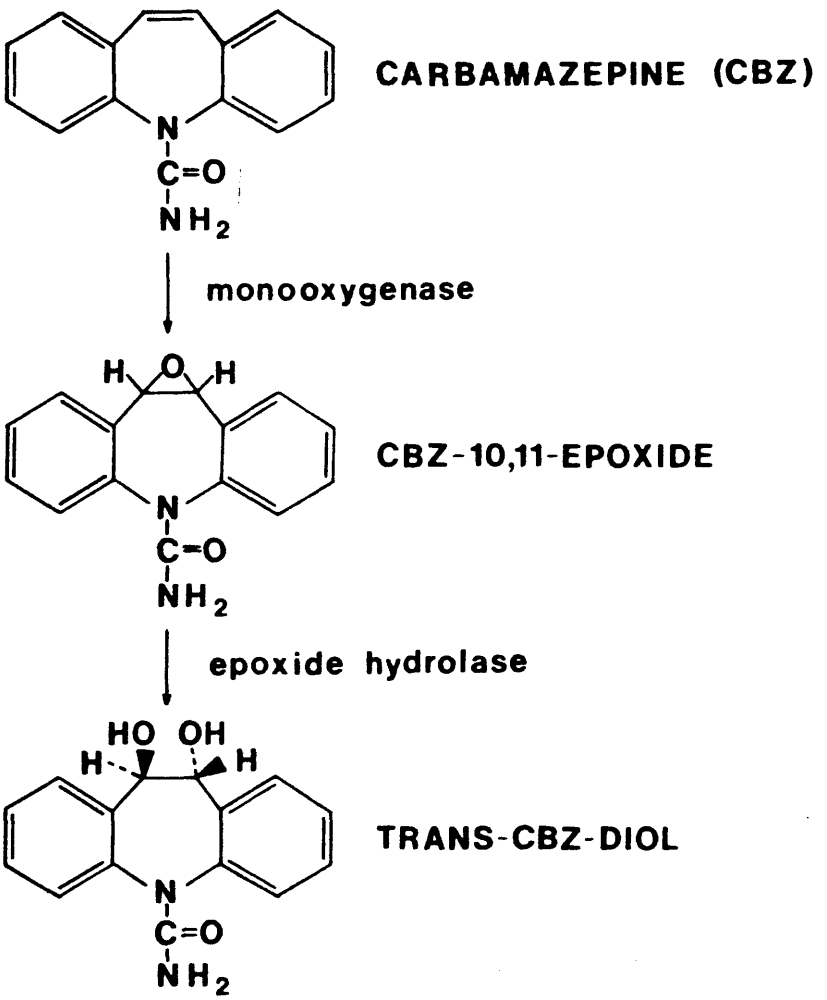


FIG. (3)

CARBAMAZEPINE: STRUCTURE AND METABOLISM

control. CBZ is 70-80% protein bound in serum. Albumin binds the drug at only one group of binding sites (Di Salle, Pacifici and Morselli, 1974), but the drug is also bound to other proteins. The half-life of CBZ in man varies tremendously and is particularly affected by co-medication. CBZ also reduces its own half-life (Rapeport et al, 1983). Half-lives of 26 hours, 12 hours and 8 hours were determined for the drug in volunteers, epileptic patients on CBZ monotherapy and epileptics taking CBZ in combination with other anticonvulsants respectively (Eichelbaum et al,1985). The mechanisms of this will be discussed later. CBZ is extensively metabolized, the metabolites being excreted in the urine and the faeces (Faigle and Feldmann, 1975). The major metabolite found in plasma is carbamazepine-10,11-epoxide (CBZ-E), fig.(3), formed through the action of hepatic mono-oxygenases on CBZ (Frigerio, Cavo-Briones and Belvedere, 1976). This compound accounts for between 10 and 20% total CBZ in plasma (Brodie, Forrest and Rapeport, 1983) and has been shown to have equipotent anticonvulsant activity with the parent drug (Frigerio and Morselli, 1985, Morselli et al, 1975). The principle excretory metabolite, trans-10,11-dihydrodihydroxycarbamazepine (trans-CBZ-diol) fig.(3), is formed by hydrolysis of CBZ-E through the action of epoxide hydrase (Oesch,1973). This compound is found in the urine, both free and as the mono-O-conjugated glucuronide, and can account for up to 60% of the daily ingested doses of carbamazepine (Eichelbaum et al, 1979). Many other minor metabolites and metabolic routes have been determined (Faigle and Feldmann 1982).

CBZ is a potent inducer of the hepatic mixed function oxidase (m.f.o) system and as such can significantly effect the

pharmacokinetics of itself and a number of other drugs. Thus prolonged treatment with CBZ results in a reduction of its half-life (Eichelbaum, 1985; Rapeport et al, 1983) and its steady state levels (Eichelbaum et al, 1975). Other drugs known to be affected in this way during comedication with CBZ include phenytoin (Hansen, Siersback-Neilsen and Skorsted, 1971) and valproic acid (Bowdle, Levy and Cutler, 1979). Similarly, drugs which induce the hepatic m.f.o. system will also reduce the half-life of CBZ (Eichelbaum et al, 1985). This has been shown to occur with phenytoin and phenobarbital (Christiansen and Dam, 1973; Christiansen and Dam, 1975). Compounds which inhibit CBZ metabolism will cause elevation of serum levels. Such an effect has been demonstrated with cimetidine, an H_2 receptor antagonist (Macphee et al, 1984). Levy and Pitick (1982) have catalogued pharmacokinetic interactions of CBZ with other drugs.

As for other anticonvulsants, the mechanism of CBZ's action is uncertain, but many workers believe that it has actions similar to phenytoin (Suria and Killam 1980). CBZ has been shown to limit high frequency sustained repetitive firing of neurons at therapeutic free serum concentrations (McLean and Macdonald, 1983). This may occur through interference with Na^+ and K^+ transport (Schauf et al, 1974). CBZ has also been shown to affect acetylcholine levels (Consolo, Bianchi and Ladinsky, 1976), to reduce catecholamine turnover (Maitre et al, 1983) and to interact with brain adenosine receptors (Marangos et al, 1983; Skerritt, Davies and Johnston, 1983). Each of these may have some bearing on the anticonvulsant efficacy of CBZ.

1.2.4 VALPROIC ACID

Valproic acid is a branched chain fatty acid, fig.(4), first synthesized in 1882 (Burton, 1882), but not identified as having anticonvulsant activity until 1963 (Meunier et al, 1963). It was first used in Europe in 1964 (Carraz et al, 1964) and in America 14 years later. The drug is generally prescribed as the sodium salt, which has greater water solubility. In man, VPA has been shown to be effective in the management of generalized tonic-clonic, absence and myoclonic seizures (Pinder et al, 1977; Bruni and Wilder, 1979).

VPA is rapidly absorbed following an oral dose and displays no site specificity for absorption. Evidence for a therapeutic range is sparse, but a target range of 50-100mg/l (0.33-0.67mM) in serum can be used. Toxicity is frequently reported at concentrations in excess of this (Mucklow, 1984). The drug is highly protein bound, on average about 90% (Patel and Levy, 1979; Bowdle et al, 1980). As total plasma VPA is increased, the free fraction of the drug rises, indicating that binding of the drug is a saturable process (Patel and Levy, 1979, Bowdle et al, 1980). Albumin is the principal protein which binds the drug and has two groups of binding sites (Gugler and Mueller, 1978). Most other drugs, including the major anticonvulsants, do not affect VPA binding due to the relatively high affinity the drug has for albumin. However salicylate can decrease the bound fraction (Schobben, Vree and van der Kleijn, 1978) as can free fatty acids (Monks and Richens, 1979; Patel and Levy, 1979) due to competitive inhibition of the binding process. The elimination half-life of VPA in man averages about 9 hours (Mattson et al, 1978), though this can be affected by concurrent medication (see later).

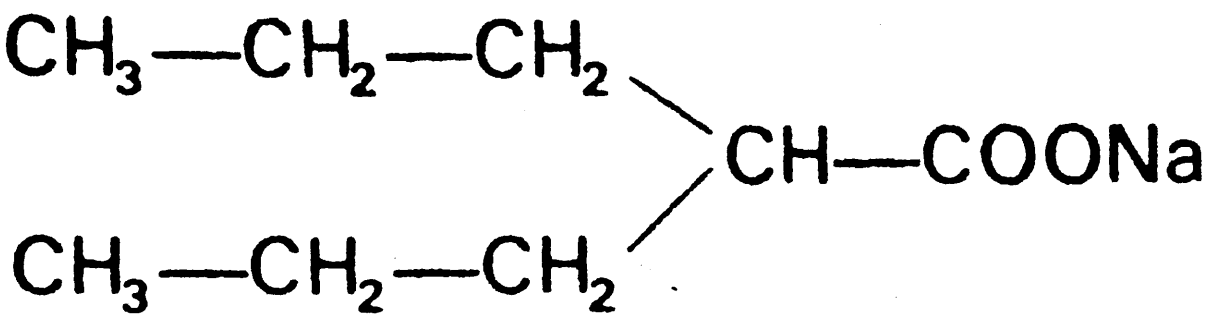


FIG. (4)

SODIUM VALPROATE

VPA is extensively metabolized primarily by β-oxidation in extra-hepatic tissues (Matsumoto, Kuhara and Yoshino, 1976) and ω-oxidation involving liver microsomal enzymes (Kochen and Scheffner, 1980). This metabolism is what would be expected of a fatty acid and explains the many metabolites found in urine and plasma (Kuhara et al, 1978). The major excretory route is in the urine and the major excretory product is the glucuronide conjugate of VPA (Vree and van der Kleijn, 1977). Less than 4% of an oral dose is excreted as the free acid (Gugler et al, 1977).

VPA frequently interacts with other drugs, affecting the pharmacokinetics of VPA and other drugs (Mattson, 1982). The tight binding of VPA may lead to the displacement of other, less tightly bound drugs, leading to either an increase in the pharmacologically active free fraction of the drug (as may occur with carbamazepine; Mattson, Mattson and Gramer, 1982) or a reduction in total concentration due to a greater rate of clearance (as occurs with phenytoin; Mattson et al, 1978). VPA also has inhibitory effects on the hepatic mixed function oxidase (m.f.o.) system. This could lead to accumulation of drugs which have to be metabolized by this pathway and is believed to be the mechanism by which VPA raises phenobarbital levels during co-medication (Kapetanovic et al, 1980). VPA does not induce its own metabolism or that of other drugs (Mattson et al, 1978) but many drugs which induce hepatic m.f.o. activity accelerate VPA metabolism resulting in a more rapid clearance and a reduced half-life (Mihaly et al, 1979; Reunanen et al, 1980).

The mechanism of action of VPA is unknown but there are three basic hypotheses (reviewed by Johnston and Slater, 1982). The first suggests that VPA operates by increasing the levels of the inhibitory neurotransmitter γ-amino butyric acid (GABA) in brain by inhibition of GABA catabolism (Godin et al, 1969; Simmler et al, 1973; Harvey, Bradford and Davison, 1975). The second hypothesis proposes that VPA exerts its effects by acting on membranes to reduce the excitability of the neuron (Slater and Johnston, 1978), while the third hypothesis suggests that VPA enhances the response of the GABA receptor in the post-synaptic cell to the binding of GABA, potentiating the inhibitory effect (Macdonald and Bergey, 1979). There is to date no data to favour any one of these hypothesis more than another and it must be remembered that much of this work was performed in vitro and as such must be interpreted with caution.

1.3 HAEM BIOSYNTHESIS

The haem biosynthetic pathway is of great interest because of the vital metabolic role played by its end-product, haem, in biological oxidative processes and oxygen transport. Variations of the basic porphyrin nucleus, of which haem is but one, are found in bacteria, yeasts and plant cells, as well as in avian and mammalian tissues, reflecting the central role played by these compounds in nature.

The steps leading to the biosynthesis of haem were initially elucidated through the use of isotopic tracer studies by Shemin (1955). These results laid the foundations for all subsequent investigations, as a result of which the pathway is now well understood and is illustrated in a typical cell in fig. (5).

As this thesis discusses the effects of drugs on the enzymes of haem biosynthesis, a short resume of the knowledge to date on each of the enzymes is presented. In general, only mammalian enzymes will be reviewed. The excretion of pathway intermediates, disorders of the pathway and the response of the pathway to interference will also be covered.

1.3.1 5-AMINOLAEVULINIC ACID SYNTHASE

(ALA-S; Succinyl CoA: Glycine C Succinyltransferase (decarboxylating); E.C.2.3.1.37).

The first and rate-limiting step of haem biosynthesis is the condensation of succinyl CoA and glycine to form ALA and is catalysed by ALA-S. The structure of ALA is shown in fig.(6). The enzyme is located in the mitochondrion, loosely attached to the inner membrane (Patton and Beattie, 1973) and requires the cofactor pyridoxal phosphate (PLP) for activity (Schulman and Richert, 1956) and also perhaps as a stabilizing factor (Beattie *et al*, 1985).

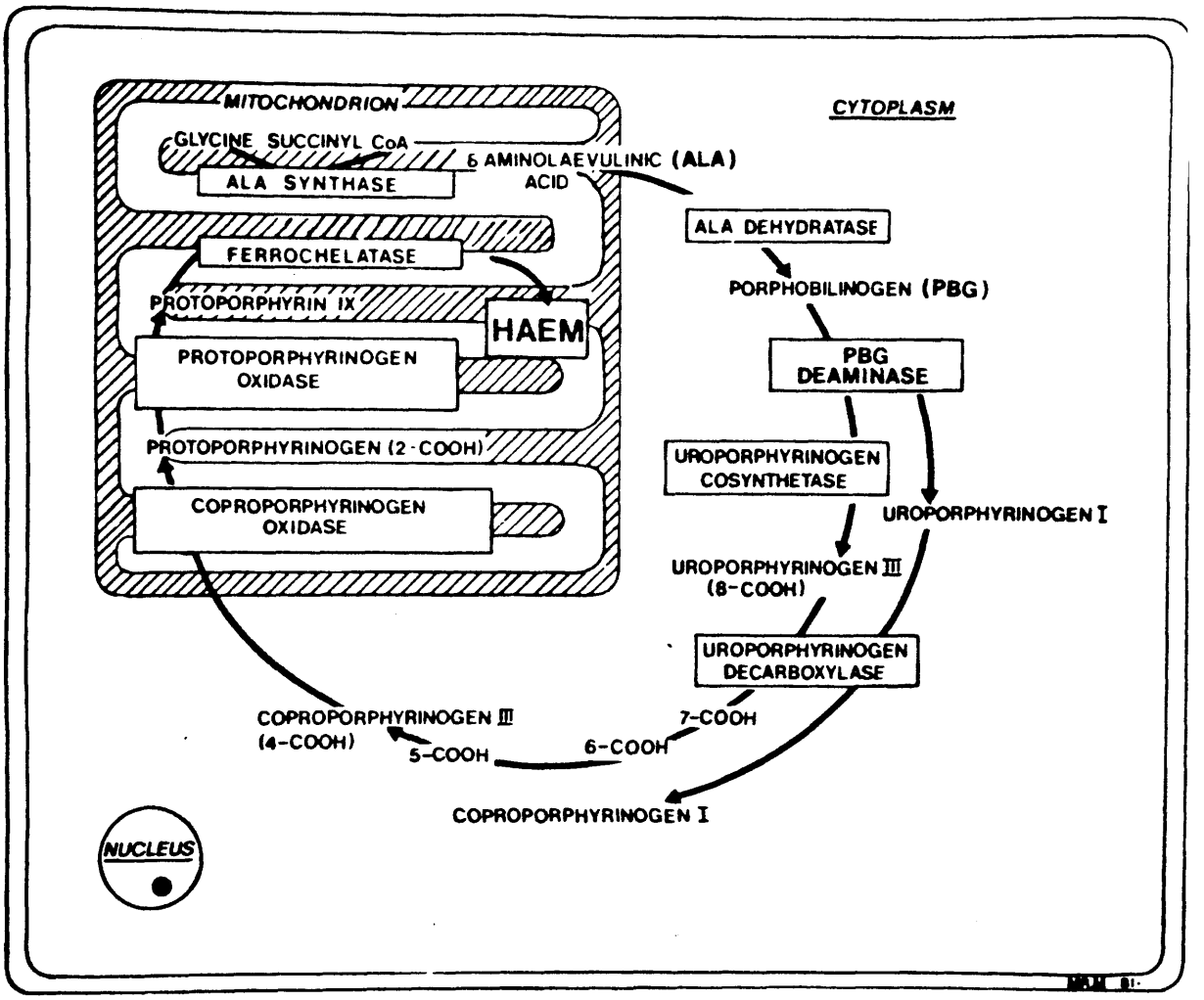


FIG. (5)

THE HAEM BIOSYNTHETIC PATHWAY

The figure shows the enzymes and intermediates of haem biosynthesis and their distribution within the cell.

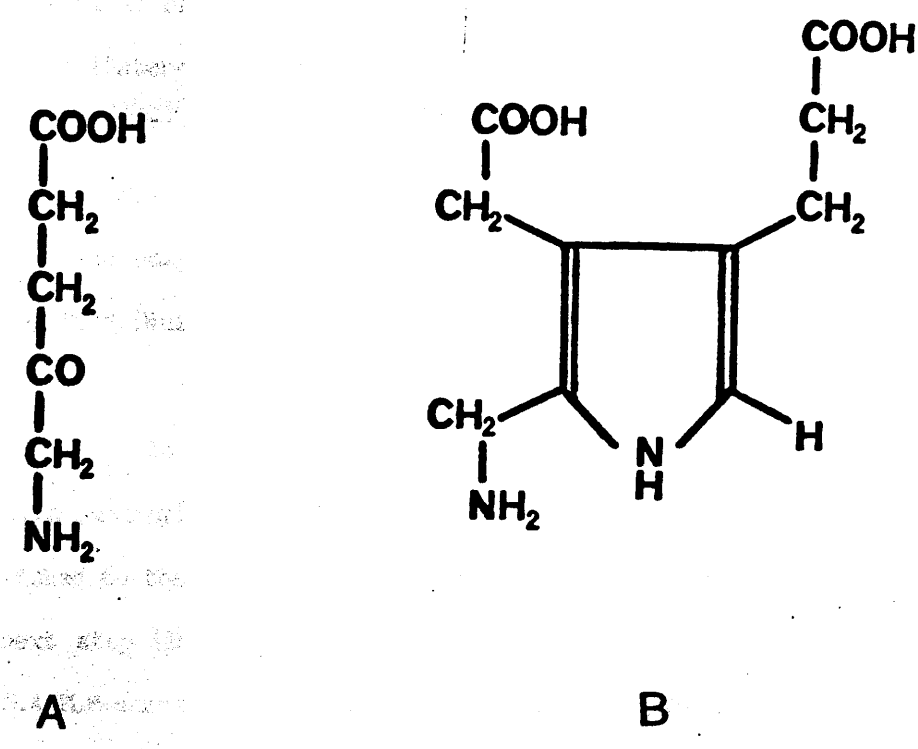


FIG. (6)

STRUCTURES ON THE PORPHYRIN PRECURSORS

A - 5-Aminolaevulinic acid (ALA)

B - Porphobilinogen (PBG)

The enzyme is synthesized in the cytoplasm as a precursor and then translocated to the mitochondria (Hayashi, Yoda and Kikuchi 1970). This has recently been demonstrated *in vitro* with the suggestion of processing of the enzyme in the course of the translocation (Yamauchi, Hayashi and Kikuchi, 1980 (B)).

ALA-S has been purified from rat liver mitochondria and shown to be a dimer of 120,000 molecular weight, composed of two identical subunits (Paterniti and Beattie, 1979). The cytosolic enzyme has also been purified and shown to be immunochemically identical to the mitochondrial enzyme (Nakakuki *et al*, 1980). The pH optimum of the purified enzyme was 7.5. The enzyme from chick liver has also been purified (Whiting and Granick, 1976).

The mechanism of the reaction is that glycine reacts with enzyme bound PLP to form a stable Schiff base carbanion. This then reacts with succinyl CoA to produce α-amino β-ketoadipic acid which remains linked to the PLP-enzyme. There are two possible mechanisms for the next step (Ahktar *et al*, 1976): either a decarboxylation to give an ALA-PLP-enzyme complex which is then hydrolysed to release ALA, or hydrolysis of the complex yielding α-amino β-ketoadipic acid which spontaneously decarboxylates to ALA.

ALA-S is the rate-limiting enzyme of haem biosynthesis and as such is strictly regulated, principally through feed-back regulation of enzyme synthesis by haem. Haem has been shown to inhibit ALA-S synthesis at the transcriptional level (Yamamoto, Hayashi and Kikuchi, 1982) at the translational level (Kikuchi and Hayashi, 1981; Yamamoto, Hayashi and Kikuchi, 1983) and at the level of translocation of the precursor ALA-S from the cytoplasm into the mitochondria

(Yamauchi, Hayashi and Kikuchi, 1980 (A), Kikuchi and Hayashi, 1981). Haem has also been shown to directly inhibit ALA-S (Whiting and Elliot, 1972; Scholnick, Hammaker and Marver, 1972) though the physiological relevance of this is doubtful (Wolfson, Bartczak and Bloomer, 1979). Steroids are also known to have regulatory effects on the levels of enzyme, though the mechanisms by which these occur are uncertain (Matsuoka, Yoda & Kikuchi, 1968; Sassa, Bradlow and Kappas, 1979). Control of flux through the pathway is also enhanced by the short half-life of the enzyme of 1 hour and the low levels of activity present in cells, (Granick and Sassa, 1971).

ALA-S activity which is normally low, can be derepressed following treatment with drugs and xenobiotics and elevated levels of the enzyme are also found in certain pathological conditions e.g. the porphyrias. The mechanisms by which these effects occur will be discussed in sub-section 1.3.9.

1.3.2 5-AMINOLAEVULINIC ACID DEHYDRATASE

(ALA-D; porphobilinogen synthase; 5-aminolaevulinate hydrolase (adding 5-aminolaevulinate and cyclizing); E.C. 4.2.1.24)

The next step in the pathway takes place in the cytoplasm. ALA-D catalyses the condensation of two molecules of ALA to form the monopyrrole porphobilinogen (PBG) with the elimination of two molecules of water. The structure of PBG is shown in fig.(6).

ALA-D has been purified from bovine liver (Wu et al, 1974) and human erythorocytes (Anderson and Desnick, 1979). Both have been shown to be octameric enzymes of molecular weights 285,000 and 252,000 respectively and to require Zn^{2+} ions for activity. The human erythrocyte enzyme binds 8 atoms of Zn^{2+} per molecule of native enzyme

(Gibbs and Jordan, 1981). The enzyme also has essential sulphydryl groups which must be maintained in a reduced state for maximum catalytic activity using reagents such as dithiothreitol, mercaptoethanol and cysteine (Shemin, 1972).

The first step in the condensation reaction is the binding of the ALA molecule which will give rise to carbon atoms 2,3,8,9 and 10 in the PBG produced to one side of the catalytic site (Jordan and Gibbs, 1985). A Schiff base is formed with a reactive amino group in the active site and a second molecule of ALA then occupies the other side of the active site. The series of steps leading to the formation of PBG are described by Shemin (1976).

ALA-D is very sensitive to inhibition by a number of compounds. Lead is an extremely potent inhibitor of the enzyme, though its effects are reversible by treatment with Zn^{2+} and sulphydryl reagents (Mauras and Allain, 1979; Moore, Meredith and Goldberg, 1980). This suggests that inhibition takes place through interaction of lead with essential -SH groups, though other factors may be involved (Sakai, Yanagihara and Ushio, 1981). Alcohol also inhibits ALA-D (Moore, McColl & Goldberg, 1985) both in human erythrocytes (Krasner et al, 1974) and in human liver (Kondo, Urata & Shimizu, 1984). Succinyl acetone, a metabolite produced in abnormal quantities by individuals with hereditary tyrosinemia, is a very potent inhibitor of ALA-D because it is a non-metabolizable substrate analogue for ALA (Tschudy, Hess and Frykholm 1981; Sassa and Kappas, 1983). Inhibition of ALA-D by these compounds may be great enough to impair haem biosynthesis and consequently lead to an increase in ALA-S activity in the manner described in 1.3.9.

1.3.3 PORPHOBILINOGEN DEAMINASE

(PBG-D; uroporphyrinogen-I-synthase; hydroxymethybilane synthase porphobilinogen ammonia-lyase (polymerizing); E.C. 4.3.1.8)

and

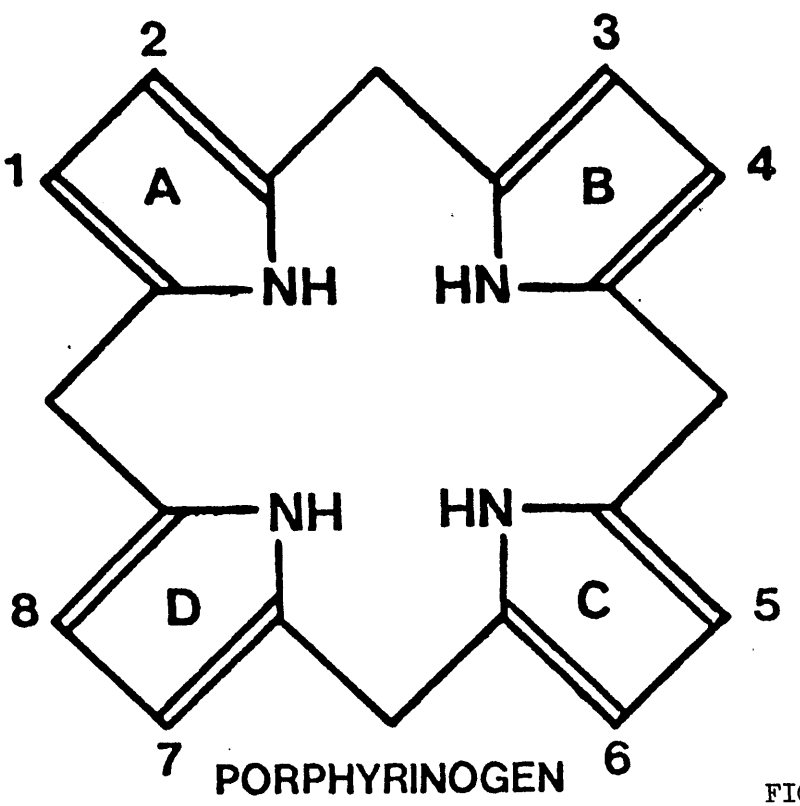
UROPORPHYRINOGEN-III-COSYNTHASE

(URO-CoS NoE.C. Number).

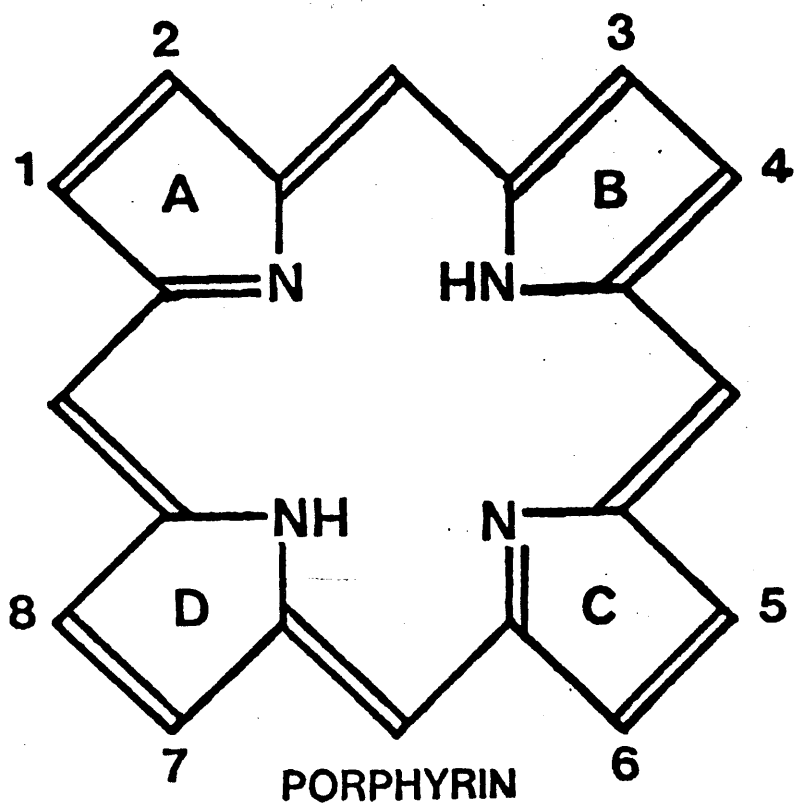
PBG-D and URO-CoS work in concert to produce the asymmetric tetrapyrrole uroporphyrinogen III (UROgen III) (fig.(7) from 4 molecules of PBG with the release of 4 molecules of ammonia. In the absence of the cosynthase UROgen I is formed. Both enzymes are cytoplasmic. PBG-D activity is normally the rate limiting factor in the conversion of PBG to UROgen III.

PBG-D has been purified from human erythrocytes and shown to be a monomeric enzyme of molecular weight 36,000 -38,000 daltons and to have a pH optimum of 8.2 (Anderson and Desnick, 1980). No purifications of the cosynthase are reported.

The mechanism of the reactions is complex. Studies on the intermediates using NMR spectroscopy (Burton et al 1979; Scott et al, 1980) have revealed that PBG-D catalyses a head to tail condensation of 4 molecules of PBG to form the linear tetrapyrrole hydroxymethylbiliane (HMB, also called preuroporphyrinogen) which is the substrate for URO-CoS in the synthesis of the asymmetrical tetrapyrrole UROgen III (Jordan et al, 1979). In the absence of URO-CoS activity, the symmetrical isomer UROgen I is formed through a rapid non-enzymatic cyclization of the highly unstable HMB($t^1/_2$ = 4.4 minutes at 37°C and pH 8.25; Burton et al, 1979). For further discussion of the mechanism and stereochemistry of the PBG-D reaction see Jones, Jordan and Ahktar (1984).



PORPHYRINOGEN



PORPHYRIN

FIG. (7)

THE STRUCTURES OF PORPHYRINOGENS AND PORPHYRINS

Porphyrin(ogen)	1	2	3	4	5	6	7	8
Uroporphyrin(ogen)III	Ac	P	Ac	P	Ac	P	P	Ac
Heptacarboxylic Porphyrin(ogen)III	Ac	P	Ac	P	Ac	P	P	Me
Hexacarboxylic Porphyrin(ogen)III	Me	P	Ac	P	Ac	P	P	Me
Pentacarboxylic Porphyrin(ogen)	Me	P	Me	P	Ac	P	P	Me
Coproporphyrin(ogen)III	Me	P	Me	P	Me	P	P	Me
Protoporphyrin(ogen)III	Me	V	Me	V	Me	P	P	Me

Ac = Acetyl
P = Propionyl
Me = Methyl
V = Vinyl

Haem is protoporphyrin IX with an Fe^{2+} ion inserted into the centre of the molecule. The series I isomers for URO-COPRO are identical to the structures shown except that the functional groups at positions 7 and 8 are reversed.

PBG-D has the lowest endogenous activity in the haem biosynthetic pathway after ALA-S (Elder, 1982) and it has been suggested for this reason that it may be a secondary control point (Brodie et al, 1977(A)). This is of interest because it implies that inhibition of PBG-D could seriously disturb flux through the pathway, especially in individuals suffering from acute intermittent porphyria where there is a genetically inherited deficiency of PBG-D activity (Meyer et al, 1972; Strand et al, 1972). This may precipitate an acute attack as described in subsection 1.3.10. With regard to this, sulphonamides (Peters et al, 1980), bilirubin (Kohashi, Tse and Piper, 1984) and carbamazepine (Yeung et al, 1983 (A)) have been reported to inhibit PBG-D activity, though the findings of Yeung et al are disputed and will be investigated in detail in this thesis.

1.3.4 UROPORPHYRINOGEN DECARBOXYLASE

(URO-D; uroporphyrinogen carboxy-lyase; E.C. 4.1.1.37).

URO-D, a cytoplasmic enzyme, catalyses the conversion of UROgen (an octacarboxylic porphyrinogen) to coproporphyrinogen (COPROgen; a tetracarboxylic porphyrinogen) (see fig.7) by 4 sequential decarboxylations.

URO-D has been purified from bovine liver (Straka and Kushner, 1983) and from human erythrocytes (Elder, Tovey and Sheppard, 1983; de Verneuil, Sassa and Kappas, 1983 (B)). The enzymes from both species are monomeric proteins and have the ability to catalyse each of the four decarboxylations which occur in the conversion of UROgen to COPROgen. Both enzyme species will convert UROgen III or UROgen I to the appropriate COPROgen isomers, though UROgen III is the preferred substrate and is more rapidly metabolized. Both appear to have at

least one essential sulphydryl group for catalytic activity and to have at least two active sites. The optimum assay pH in each case is 6.8. The bovine liver enzyme has a molecular weight of around 57,000 whilst the human erythrocyte enzyme has a molecular weight of 46,000. URO-D has also been purified from chicken erythrocytes (Kawanishi, Seki and Sano 1983).

Following binding of UROgen to the enzyme, the four decarboxylations appear to occur in sequence, the first being the rapid removal of the carboxyl group of the carboxymethyl constituent of the D ring, yielding heptacarboxylic (7-COOH) porphyrinogen, followed by the slower elimination of the carboxyl groups on the A,B and finally C rings to form hexacarboxylic-(6-COOH), pentacarboxylic-(5-COOH) and tetracarboxylic-(COPRO) porphyrinogens respectively (Jackson et al, 1976). The structures of these are shown in fig.(7).

URO-D is sensitive to inhibition by polyhalogenated aromatic compounds (Sinclair et al, 1984; Rifkind et al, 1985; Sano, Kawaniski and Seki, 1985). Iron also plays a role in the inhibition of this enzyme (Smith and Francis,1983; de Verneuil, Sassa and Kappas,1983(A); Ferioli, Harvey and de Matteis, 1984; Mukerji, Pimstone and Burns, 1984). Liver disease such as is found in alcoholism may also lead to depressed URO-D activities (Moore, McColl and Goldberg, 1985). All of these may produce a cutaneous hepatic porphyria syndrome, identical to that found in the inherited deficiency of URO-D except that in this case the lesion is confined to the liver whereas in the inherited situation it is present in all tissues.

1.3.5 COPROPORPHYRINOGEN OXIDASE

(COPRO-O; coproporphyrinogen: oxygen oxidoreductase (decarboxylating); E.C.1.3.3.3).

The pathway now re-enters the mitochondria, where COPROgen III undergoes two oxidative decarboxylations to produce protoporphyrinogen IX. (PROTOgen IX), fig.(7). The reaction is catalysed by COPRO-O, an enzyme located in the intermembrane space of the mitochondria (Elder and Evans, 1978).

The enzyme reaction occurs in two steps (Ahktar et al, 1976) and appears to follow a specific sequence, the propionic acid on the A ring being decarboxylated before that on the B ring (Jackson et al, 1976). The enzyme requires molecular oxygen as a hydrogen acceptor for this reaction (Sano and Granick, 1961). COPROgen I is not a substrate for the enzyme (Porra and Falk, 1964; Batlle, Benson and Rimington, 1965).

1.3.6 PROTOPORPHYRINOGEN OXIDASE

(PROTO-O; protoporphyrinogen IX dehydrogenase; E.C.1.3.3.4).

The reaction catalysed by PROTO-O is the oxidation of PROTOgen IX to protoporphyrin IX (PROTO IX). This reaction can occur spontaneously and for this reason it was relatively recently that the existence of this enzyme was established. Jackson et al (1974) demonstrated by isotopic labelling studies that the reaction is indeed enzyme catalysed in vivo and occurs in a stereospecific manner: 6 hydrogen atoms are removed from one side of PROTOgen IX only. The enzyme requires oxygen and is bound to the inner mitochondrial membrane (Poulson and Polglase, 1975; Poulson, 1976).

1.3.7 FERROCHELATASE

(FERRO-C; haem synthetase; protohaem ferro-lyase; E.C. 4.99.1.1.).

The final step in the biosynthesis of haem takes place within the mitochondrion and is catalysed by FERRO-C. Ferrous ions (Fe^{2+}) are inserted into PROTO IX with concomitant removal of two hydrogen atoms. FERRO-C is attached to the inner mitochondrial membrane (McKay et al, 1969) and requires lipid for activity (Simpson and Poulson, 1977; Taketani and Tokunaga, 1981).

The enzyme has been purified from rat liver (Taketani and Tokunaga, 1981) and from bovine liver (Taketani and Tokunaga, 1982) and shown to have molecular weights of approximately 240,000 and 200,000 respectively and to be oligomers of 4 or 5 identical subunits.

FERRO-C activity is severely inhibited by treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC), producing a form of porphyria in experimental animals. The actual inhibitor is formed by a transmethylation from DDC to the porphyrin moiety of cytochrome P450 (Ortiz de Montellano, Beilan and Kunze, 1981; Tephly et al, 1980). Lead (Campbell et al, 1977) and alcohol (Brodie et al, 1979), also reduce FERRO-C activity.

1.3.8 THE EXCRETION OF INTERMEDIATES IN THE HAEM BIOSYNTHETIC PATHWAY

The intermediates of haem biosynthesis, ALA,PBG and the oxidized forms of the various porphyrinogens are normally excreted in small quantities in urine. The structures of these compounds were shown in figs. (6) and (7). Porphyrins found in normal urine are predominantly COPRO but traces of URO and the other intermediates are also found. Most of the URO-I produced and all of the COPRO-I

produced is excreted in this way because as explained in 1.3.4 and 1.3.5, URO-I is a poor substrate for URO-D and COPRO-I is not a substrate for COPRO-O.

When the pathway is subjected to stress, for example by inhibition of one of the enzymes, an alteration in the excretion profile of these precursors is found. Thus lead-poisoning, where ALA-D and FERRO-C activities are reduced, leads to increased excretion of ALA and PROTO, whereas the depression of URO-D sometimes found in alcoholic liver disease may result in an increase in the excretion of URO and 7-COOH porphyrins. The porphyrias, genetically inherited defects of haem biosynthetic enzymes, also result in abnormal urinary and faecal excretion of porphyrins and precursors. The changes found are discussed in detail in 1.3.10.

1.3.9 DEREPRESSION OF 5-AMINOLAEVULINATE SYNTHASE

ALA-S, the rate controlling step in haem biosynthesis, can be derepressed by treatment with drugs or in pathological conditions such as the porphyrias. The primary control of the levels of ALA-S activity is through feed-back inhibition of synthesis by haem, as was discussed in 1.3.1. The current concept of regulation of this process is that the rate of enzyme synthesis is governed by a small, rapidly turning-over "free" haem pool within the cell. If this pool is depleted through decreased synthesis, increased utilization or increased destruction of haem, ALA-S synthesis will be induced. The circumstances giving rise to such changes in intracellular haem will now be discussed.

Decreased synthesis of haem can occur when the activity of one or more of the enzymes in the pathway is reduced, due to inhibition by some factor or a genetically inherited defect. A classic example of such inhibition is found in lead-poisoning where ALA-D and FERRO-C activities are affected. Genetically inherited defects constitute the porphyrias and will be discussed in 1.3.10. In these situations, the reduced availability of pathway intermediates after the block leads to lower levels of haem output than normal. However, these levels, though lower than normal, may be insufficient in themselves to trigger derepression of ALA-S and often an additional reduction of intracellular haem by either of the remaining mechanisms is required. The presence of such a block, however, greatly increases the sensitivity of ALA-S to derepression in these circumstances.

Increased utilization of haem is the commonest mechanism by which ALA-S activity is increased. Many drugs lead to derepression of ALA-S. The vast majority of these are also lipid soluble and metabolized prior to excretion by the microsomal mixed function oxidase system. This occurs primarily in the liver (Lu, Kuntzman and Conney, 1976). Part of this system is a group of inducible haemoproteins known as the cytochrome P450s (see Dus, 1982 for a discussion of the active site and Rabin, 1983 for a discussion of the nature and inducibility of cyt P450). The synthesis of these proteins utilizes 65% of haem produced in the liver (Sassa and Kappas, 1981). The cyt P450s are responsible for the actual oxidative metabolism of compounds processed by the microsomal mixed function oxidase system. Some of these drugs, such as phenobarbitone, induce de novo synthesis of the apoprotein (Hardwick, Gonzalez and Kasper,1982) which will bind haem and

consequently reduce the intracellular haem concentration thus causing derepression of ALA-S. Others are "suicide substrates", that is, they are metabolized by cyt P450 but destroy the haem moiety in the process (Ortiz de Montellano et al, 1981; Ortiz de Montellano and Correia, 1983) leading to the synthesis of new cyt P450 to replace what was lost. This destruction of haem is the third way in which intracellular haem is depleted. Two classic examples of compounds which lead to derepression of ALA-S through increased haem destruction are 2-allyl-2-isopropylacetamide (De Matteis, 1971) and DDC, which has already been discussed (1.3.7).

As was mentioned in the discussion on ALA-S in 1.3.1, steroids can also cause derepression of ALA-S, not necessarily by mechanisms which involve haem (Anderson, Freddara and Kappas 1982; Thompson et al, 1984 (A). Fig.(8) summarises the different sites at which drugs may act to produce derepression of ALA-S.

Derepression of ALA-S does not present a problem in normal individuals, though a slight increase in urinary ALA and PBG may be detected. However if there exists a block in the pathway as is found in the porphyrias or conditions such as lead poisoning, extensive accumulation of ALA, PBG and other porphyrins may occur with the various clinical problems associated with high levels of these compounds. The extent of the increase in ALA-S activity is likely to be greater in these individuals due to the inherent instability of control, leading to even greater production of the porphyrins and their precursors. Thus any drug or compound which can cause derepression of ALA-S will precipitate an acute attack of porphyria in susceptible individuals.

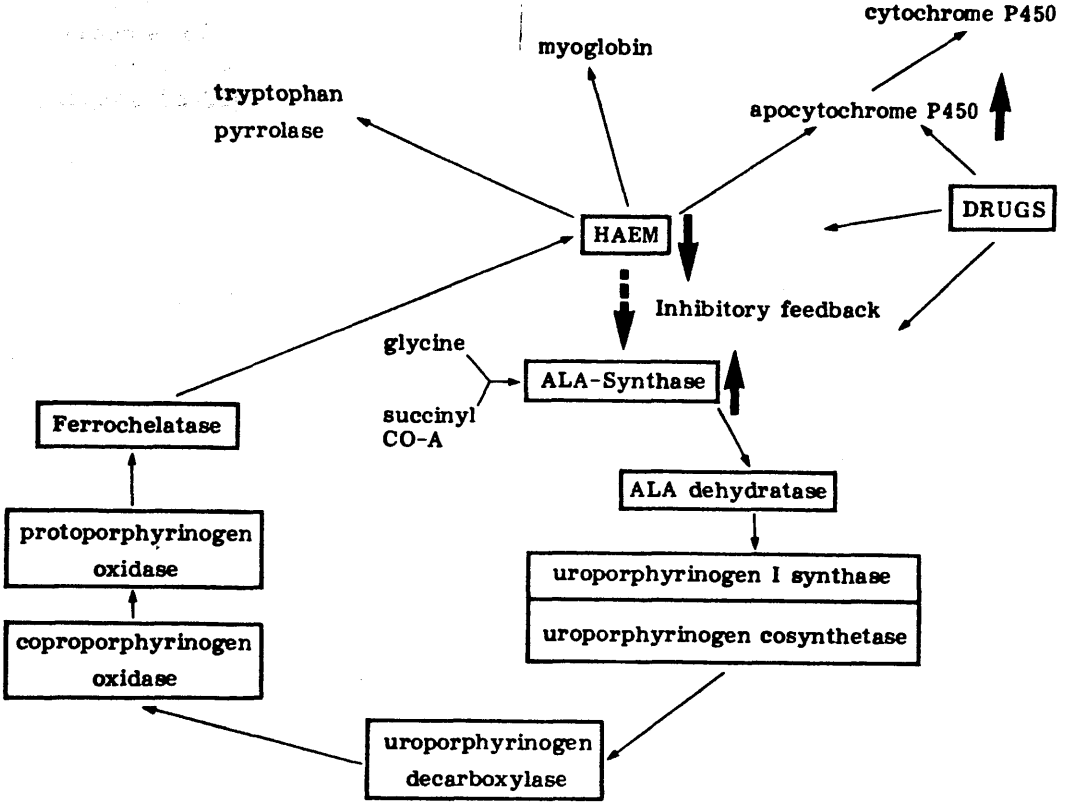


FIG. (8)

SITES OF INTERACTION OF DRUGS WITH HAEM BIOSYNTHESIS

Many drugs increase the rate of haem biosynthesis through derepression of ALA-S, the rate limiting enzyme of the pathway. This derepression may be brought about by a reduction in haem levels which could occur as a direct or indirect result of the drugs as shown. Alternatively, certain drugs may directly derepress ALA-S. The mechanisms are discussed more fully in subsection 1.3.9.

1.3.10 THE PORPHYRIAS

The porphyrias are a group of metabolic disorders characterized by over-production and excretion of porphyrins and their precursors. They may be conveniently divided into two classes: acute porphyrias and non-acute porphyrias, the characterization being made on the presence of acute generalized neuropathies in the former which are absent in the latter. Each of the porphyrias is attributable to a particular enzymic deficiency in the haem biosynthetic pathway which may be either genetically transmitted or acquired through exposure to a foreign agent. Deficiencies for each of the enzymes in the pathway from ALA to haem are known and each gives rise to a particular form of porphyria. For a review of the defective enzymes see Elder (1983) and Rimington (1985). Table (1) lists the site of the enzymic lesion and classification (acute/non-acute) of each of the porphyrias. The abnormal porphyrin and precursor excretion patterns found in all of the porphyrias is a consequence of the high levels of ALA-S activity found in the tissues of patients with these diseases. This occurs because the enzyme deficiency may compromise haem output, leading to derepression of ALA-S (as described in 1.3.9), in an effort to satisfy the cell's haem requirements. This derepression of ALA-S probably occurs in all tissues: in acute intermittent porphyria increased ALA-S activity has been demonstrated in both liver (Strand *et al*, 1970) and leucocytes (McColl *et al*, 1982; Brodie *et al*, 1977 (A). The vast majority of the porphyrins and precursors excreted, however, originate from the liver.

TABLE (1)

SITES OF THE ENZYMIC LESIONS IN THE PORPHYRIAS

Enzyme Deficiency	Porphyria Resulting	Acute or Non-Acute
5-Aminolaevulinic acid dehydratase	Plumboporphyria	Acute
Porphobilinogen deaminase	Acute intermittent porphyria	Acute
Uroporphyrinogen III cosynthase	Congenital erythropoietic protoporphyria	Non-acute
Uroporphyrinogen decarboxylase	Porphyria cutanea tarda	Non-acute
Coproporphyrinogen oxidase	Hereditary coproporphyria	Acute
Protoporphyrinogen oxidase	Variegate porphyria	Acute
Ferrochelatase	Erythropoietic protoporphyria	Non-acute

The clinical manifestations of the porphyrias varies between the two groups: acute and non-acute (see table (1)) The acute porphyrias are characterized by acute attacks during which the patient almost always suffers severe abdominal pain, vomiting, constipation and tachycardia. There may also be neuropsychiatric disturbances including epilepsy, psychoneurosis and muscle weakness or paralysis. Acute attacks can last from several days to several months and are usually interspersed with periods of remission during which the clinical features subside. Often there are no clinical symptoms although biochemical analysis reveals the presence of the disease. This is described as latency. The clinical symptoms in the non-acute porphyrias are photosensitive skin lesions due to deposition of porphyrin in the upper epidermal layer of the skin. The severity of these lesions varies between the different porphyrias. There may be clinical evidence of liver disease. For further reading on the clinical presentation of the porphyrias see Goldberg and Moore (1980), and Disler and Moore (1985).

There now follows a short resume of the biochemical features of each of the porphyrias.

Plumboporphyria

This extremely rare disease, due to low levels of ALA-D activity (2% of normal), is inherited in an autosomal recessive manner (Bird et al, 1979; Doss et al, 1982). Recently, de Verneuil et al (1985) have demonstrated that low activity is due to a structurally modified enzyme. The disease is characterized by excessive production and excretion of ALA.

Acute Intermittent Porphyria (AIP)

The commonest of the acute hepatic porphyrias, AIP is characterized by a 50% reduction in the activity of PBG-D, and has been shown by familial studies to be inherited as an autosomal dominant trait. The enzyme has been shown to be defective in all tissues studied to date including liver (Strand et al, 1970), erythrocytes (Strand et al, 1972), epidermal cells (Bickers et al, 1977), cultured skin fibroblasts (Bonkowsky et al, 1975 (B), cultured lymphocytes (Sassa, Zalar and Kappas, 1978) and cultured amniotic cells (Sassa et al, 1975). Sassa et al, (1978) demonstrated that the low activity is due to a defect in the synthesis of PBG-D rather than a defective protein. The disease exhibits a high degree of latency, the majority of cases only coming to light when some aggravating factor precipitates an acute attack. The most common factors are drug ingestion and endogenous or exogenous steroid hormones, all of which lead to derepression of ALA-S. However in both the latent and remission phases of the acute disease, increased urinary excretion of ALA, PBG and uroporphyrin (URO) is observed (Doss, 1978; Moore and Disler, 1985). In the acute attack all of these are grossly elevated as a consequence of derepression of ALA-S. The factors governing latency are poorly understood -individuals may have similar biochemical profiles to patients in attack, yet can be totally asymptomatic.

Congenital Erythropoietic Porphyria (EP)

This is a very rare porphyria, but was probably the first to be discovered (Schultz, 1874). It is characterized by a deficiency of URO-CoS (Nordmann and Deybach, 1982) and is inherited as an autosomal

recessive trait. ALA-S is derepressed but so too is PBG-D (Moore et al, 1978), therefore no precursors accumulate, but very large amounts of URO-I and COPRO-I are excreted in the urine (Moore and Disler, 1985).

Porphyria Cutanea Tarda (PCT)

The underlying defect in PCT (or cutaneous hepatic porphyria) is a deficiency of URO-D. Three forms of the disease are found: familial, sporadic and toxic. Familial PCT is hereditary and the deficiency is found in liver and erythrocytes in a pattern consistent with autosomal dominant inheritance (Kushner et al, 1976). In the case of sporadic and toxic PCT, the defect is confined to the liver and may be caused by hepatic siderosis, alcoholism or ingestion of polyhalogenated aromatic substances (Mukerji, Pimstone and Burns, 1984; Felsher, Jones and Redeker, 1983; McColl et al, 1981; Sano, Kawanishi and Seki, 1985; de Verneuil, Sassa and Kappas, 1983 (A)). Total urinary porphyrin excretion is elevated due to the depression of ALA-S and to the presence of large amounts of URO and 7-COOH porphyrin (Moore and Disler, 1985; Doss, 1978).

Hereditary Coproporphyria (HC)

COPRO-O is the defective enzyme in HC, only 50% of normal activities have been found in individuals with the disease (Grandchamp and Nordmann, 1977). The enzyme has been shown to be defective in liver (Hawk et al, 1978) leucocytes (Brodie et al, 1977 (C) cultured fibroblasts (Elder et al, 1976) and lymphocytes (Nordmann et al, 1977) and to be inherited as an autosomal dominant trait. Individuals with 50% activity are heterozygous for the defective allele, Homozygosity has also been reported where essentially no activity is present (Grandchamp, Phung and Nordmann, 1977). Precursor excretion varies

in remission and in attack and as for AIP, there is a high degree of latency. However in attack large quantities of COPRO are found in urine and faeces and there is increased excretion of ALA and PBG (Doss, 1978; Moore and Disler, 1985).

Variegate Porphyria (VP)

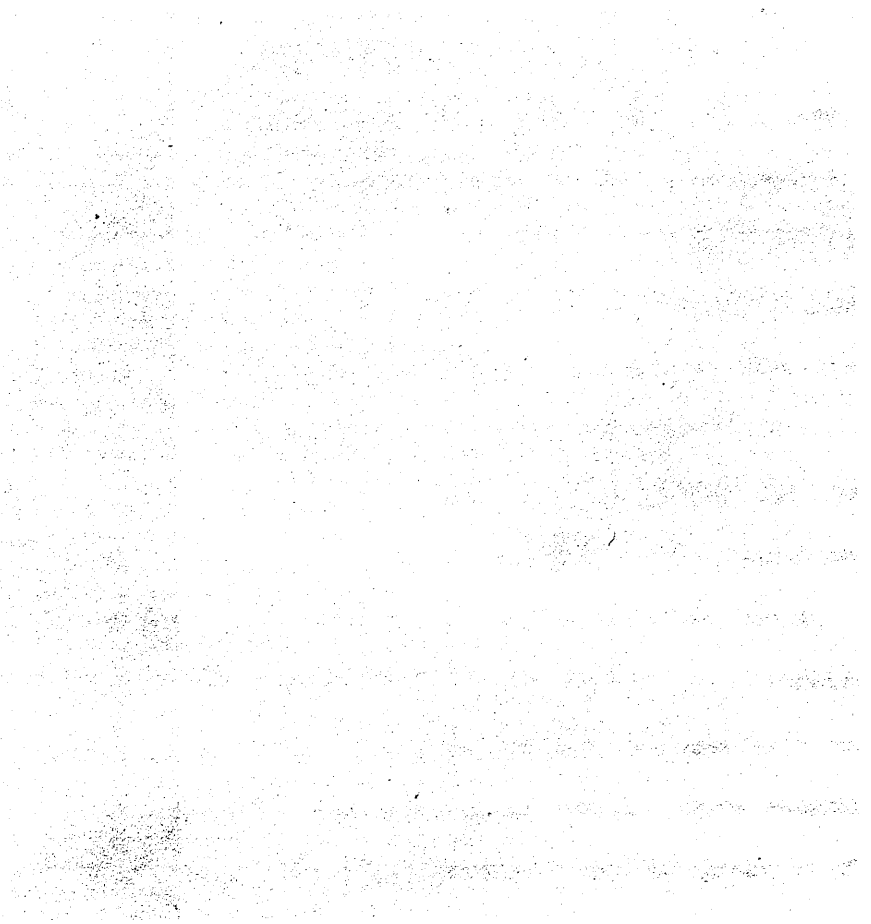
The defect in (VP) lies in the oxidation of PROTOgen. PROTO-O is reduced to 50% in patients with VP (Brenner and Bloomer, 1980; Viljoen et al, 1983) and there is evidence that FERRO-C activity may also be reduced (Siepker and Kramer, 1985). The condition is inherited as an autosomal dominant trait and as for AIP and HC, the disease has acute and remission phases, the usual precipitating factors being responsible for induction of an acute attack. In attack there is elevated ALA, PBG and urinary porphyrin excretion. Faecal protoporphyrin excretion is also grossly elevated (Doss,1978; Moore and Disler, 1985).

Erythropoietic Protoporphyria (EPP)

The defective enzyme in EPP is ferrochelatase and the defect is inherited as an autosomal dominant trait. The enzyme defect has been demonstrated in hepatocytes (Bonkowsky et al, 1975 (A), erythrocytes (Bottomley et al, 1975), leucocytes (Brodie et al, 1977 (D) and cultured fibroblasts (Bonkowsky et al, 1975 (A). The disease is characterized by very high erythrocyte and faecal protoporphyrin (Moore and Disler, 1985). Recent work has suggested an additional deficiency of PROTO-O in this disease (Siepker and Kramer, 1985).

Other conditions which may result in over-production of porphyrins and precursors

In both lead poisoning and hereditary tyrosinemia there is increased ALA excretion (Doss, 1978; Moore and Disler, 1985) due to reduced ALA-D activity. Additionally in lead poisoning, as a consequence of COPRO-O and FERRO-C inhibition, there is an increase in erythrocyte protoporphyrin (Moore and Disler, 1985).



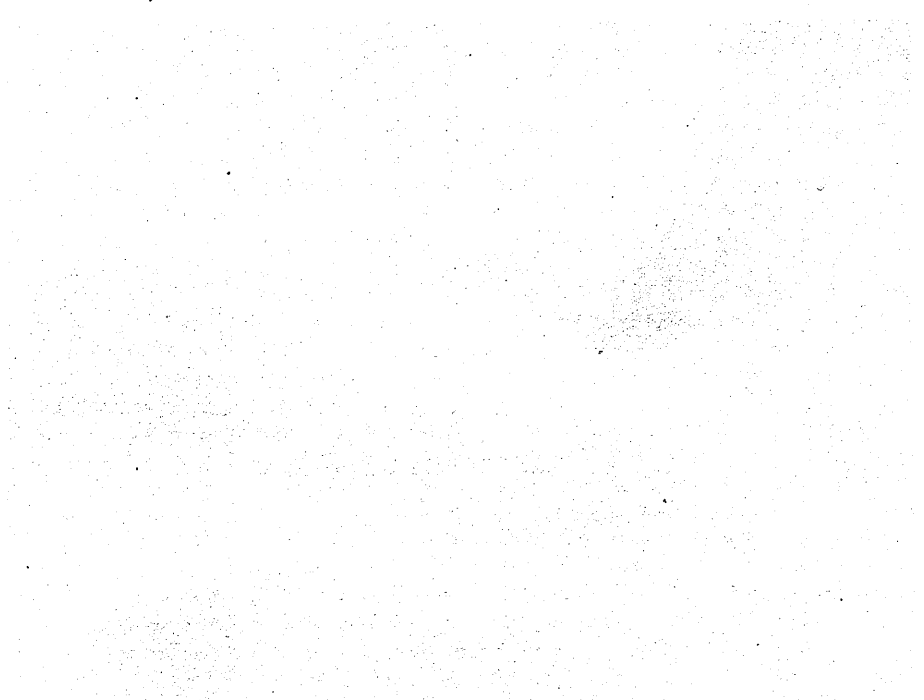
1.4 OBJECTIVES OF THIS THESIS

As discussed in sub-section 1.3.10, porphyria can be accompanied with neurological complications, including epileptic seizures. Seizure control in these patients may be more difficult then in non-porphyric individuals because the stimulatory action of many drugs on haem biosynthesis upsets the delicate balance which exists in porphyria (see sub-section 1.3.9). It is difficult to prescribe a drug which will control the epilepsy but which will not aggravate the porphyria. Controversy exists as to which drugs are safe and which are not, much of the evidence being gathered from non-human systems including rat liver, rat hepatocyte culture and chick hepatocyte culture. Alternatively evidence may be provided from single case reports. With regard to the four anticonvulsants discussed earlier in this chapter, opinions can be divided. There is a consensus that barbiturates such as phenobarbitone, are to be avoided in epileptic patients with porphyria due to the extremely marked derepression of ALA-S brought about by these drugs. However whereas as some authors decry the use of carbamazepine (CBZ) (Larson et al, 1978, Reynolds and Miska, 1981; Bonkowsky, 1981) others advocate it (Lai, 1981, and Peters, 1981). Sodium valproate (VPA) is usually not recommended (Bonkowsky et al, 1980; Reynolds and Miska, 1981; Merino and Lozano, 1980; Doss et al, 1981) though others disagree (Sergay, 1979 and Brodie et al, 1977, B). However no definitive controlled human studies have been done. Phenytoin is also not generally recommended (Larson et al, 1978; Magnussen et al, 1975). More recently McColl et al (1980) have shown that phenytoin causes derepression of ALA-S in man and is therefore unsafe. This thesis was aimed at establishing

the long term effects of anticonvulsant drugs particularly CBZ and VPA, on haem biosynthesis in man, to assess the safety of these drugs in porphyria. CBZ was of primary interest for the reasons outlined below.

Recent reports have announced the appearance of an acute intermittent porphyria (AIP) syndrome in non-porphyric individuals being treated with CBZ. This was first noted by Yeung et al, (1983) in an epileptic patient being treated with the drug. The patient exhibited raised ALA-S activity, reduced PBG-D activity, grossly elevated excretion of urinary ALA, PBG and porphyrin (particularly uroporphyrin) and acute abdominal pain. These symptoms, which all vanished on withdrawal of CBZ, are typical of the acute phase of AIP. The interpretations of the findings, particularly as regards PBG-D activity, have been disputed by Doss and Shafer, (1983); Rideout et al, (1983) and Shanley, (1983). However all these features (with the exception of abdominal pain), were reported to occur to a lesser extent in volunteer subjects given CBZ (Rapeport et al, 1984). In this case, the overall biochemical picture was the same as that found in the latent phase of AIP. This aroused interest in the possible use of CBZ treatment as a model for studying AIP. Thus in addition to the earlier stated objectives, this thesis was also aimed at investigating in more detail the effects of CBZ on haem biosynthesis and the mechanisms whereby these occurred. The settling of the dispute as regards the effects of CBZ on PBG-D was of particular concern. It was also hoped to assess the viability of CBZ treatment as a model for studying AIP.

In order to achieve these objectives haem biosynthesis was primarily studied in peripheral blood cells from epileptic patients receiving long-term anticonvulsant therapy. It was assumed that enzyme activities in these cells would reflect the levels of the corresponding hepatic enzymes which are quantitatively much more important. In addition to this, haem biosynthesis was also studied in rats, human lymphocyte cell culture and in in vitro systems. The effects of anticonvulsants on haem biosynthesis in these systems were not studied for their own sake but solely to try and further understand the effects of these drugs on haem biosynthesis in man.



CHAPTER 2

CHAPTER 2

2. MATERIALS AND METHODS

This chapter describes the materials and methods used in this thesis and consists of two sections. The first of these covers the materials and equipment used and the second describes the methodology. In order that a full understanding of what was done is possible, a brief summary of each of the methods used is provided, even when the technique was performed exactly as referenced. When departures from referenced methods were made, the exact experimental details are given.

2.1 MATERIALS AND EQUIPMENT

2.1.1 MATERIALS

Radioisotopes 4-^{14}C ALA, 2-^{14}C glycine, 2-keto (5-^{14}C) glutaric acid and 2,3-^{14}C succinic acid were obtained from Amersham International plc, Amersham, U.K.

Biochemicals ALA, PBG, reduced glutathione, dithiothreitol, 2-ketoglutaric acid, succinic acid, 5,5'-dithiobis (2-nitrobenzoic acid), dimethyl sulphoxide, NADH, NAD^+, NADPH, GTP, coenzyme A and coproporphyrin standard were obtained from Sigma. Pyridoxal phosphate was from Koch Light Laboratories. Porphyrin methyl esters were obtained from Porphyrin Products, Utah, U.S.A. p-Dimethylaminobenezaldehyde was obtained from Merck.

Enzymes Succinyl CoA synthetase was obtained from Boehringer-Mannhiem (GMBH). α-ketoglutarate dehydrogenase was obtained from Sigma.

Drugs CBZ, CBZ-E and H_2-CBZ were obtained from Geigy Pharmaceuticals. Primidone was obtained from Imperial Chemical Industries Ltd. Phenobarbitone was from May and Baker.

Cell Culture Materials RPM1 1640 medium, L-glutamine and Lux tissue culture flasks were obtained from Flow Laboratories. Foetal calf serum and Penstrep were from Gibco. Phytohaemagglutinin, pokeweed mitogen, trypan blue and Histopaque were obtained from Sigma.

All other chemicals and reagents were obtained from BDH. Any materials whose origins are not listed here are described in the text.

2.1.2 EQUIPMENT USED IN ANALYSES

Routine analysis For spectrophotometric measurements a Pye Unicam SP8-200 UV/VIS double beam spectrophotometer was used and for spectrofluorometric detection a Perkin Elmer 3000 Fluorescence Spectrometer was used.

Cell Culture Studies All manipulations were carried out in a Hepaire vertical laminar flow cabinet. Cultures were incubated in an LEEC automatic CO_2 incubator Model GA2.

HPLC Analyses were carried out on a Hewlett Packard system, comprising a 10848 Liquid Chromatograph, a 79831A injector and a 79850B LC terminal. The method of detection used depended on the nature of the analyses. For spectrophotometric detection, the aforementioned system was linked to a Kontron Uvikon 7305 LC UV spectrophotometer and for spectrofluometric detection to a Perkin Elmer 3000 fluorescence spectrometer equipped with a Perkin Elmer LC flow cell.

Emit The Emit system used consisted of a Syva CP 5000 Plus Clinical Processor linked to a Gilford Stasar III Spectrophotometer.

Equilibrium Dialysis Dianorm Equilibrium Dialysis system. Diachema, Zurich, Switzerland.

Lead Determinations Analyses were performed using a Perkin Elmer 703 Atomic Absorbtion spectrophotometer controlled by a Perkin Elmer HGA 500.

2.2 METHODS FOR DETERMINATION OF ENZYME ACTIVITIES

2.2.1 5-AMINOLAEVULINIC ACID SYNTHASE E.C. 2.3.1.37

A number of assays were examined for use in the determination of 5-aminolaevulinic acid synthase (ALA-S) activity in (i) human leucocytes (ii) rat liver and (iii) cultured human lymphocytes. All the assays used were radiochemical and involved incubation of the enzyme with a radioactive precursor and then isolation and quantification of the radioactive ALA generated. The three assays tried in each of the aforementioned systems were as follows:

(a) the substrate was 2-^{14}C glycine and the method was modified from Dowdle, Mustard and Eales (1967) and Freshney and Paul (1970);

(b) 2-keto (5-^{14}C) glutaric acid was used as substrate using a method adapted from Tikerpae, Samson and Lim (1981);

(c) taken from Fitzsimmons (1984) utilising 2,3^{14}C succinic acid as the radioactive precursor.

In method (a), the assay products were separated by electrophoresis. The assay products in method (b) and (c) were separated by HPLC following derivativization. For each of the tissues studied the three basic methods described were assessed and the most suitable assay for each adopted for routine use.

(i) Human leucocyte ALA-S

Leucocytes were isolated by centrifuging fresh, heparinised whole blood at 2,500g for 30 minutes then removing the layer of white cells (the buffy coat) with a pipette. Cells were washed twice in ice-cold isotonic saline then assayed for ALA-S activity exactly as described

by Brodie et al (1977 (C)), using method (a). Briefly, leucocytes were disrupted by sonication then incubated for 1 hour in buffer containing radioactive glycine, sodium malate (from which succinyl COA is formed), magnesium chloride and EDTA (to inhibit ALA dehydratase) and sucrose, pyridoxal phosphate and 2-mercaptoethanol to stabilize the enzyme. The reaction was stopping by adding trichloroacetic acid containing ALA. Precipitated protein was removed by centrifugation, aliquots of supernatant were spotted on to chromatography paper and ALA was then separated from glycine by high voltage electrophoresis. The ALA and glycine spots were identified by reaction with ninhydrin, cut out and combusted in a sample oxidizer, collected into scintillation fluid and then finally counted in a liquid scintillation spectrophotometer. Results were expressed as pmoles ALA produced/hour/mg protein.

The use of system (c), (^{14}C succinate), only gave appreciable counts when succinyl CoA synthetase was added. Unfortunately incorporation of this step led to increased background counts, a problem also encountered by De Matteis and Hollands (1982) and other workers quoted therein. Similar problems were encountered with α-ketoglutarate.

(ii) Rat Liver ALA-S

After excision and perfusion of livers as described in 2.5.2, 1g of liver was homogenized in 5ml of ice-cold isotonic saline using a Polytron homogenizer. ALA-S was assayed in the homogenate as described by Thompson et al (1984(B))

which was essentially the method quoted for leucocyte ALA-S. Results were expressed as pmol ALA produced/hour/mg protein.

Comparable results were found using α-ketoglutarate and succinate as radio-labelled substrates and coupling enzymes were not required. However the method described was used as it was convenient to have similar assays for the two systems covered so far.

(iii) ALA-S in cultured human lymphocytes

For this assay to be of use, it would be required to measure ALA-S activity in 10-15 million cells. Using method (a) and double this number of cells, the counts incorporated into ALA were barely distinguishable from background. This method was therefore not suitable. As was the case with leucocytes, no activity at all was detectable using either systems (b) or (c). When coupling enzymes were added, (to generate succinylCoA, the substrate for the reaction) the background counts became so high that it was not possible to ascertain the presence of labelled ALA. Thus none of the methods were suitable for the routine determination of ALA-S activity in cultured human lymphocytes. No measurements of ALA-S were made in this system.

2.2.2 5-AMINOLAEVULINIC ACID DEHYDRATASE E.C. 4.2.1.24

5-aminolaevulinic acid dehydratase (ALA-D) activity is measured by a colorimetric assay, the basis of which is as follows: enzyme is incubated with ALA, then the reaction is terminated and the amount of

PBG formed determined by mixing with an equal volume of Ehrlichs reagent, para-dimethylaminobenzaldehyde (pDMAB), and measuring the extinction, at 555nm, of the coloured PBG-pDMAB complex (ϵmM=62).

Enzyme activity was determined in (i) human erythrocytes (ii) rat liver (iii) mitogen-stimulated human lymphocytes and (iv) in vitro studies.

ALA-D activity is readily lost due to oxidation of essential thiol groups or loss of Zn^{2+} ions, therefore dithiothreitol (DTT) and zinc chloride ($ZnCl_2$) were routinely included in assays, with the exception of assays in human erythrocytes. In this system, activity was measured in fresh blood (circulating activity) and in the same blood after treatment with these activators (restored activity).

The enzyme is sensitive to inhibition by heavy metal ions, therefore all glassware used was acid-washed in 50% nitric acid and disposable plastic tubes were used for the assays.

The methods used were modified from the European Standardised method (Berlin and Schaller, 1974). Optimum activation or restoration of erythrocyte enzyme was achieved by following the procedure of Fujita, Orii and Sano (1981). The assay for ALA-D activity in mitogen-stimulated lymphocytes also incorporated some features described by Sassa (1982).

(i) Human erythrocytes

All reagents were equilibrated at 37°C prior to assay. Three tubes were required per sample: one blank and two tests. 0.1ml of whole heparinised blood, kept on ice and not more than 3 hours old, was added to each tube and haemolysed by adding 0.65ml of water, mixing and incubating

for 3 minutes at 37°C. The reaction was then started by adding to all tubes, 0.5ml sodium phosphate buffer (0.1M,pH6.8) to which ALA had been added to give a 10mM solution. The blank was terminated immediately with 0.5ml of trichloroacetic acid (TCA) solution (10% W/V) containing mercuric chloride ($HgCl_2$,5mM) and the others were terminated in the same way after 30 minutes at 37°C. Precipitated protein was removed by centrifugation at 1,500g for 15 minutes, after which the supernatant was decanted and re-centrifuged. 0.5ml of this final supernatant was mixed with 0.5ml of Special Ehrlichs solution (2.5g pDMAB and 0.1g $HgCl_2$ were dissolved in 50ml glacial acetic acid, 24.5ml perchloric acid was added, the mixture cooled then made up to 100ml with glacial acetic acid). After waiting 5 minutes for colour development the absorbance at 555nm was read against the blank in 1cm path-length semi-micro cuvettes, and the amount of PBG produced determined as described. Results were expressed as nmoles PBG produced/hour/ml erythrocytes.

Assay of restored ALA-D activity was performed by adding DTT (10mM) and $ZnCl_2$(0.1mM) to the water used in the haemolysis step and incubating at 37°C for 10 minutes prior to assay exactly as described above.

(ii) Rat liver

Livers were removed as described in 2.5.2 and 4g homogenized in 10ml of ice cold potassium phosphate buffer, (0.15M,pH7.4) containing DTT (1mM), using 5 strokes of a

motor driven Potter-Elvehjem homogenizer. After centrifugation at 1,500g for 10 min at 4°C, the supernatant was spun for 25 minutes at 22,500g, also at 4°C. The resultant supernatant was assayed for ALA-D activity as described previously for restored human erythrocytes except that 0.4ml of homogenate was added per tube and 0.35ml of water containing DTT and $ZnCl_2$ was used to activate the enzyme. Results were expressed as μmoles PBG produced/hour/g protein.

(iii) Cultured human lymphocytes

Mitogen-stimulated lymphocytes were prepared as described in 2.6. 7.5-10 x 10^6 cells were lysed by adding 0.25ml of water containing DTT (10mM) and $ZnCl_2$ (1mM) and incubating for 1 minute at 37°C. 0.25ml of 10mM ALA in 50mM potassium phosphate buffer, (final pH=6.3), was added and the mixture incubated in a covered water bath at 37°C for 1 hour, after which the reaction was terminated with 0.25ml of TCA (10% W/V) containing $HgCl_2$(5mM). Tubes were left in the dark for 10 minutes to allow complete precipitation of protein, then spun at 1,500g for 15 minutes. 0.6ml of the supernatant was mixed with 0.6ml modified Special Ehrlichs solution (Sassa, 1982) against a blank in 1cm path-length semi-microcuvettes after 15 minutes and the extinction at 555nm measured. Blanks were prepared exactly as for samples except that no cells were present. The amount of PBG produced was calculated as described and activity was expressed as pmoles PBG produced/hour/10^6 cells.

(iv) In vitro studies

In these experiments, the effects of metabolism of carbamazepine on ALA-D in vitro were studied. ALA-D activity was measured by taking 0.1ml from the reaction mixtures described in 2.8 and assaying it exactly as described for restored or activated human erythrocytes. Activity was expressed as a percentage of the original starting activity.

2.2.3 PORPHOBILINOGEN DEAMINASE (EC 4.3.1.8)

Porphobilinogen deaminase (PBG-D) activity is measured by fluorometric determination of uroporphyrin I(URO-I). URO-I is formed by oxidation of uroporphyrinogen I which in turn is formed by rapid non-enzymatic cyclization of hydroxymethylbilane,the end product of the sequential head-to-tail condensation of 4 molecules of PBG by PBG-D. .The enzyme was measured in human erythrocytes, rat liver and cultured human lymphocytes. For the assay in human erythrocytes, the substrate (PBG) is either added directly or produced in situ from ALA by ALA dehydratase (present in the erythrocytes). This latter enzymatically coupled method will be referred to as measurement of URO-I synthase (URO-I-S) activity to distinguish the two. (This was the nomenclature used in the paper from which this method was taken).

Uroporphyrinogen-I Synthase Assay in Human Erythrocytes

The assay was performed exactly as described by Piepkorn, Hamernyik and Labbe (1978). Briefly, blood was haemolysed and incubated for 30 minutes with dithiothreitol (DTT) and zinc sulphate in order to maximise ALA-D activity. ALA was added and the mixture

incubated for 1 hour before termination with trichloroacetic acid (TCA). UROgen I formed in the assay was converted to URO-I by oxidation catalysed by the added TCA. After centrifugation to remove precipitated URO-I protein was quantified in 1cm path length cuvettes by fluorimetry (excitation wavelength 396nm, emission wavelength 594nm; both with a 10nm band pass). Standardisation was achieved using a solution of coproporphyrin of known concentration. A correction factor was also calculated from a standard curve of URO-I fluorescence versus concentration. After making the necessary corrections, results were expressed as nmol URO produced/hour/ml/RBCs.

The effects of increasing ALA-D activity on erythrocyte URO-1-S activity were investigated by adding purified ALA-D to whole blood. 50µl of ALA-D, purified as described in section 2.9.2, was diluted to 5ml with sodium phosphate buffer (0.1M,pH7.4) containing dithiothreitol (10mM) and $ZnCl_2$(1mM). This gave a solution containing 1.36×10^{-2} units of ALA-D activity/ml (1 unit was defined as the amount of enzyme which converts 1 µmole of ALA to 0.5 µmol PBG/min). An aliquot of this, usually 50µl (6.8×10^{-4} units), was added to the URO-1-S assay at the start of the preincubation period. In order to assess if the purified enzyme itself contained any URO-1-S activity, an aliquot was assayed for activity exactly as described previously for an aliquot of blood. This revealed that there was slight URO-1-S activity present, but only enough to increase the average URO-1-S result by 0.6 nmol URO/hour/ml RBC for a 50µl aliquot of added ALA-D. Results were adjusted accordingly.

Porphobilinogen Deaminase

PBG-D activity was measured in:

(i) human erythrocytes

(ii) rat liver and

(iii) cultured human lymphocytes

The methods used for all 3 assays were modified from Anderson and Desnick (1982). PBG, the substrate for the assay, was prepared as described in 2.9 or purchased from Sigma. Both preparations gave identical activities although the material synthesized in 2.9 gave higher blank readings.

(i) Human Erythrocytes

All reagents were equilibrated at 37°C prior to assay. 0.2ml of whole blood was haemolysed by the addition of 0.8ml of haemolysis buffer (sodium phosphate buffer (1mM, pH7.6) containing DTT (1mM), magnesium chloride (1mM) and Triton X-100 (0.05% v/v)).

For each sample, three plastic tubes were required, one for a blank and two duplicates for assays. 1.8ml of assay buffer, (TRIS-HCl 0.1M, pH8.1) containing DTT (0.1mM)), was added to all three tubes; 0.1ml of haemolysate was added to the two assay tubes.

After preincubation at 37°C for 3 minutes, 0.5ml of assay buffer containing PBG (1mM) was added to all tubes and the tubes mixed followed by incubation at 37°C for 1 hour in the dark. After termination with 0.35ml of TCA (40% w/v), 100µl of haemolysate was added to the blank and all tubes exposed to ultra-violet light for 20 minutes to oxidise UROgen to URO. The blank was prepared in this way to take

into account non-enzymatic polymerization of PBG with subsequent cyclization. Precipitated protein was removed by centrifugation for 15 minutes at 1,500g and the concentration of URO determined by fluorimetry in 1cm path-length cell (excitation wavelength of 405nm and emission wavelength of 595nm, both with a 10nm band pass).

Standardisation was performed with a solution of coproporphyrin of known concentration and results calculated by applying a correction factor obtained from a standard curve of URO fluorescence versus concentration. Results were expressed as nmoles URO produced/hour/ml erythrocytes.

(ii) Rat liver

Liver homogenates were prepared exactly as described in 2.2.2 for rats. After the 10,000g step, 0.5ml of homogenate was added to 1ml of haemolysis buffer previously described. At this stage samples were stable in the refrigerator for at least 2 days. The homogenates were then treated exactly the same as blood haemolysates and assayed as described previously. Results are expressed as nmoles URO produced/hour/g protein.

(iii) Cultured human lymphocytes

2-4 x 10^6 cells (cultured as described in 2.6) were spun down at 500g and lysed by freeze-thawing 3 times in 0.25ml of sodium phosphate buffer (100mM, pH7.6) containing PBG (0.2mM). Tubes were then incubated in the dark for 1 hour at 37°C after which the reaction was terminated by the addition of 2.75ml of TCA (10% w/v). A blank was prepared

by omitting cells from 1 tube. Samples were then treated as described for rat and human assays except that a different correction factor was required, due to the altered fluorescence of URO in high concentrations of TCA. Activity was expressed as pmoles of URO produced/hour/10^6 cells.

2.2.4 UROPORPHYRINOGEN DECARBOXYLASE E.C. 4.1.1.37

The assay used for determination of uroporphyrinogen decarboxylase (URO-D) consisted of several stages. These were (a) generation of uroporphyrinogen (URO-gen) substrate (b) incubation for determination of URO-D activity, (c) termination of the assay and (d) analysis of the reaction products by fluorimetry and HPLC.

The method used was adapted from Brodie et al (1977 (C)) which was developed from an assay for PBG-D (Frydman and Feinstein, 1974). Some features from Alleman et al (1982) were also incorporated into the method.

URO-D activity was measured in (i) human erythrocytes and (ii) rat liver.

(i) Human erythrocytes

4-5ml erythrocytes were isolated from fresh heparinised blood by centrifugation and washed twice with 5ml ice-cold saline. 0.5ml erythrocytes was mixed with 0.5ml assay buffer (sodium phosphate buffer (83mM, pH7.65) containing reduced glutathione (3.2mM)) and an aliquot taken from determination of packed cell volume prior to haemolysis by freeze-thawing 3 times in acetone dry ice. 0.2ml haemolysate was added to each of 3 tubes containing 1ml of assay buffer.

UROgen was synthesized by adding 0.1ml of 1.0mM PBG in assay buffer to all tubes and incubating at 37°C in the dark for 1 hour. URO-D activity was then measured by adding 0.2ml potassium dihydrogen orthophosphate solution (0.4M) to change the pH from one which favours PBG-D activity to the optimum assay pH for URO-D. Two of the four tubes were removed at this stage and designated zero times. One of these was frozen and 10ml of 2:1 (v/v) ethyl acetate: acetic acid was added to the other. After 1 hour at 37°C, the remaining tube was frozen. After centrifugation to remove protein (1,500g, 15 minutes) porphyrins were extracted from the tube to which ethyl acetate: acetic acid had been added by washing with 5% HCl until no fluorescence was detectable in the acid extract when viewed under U.V. light (typically 4 x 5ml 5% HCl). The amount of porphyrin extracted was quantified by spectrofluorimetry (excitation 396nm, emission 594nm, both with 10nm band pass) following calibration of the fluorimeter with a coproporphyrin solution of known concentration. The contents of the other tubes (one zero time and one assay) were freeze-dried and the porphyrins separated (after preparation of methyl ester derivatives) and the relative amounts of each determined by HPLC as described in section 2.3.4. (see fig. 9).

Combining the two sets of data - the quantity and composition of porphyrins in the mixture - for the zero time and after 1 hour's incubation allowed calculation of the number of decarboxylations catalysed by the enzyme over the

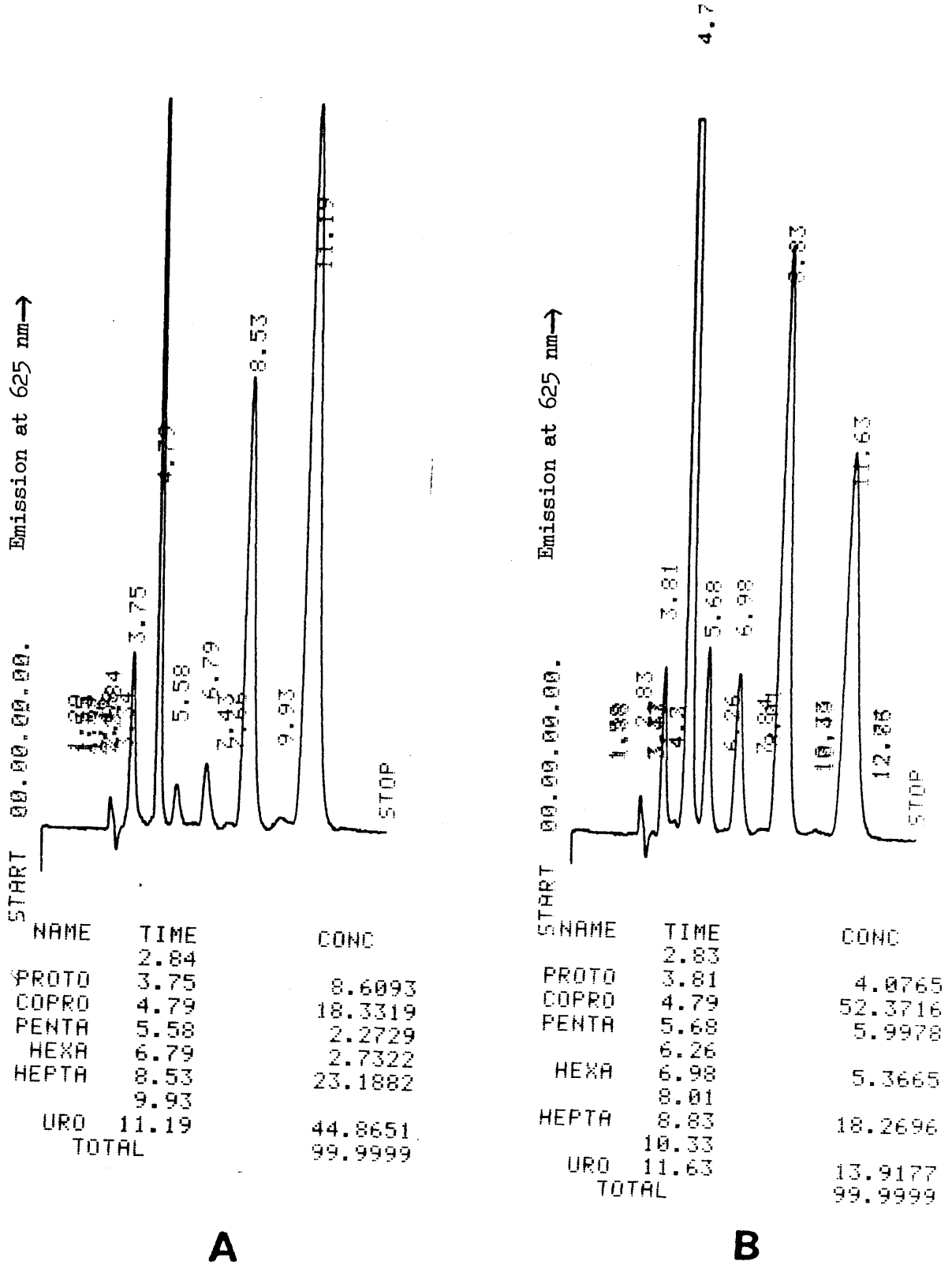


FIG. (9)

HPLC ANALYSIS OF THE SUBSTRATE AND REACTION PRODUCTS IN THE URO-D ASSAY IN HUMAN ERYTHROCYTES

Trace A shows substrate generated from PBG in the first stage of the assay. The major peak is uroporphyrin.

Trace B shows the reaction products after the second stage of the assay. The majority of the uroporphyrin has been converted to coproporphyrin and intermediates.

The assay details are given in sub-section 2.2.4.

assay period. The calculation assumed that each of the porphyrins had equivalent fluorescence at the settings used. Activity was expressed as nmoles decarboxylations/hour/l erythrocytes.

(ii) Rat Liver

Livers were removed as described in 2.5.2 and 3g homogenized in 10ml assay buffer just described. 0.2ml was then assayed exactly as for erythrocyte assays and activity expressed as μmoles decarboxylations/hour/g protein.

2.3 PORPHYRIN PRECURSORS AND PORPHYRINS

2.3.1 5-AMINOLAEVULINIC ACID IN URINE

ALA was measured in urine using test kits supplied by Bio-rad Laboratories. The procedure followed was exactly as described in Bio-rad Laboratories Technical Bulletin No.4208 (1979). The basis of the method was that urine from which PBG was removed as described in 2.3.2 was passed through a column of cation-exchange resin on which the ALA was retained. ALA was then eluted and condensed with acetylacetone. The pyrrole produced was mixed with para-dimethylaminobenzaldehyde and the resultant coloured complex quantified by spectrophotometric measurement. Results are calculated from the molar extinction coefficient of the complex at 555nm (ϵmM=62) and expressed as nmoles ALA excreted/l or /24 hours.

2.3.2 PORPHOBILINOGEN IN URINE

PBG was measured in urine using test kits supplied by Bio-rad Laboratories. The procedure followed was exactly as described in Bio-rad Laboratories Technical Bulletin No.4208 (1979). PBG was removed from urine by retention on an anion exchange resin. It was then eluted from the resin, complexed with para-dimethylaminobenzaldehyde and quantified as described for the complex formed in 2.3.1. Results are expressed as nmoles PBG excreted/l or /24 hours.

2.3.3 TOTAL PORPHYRIN IN URINE

Total porphyrin in urine was determined using test kits supplied by Bio-rad Laboratories. The procedure followed was modified from Bio-rad Laboratories Technical Bulletin No.4003 (1981).

Method

Columns from the Bio-rad kit were shaken to resuspend the ion exchange resin and allowed to drain. 1ml of urine was added and allowed to run through after which the column was marked with 10ml of distilled water. Porphyrins were then eluted into a test-tube with 2 x 2ml of 3M HCl and the fluoresence of the sample determined in a fluorimeter (excitation wavelength 396nm, emission wavelength 594nm; both set with a 10 nm band pass. Concentration of porphyrin was calculated by reference to a coproporphyrin standard of known concentration. Results are expressed as nmoles porphyrin excreted /l or /24 hours.

2.3.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF PORPHYRINS

Using HPLC it is possible to individually resolve uroporphyrin, hepta-,hexa- and penta-carboxylic porphyrins, coproporphyrin and protoporphyrin. Such resolution cannot be achieved using methods whereby porphyrins are extracted from mixtures by the use of different solvents (e.g. Rimington, 1971).

For HPLC analyses two methods were used:

(a) "Free porphyrin method"; porphyrin was subjected to HPLC after a preliminary extraction step, and

(b) "Porphyrin methyl ester method"; porphyrin was extracted, derivatized by esterification, re-extracted and analysed.

In both cases, individual porphyrins were expressed as % of the total porphyrin content of the sample. To do this, it was necessary to apply correction factors to the peak areas. These factors were calculated from injection of a sample containing known amounts of each of the relevant porphyrins (this is necessary because of the different fluorescence characteristics of each of the porphyrins).

Free porphyrin method for porphyrins in urine

The method was essentially as described by Jordan (1984).

Porphyrins were extracted from 1ml of urine as described in 2.3.3, or by retention, washing and elution from C18 Sep-Paks (Waters Associates) as described by Muraca and Goosens (1984). 0.1ml of porphyrin in 10% HCl was then injected onto the HPLC system described in 2.1.2 linked to a Waters Bondapak C-18 column (300 x 4mm). A linear gradient of acetonitrile in sodium phosphate buffer (10mM, pH7.5) was run over 25 minutes. Acetonitrile was then increased to 65% for 10 minutes before returning to starting conditions. The flow rate was 1 ml/minute and detection was by fluorescence (excitation at 405nm, emission at 595nm).

This method has the advantage of requiring only a small sample, but the drawback of taking a long time to run. This precludes the preparation of many samples at once since certain porphyrins (particularly uroporphyrin) are unstable in acid. A further disadvantage with this method is that if the column is not sufficiently re-equilibrated between each analysis, retention times and peak resolution are affected. The method used for all HPLC analysis was therefore the esterification method now described.

Porphyrin methyl ester method

The method used was essentially that of Seubert and Seubert (1982) with modifications depending on the starting material. The method will be dealt with in two sections:

(a) extraction, esterification and preparation of HPLC

(b) HPLC analysis of esters

(a) Extraction, esterification and preparation for HPLC

(i) Human urine

200ml of urine from a 24 hour urine collection was adjusted to pH5-7 with glacial acetic acid. 2.0g of talc were added and the mixture left to stir in the dark for 15 minutes. The talc with absorbed porphyrins was removed by filtration under suction through a sintered glass filter funnel and dried at 90°C for 1 hour. Porphyrins were recovered by adding 25ml of methanol/concentrated sulphuric acid (95/5, v/v) to the funnel and collecting the filtrate in a large tube. Methyl esters of the porphyrins were formed by heating this solution and the funnel at 60°C for 1 hour or by leaving both at room temperature in the dark for 16 hours. Both esterification procedures yielded identical results. The talc left in the funnel was washed with 2 x 8ml of chloroform to remove residual porphyrin ester and the washes added to the previously prepared filtrate. 10ml of water was added to separate the organic and aqueous phases after which the upper aqueous phase was removed and discarded. The chloroform left was washed with 15ml of

water, 15ml of 3% sodium bicarbonate solution and finally 2 x 15 ml of water. It was then shaken with 0.1g of anhydrous sodium sulphate and filtered before evaporating to dryness under air. The residue was dissolved in 0.1ml of chloroform to which was added 0.9ml mobile phase (to be described). This was filtered through 0.2μm filters (Gelman Sciences) and 0.1ml was injected.

(ii) Rat Urine

Exactly as for human urine except that only 10ml of urine mixed with 0.5g of talc was used. Porphyrin esters were dissolved in 20μl of chloroform and then 0.18ml of mobile phase was added. Samples were filtered as described above and 0.1ml was injected.

(iii) Porphyrins from cell culture studies

The starting material was porphyrin in acid prepared as described in 2.6.4. The pH of the solution was adjusted to between pH5 and 7 with saturated sodium acetate after which 0.1g of talc was added. The procedure was essentially that described for human urine except that the volumes of all reagents were reduced by a factor of 5. Porphyrin esters were dissolved in 10μl of chloroform to which was added 90μl of mobile phase - all of this was injected.

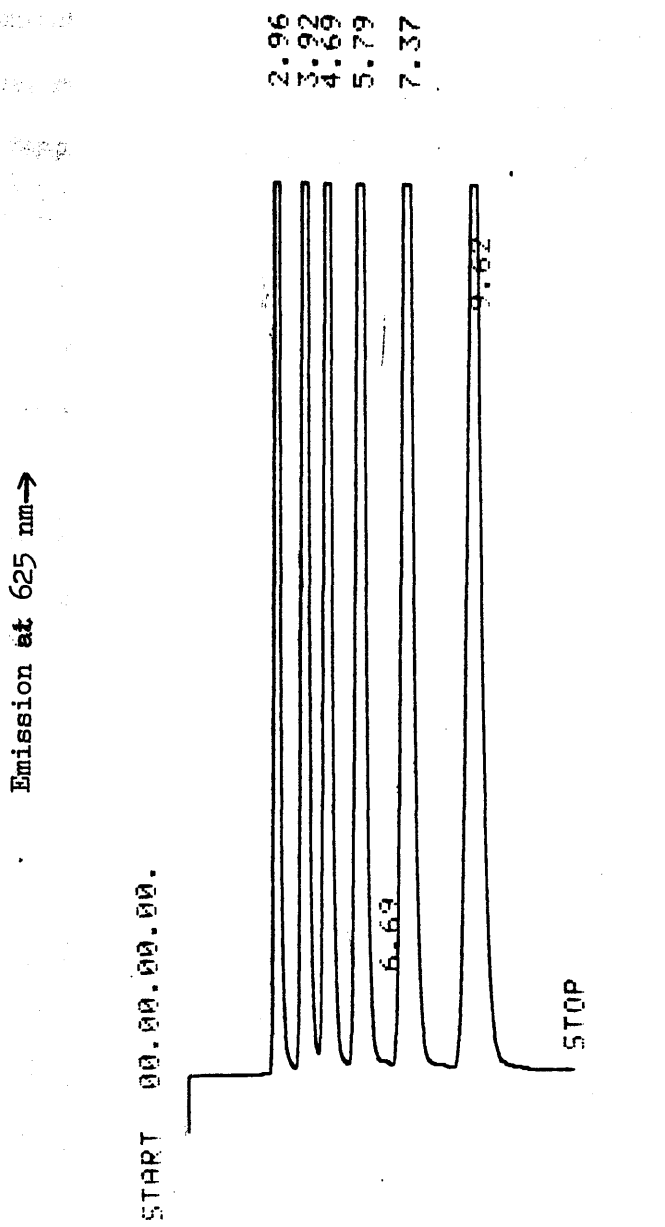
(iv) Porphyrins from uroporphyrinogen decarboxylase assays - HPLC analysis of porphyrin composition

HPLC analysis of porphyrins from URO-D assays was performed using the same system as for the other porphyrins, but the esterification procedure was modified from Alleman et al (1982). Freeze-dried samples were dissolved in 1ml

of chloroform. Esterification was achieved by adding 5ml of methanol/concentrated sulphuric acid, (90/10,v/v), and incubating the tube for 1 hour at 40°C. The solution was transferred to a larger tube and 9ml of water, then 18ml of chloroform were added. The upper aqueous phase and protein at the solvent interface were removed by suction and discarded. The chloroform was washed with 15ml of water, 15ml of 5% sodium bicarbonate, 2 x 15ml of water, filtered, then evaporated to dryness. The residue was dissolved in 25µl of chloroform, 250µl of mobile phase was added and the solution filtered through a 0.2µm filter (Gelman Sciences). 100µl were injected.

(b) High performance liquid chromatography of porphyrin methyl-esters

Chromatography was performed using the HPLC system described in 2.1.1 and an HP 79915 Si Opt. 174 column (200 x 46mm) from Hewlett Packard. The solvent used was n-heptane/ethyl acetate/chloroform/methanol (60/25/12.5/2.5) at a flow rate of 1.2 ml/min. Detection was by fluorescence at 625 nm, excitation wavelength was 405 nm, (both with 10 nm band pass). The chromatogram produced following injection of a solution of porphyrin methyl ester standards is shown in fig. (10).



NAME	TIME	CONC
PROTO	2.96	16.6607
COPRO	3.92	16.5944
PENTA	4.69	16.6738
HEXA	5.79	16.7435
HEPTA	7.37	16.7558
URO	9.62	16.5714
TOTAL		99.9999

FIG. (10)

HPLC CHROMATOGRAM OF PORPHYRIN STANDARDS

2.4 MEASUREMENT OF DRUG CONCENTRATIONS

Many of the experiments undertaken required determination of drug concentrations. Samples in which drugs were measured were human plasma, rat serum, cell culture media and *in vitro* drug metabolism.

Samples were prepared as described in the relevant sections and stored at -18°C for up to 2 months prior to analyses with no effects on drug levels. For determination of "total" carbamazepine (CBZ) in human plasma and rat serum, and of phenytoin, valproic acid and phenobarbitone in human plasma, the EMIT system (Syva) was used, following the procedure in the appropriate EMIT instruction booklet. Certain experiments necessitated the determination of concentrations of both CBZ and its epoxide metabolite (CBZ-E). These were measured by an HPLC method modified from Meijer (1981).

Throughout this thesis, drug concentrations are expressed in mg/l rather than in molar units, the reason being that these are the units normally used to report drug concentrations in this hospital.

2.4.1 DRUG CONCENTRATION BY EMIT

The equipment used is outlined in 2.1.2 and all necessary reagents are supplied in kit form by Syva. The principle of the assay is that a sample containing a given drug is mixed with antibody to that compound and then a precise quantity of drug labelled with glucose-6-phosphate dehydrogenase (G-6-PDH) is added. Labelled and unlabelled drug compete for the antibody and the amount of unlabelled drug present will determine the extent of inactivation of G-6-PDH. The residual activity, as determined by the spectrophotometric measurement of the rate of NADH production from NAD^+, is directly related to the concentration of drug present. Calibration with

standards is necessary, after which the concentration of unknown samples can be calculated from a standard curve of reaction rate versus concentration of drug. Results for all drugs measured are expressed as mg/l.

2.4.2 CARBAMAZEPINE AND CARBAMAZEPINE 10,11 EPOXIDE BY HPLC

The assay involves 3 steps:

(i) preparation of standards

(ii) extraction of drug and

(iii) chromatography

(i) Preparation of standards

CBZ in methanol was added to blank plasma to give concentrations of 20,15,10 and 5μg/ml. CBZ-E standards were likewise prepared to give final concentrations of 10, 7.5, 5 and 2.5μg/ml. These were then extracted in tandem with the unknown samples as described below.

(ii) Extraction

CBZ and CBZ-E were extracted with 7ml of chloroform from 1 1 ml of sample to which 50μl of 0.5mg/ml 5-(-p-methylphenyl)-5-hydantoin (Supelco Inc.) in methanol had been added as internal standard. After thorough mixing, samples were centrifuged at 1500g for 10 min and the upper aqueous phase removed by suction. The chloroform extract was decanted into clean tubes and evaporated to dryness under air.

(iii) Chromatography

Chromatography was performed using the HPLC system described in 2.1.2 and a Spherisorb 5 ODS column (250 x 4.6mm) from Phase Separations. The solvent used was water/methanol/acetonitrile

(56/24/20) at a flow rate of 1.0 ml min. Detection was by UV absorbance at 230 nm. Dried samples were reconstituted in 200 µl of mobile phase and 50µl was injected onto the column. Drug concentrations were calculated from the ratio of peak rea of the drug to the peak area of the internal standard. Calculations were automatically performed after calibration with standards of known concentration by the computer terminal linked to the system. Results of both CBZ and CBZ-E are expressed as mg/l.

Fig. (11) shows the chromatogram obtained following injection of a standard sample onto the column.

2.4.3 DETERMINATION OF FREE CARBAMAZEPINE BY EQUILIBRIUM DIALYSIS

Free CBZ levels were determined by equilibrium dialysis using the equipment described in section 2.1.2. Dialysis was performed in teflon cells. 0.9ml plasma was injected into one half of the cell and 0.9ml potassium phosphate buffer (0.13M,pH7.4) into the other half. The two halves were separated by a dialysis membrane (Spectrapor, mol.wt. cut-off=6000-8000) and the assembly was rotated for 24 hours at 37°C. The respective compartments were then emptied and the concentration of free and bound CBZ in each fraction determined by EMIT as described in section 2.4.1.

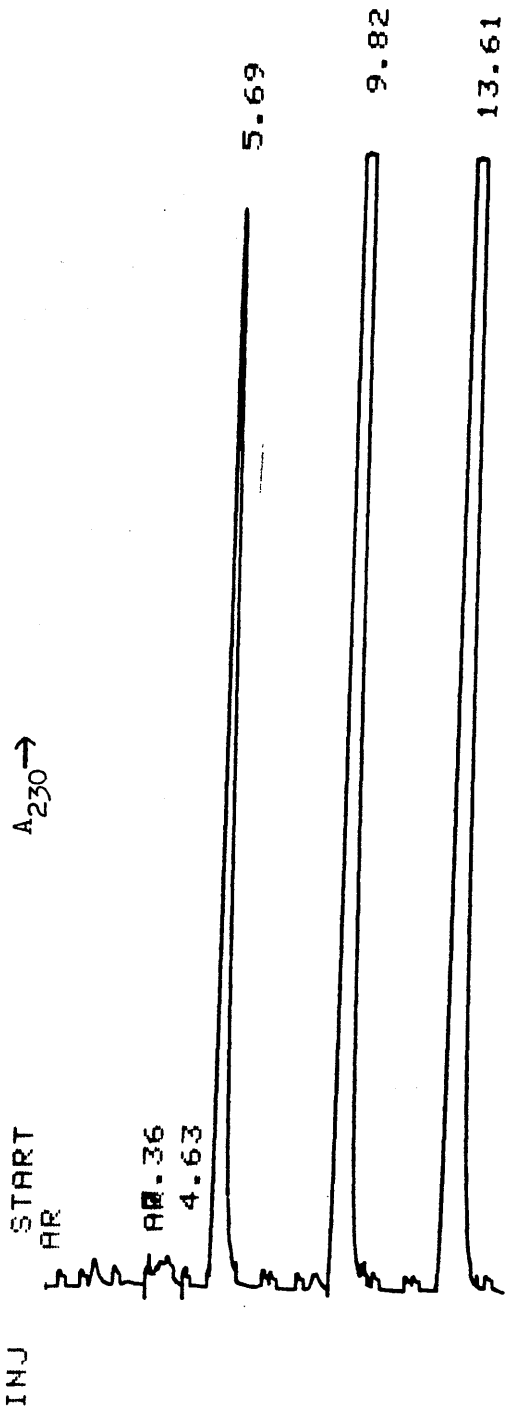


FIG. (11)

ANALYSIS OF CBZ AND CBZ-E BY HPLC

The figure is a chromatogram showing the resolution of CBZ, CBZ-E and internal standard (I.S.) obtained by HPLC. The sample shown is a standard prepared by adding CBZ, CBZ-E and I.S. to blank plasma. The analysis was then performed as described in sub-section 2.4.2.

Peak	Retention time
CBZ-E	5.69
CBZ	9.82
I.S.	13.61

2.5 ANIMALS

Animals used in experiments were male Sprague-Dawley rats (150-250g). Rats were fed and watered ad libitum with Oxoid 41B diet and tap water.

2.5.1 ADMINISTRATION OF CARBAMAZEPINE TO RATS

CBZ was administered orally twice daily (at 9.00 a.m. and 9.00 p.m.) as a suspension of ground tablets (Tegretol) in cottonseed oil (40 mg CBZ/ml). Control animals were given identical doses of cottonseed oil containing ground placebo tablets.

2.5.2 SACRIFICE AND REMOVAL OF LIVER AND BLOOD

Rats were fasted for 24 hours prior to sacrifice to minimise differences in enzymes which may arise due to differences in the nutritional status of the animal. The animals were sacrificed 2 hours after the last dose by cervical dislocation, the livers excised and perfused with ice-cold potassium chloride (0.15M) then stored in this solution prior to processing as described in 2.2 for the relevant enzyme assays. Blood was collected from the hepatic vein prior to removal of livers and allowed to clot before centrifugation at $4^{o}C$ and removal of serum for drug analyses described in 2.4.

2.5.3 COLLECTION OF URINE

Urine was collected from individual rats over 24 hour periods by keeping the animals in metabolic cages.

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2.6 CELL CULTURE STUDIES

The effects of CBZ and CBZ-E on some of the enzymes of haem biosynthesis were studied in cell culture. The most convenient system for these experiments was mitogen-stimulated human lymphocytes the preparation and maintenance of which is now described.

2.6.1 PREPARATION OF LYMPHOCYTES

Lymphocytes were prepared from the buffy coat obtained from 1 pint of blood. The buffy coat was diluted with 3 volumes of Hanks Balanced Salts solution (HBSS). 25ml aliquots were layered onto 10ml of Histopaque in 35ml sterile screw-top tubes and centrifuged for 30 minutes at 400g. The lymphocyte rich interphase from each tube was removed with a sterile pasteur pipette, resuspended in 20ml of HBSS and centrifuged for 10 minutes at 300g. The resultant pellets were combined and washed twice more before suspending in RPM1 1640 medium with the following supplements: foetal calf serum (10%), sodium bicarbonate (0.75mg/ml), L-glutamine (2mM), Penstrep (100 U/ml), pokeweed mitogen (2.5 μg/ml) and phytohaemagluttinin (5μg/ml). Cell density was determined using a Hawksley Cristallite improved Newbauer Counting Chamber and viability was determined by exclusion of trypan blue. Viability was usually in excess of 90%.

2.6.2 CULTURE CONDITIONS

Typically 10 or 15ml of medium containing 7.5×10^5 cells/ml was placed in a 50ml Lux tissue culture flask and incubated at 37°C in an LEEC automatic CO_2 incubator model GA2 with an atmosphere of 5% CO_2 in air. Cultures were maintained under these conditions until required for assays, but no cultures were kept for more than 5 days.

2.6.3 ADDITION OF DRUGS

Additions to cultures were made by dissolving the material to be added in dimethylsulfoxide (DMSO) and adding 10µl of the solution to flasks. If less than 10µl was to be added, the difference was made up with DMSO. Stock solutions of CBZ, and CBZ-E (30 and 15mg/ml) respectively) were prepared and stored at 4°C.

2.6.4 ENZYME ASSAYS AND PORPHYRIN FORMATION

Enzyme assays are described under the relevant headings in section 2.2. The formation of porphyrins from ALA was also investigated using a method modified from Sassa et al (1978).

Method for porphyrin formation

7.5 x 10^6 cells, cultured as described for 4 days, were pelletted by centrifugation at 300g for 10 minutes, resuspended in 10ml HBSS and recentrifuged. The cells were then suspended in 10ml of the medium described in section 2.6.2 but omitting the foetal calf serum and mitogens. The medium also contained ALA (0.6mM) and calcium magnesium ethylenediaminotetra-acetic acid (CaMgEDTA)(5mM). CaMgEDTA was prepared from calcium chloride, magnesium chloride and disodium EDTA as described by Sinclair and Granick (1977). After incubation for 24 hours at 37°C in an atmosphere of 5% CO_2, the cells were collected by centrifugation at 300g for 10 min. Porphyrin was extracted from the pelletted cells by agitation with 1ml of 0.5N perchloric acid - 50% methanol solution. This was then transferred to the flask from which the cells had come to extract porphyrin from cells which may remain in the flask. After centrifugation at 1500g for 10 minutes to remove precipitated protein, the supernatant was

diluted to 3ml with hydrochloric acid (5%) and the fluorescence measured in 1cm path length cuvettes, (excitation: 396nm, emission:594nm, band-pass:10nm). Standardisation was performed using a solution of coproporphyrin of known concentration. Porphyrin in the medium from which cells had been removed was isolated and measured as described in section 2.3.3 for urine except that 10ml of medium was added to the columns. Results were expressed as ng porphyrin produced/24 hours/10^6 cells.

For HPLC analysis of porphyrins in these extracts, the solutions were adjusted to pH6 with saturated sodium acetate. The subsequent steps are described in section 2.3.4.

2.7 FURTHER INVESTIGATIONS ON 5-AMINOLAEVULINATE DEHYDRATASE IN MAN

In the course of these studies, it was established that ALA-D activity in human erythrocytes was affected by treatment with CBZ. This section describes several methods used to further study the effect. One such technique has already been covered: the effects of including dithiothreitol and zinc chloride in the assay to restore non-active ALA-D (2.2.2). Other experiments looked at the effects of dialysis on activity. Parameters such as apparent Km values of erythrocyte ALA-D for its substrate and the optimum assay pH were determined. Attempts to reproduce the effect by adding CBZ and some metabolites to blood were also made. The methodology for these experiments now follows.

2.7.1 DIALYSIS OF ERYTHROCYTES

Red cells were separated from fresh, heparinised blood by centrifugation at 1,500g, washed twice with ice-cold isotonic saline and 2ml then placed in dialysis tubing (Visking size 5-24/32") either as they were, after reconstitution with dialysis buffer (50% v/v) or after haemolysis by sonication. The samples were dialysed against 2 litres of sodium phosphate buffer (0.15M,pH7.0) containing DTT (2mM) and $ZnCl_2$ (0.1mM) for 24 hours at 4°C, then assayed as described in section 2.2.2. Controls were prepared by separating and treating red cells as described above and storing them for 24 hours at 4°C without dialysis. Results were expressed as percentages of controls.

2.7.2 DETERMINATION OF APPARENT Km VALUES FOR ERYTHROCYTE ALA-D

The apparent Km of ALA-D for ALA was measured to determine whether or not the altered enzyme activity was a result of altered substrate affinity.

The assay was performed as described in section 2.2.2 for untreated human erythrocytes, but a range of ALA concentrations was used, (0.5, 1.0, 5.0 and 10.0mM). The final pH of each solution was 6.6. For each concentration of ALA used, ([S]) the assays were terminated at 0,5,10,15,20 and 30 minutes (or in cases where activity was normal, after 0,4,8,12 and 16 minutes) and the amount of PBG formed after this time determined.

Plots of A_{555} versus time for each concentration were made, fig.(12). From these the rate of reaction (V) at each concentration of ALA substrate ([S]) was calculated and expressed as $V = A_{555}/30$ minutes. Plots of 1/V versus 1/[S] were constructed and apparent Km values determined by taking the negative reciprocal of the x-axis intercept fig.(13) according to the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{Km}{Vmax.[S]} + \frac{1}{Vmax}$$

2.7.3 OPTIMUM ASSAY pH OF ERYTHROCYTE ALA-D

The optimum assay pH of ALA-D was determined in patients taking CBZ to investigate the possibility that the alteration of activity may be due in part to an altered pH activity profile, as is the case with lead (Chiba, Tashiro and Kikuchi, 1977; Farant and Wigfield, 1984).

The assay was performed as described in section 2.2.2 for untreated erythrocytes except the pH of the ALA solution added to the blood haemolysate was varied. Buffers of the required pH were prepared by mixing appropriate volumes of disodium hydrogen phosphate (0.1M) containing ALA (10mM) and sodium dihydrogen phosphate (0.1M), also containing ALA (10mM). Buffers of pH 5.6, 5.8, 6.0, 6.2, 6.3, 6.4, 6.5, 6.6 6.7, 6.8, 7.0, 7.2, 7.4, 7.6 and 7.8 were prepared in

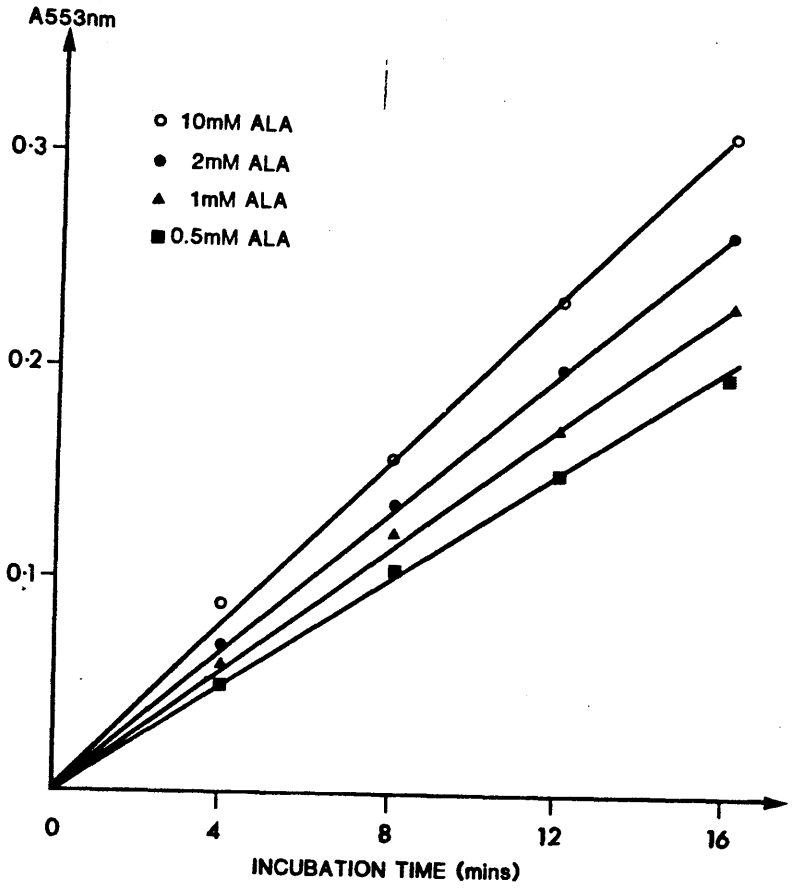


FIG. (12)

THE RATES OF REACTION AT VARIOUS SUBSTRATE CONCENTRATIONS IN THE ERYTHROCYTE ALA-D ASSAY

The figure shows the data used for the calculation of the apparent km of erythrocyte ALA-D for ALA in fig. (13). This was the method by which the apparent Km values presented in table (11)were calculated.

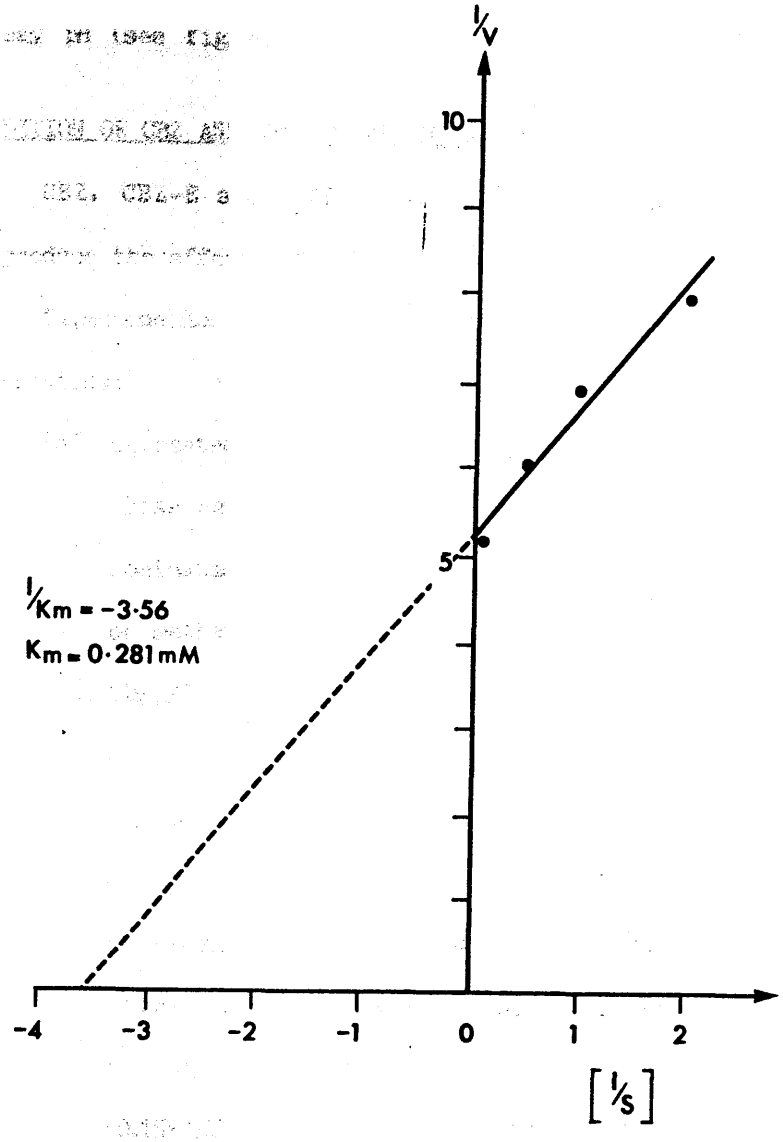


FIG. (13)

ESTIMATION OF THE APPARENT Km OF HUMAN ERYTHROCYTE ALA-D FOR ALA

The figure shows the calculation of the apparent Km of the enzyme for ALA by plotting the data from fig. (12) on a double reciprocal plot.

this way and these produced final pH values of 6.0, 6.1, 6.25, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 6.95, 7.1, 7.3, 7.5, 7.6 and 7.75 respectively when added to blood haemolysates. Results were calculated as normal and enzyme activity was plotted against the final assay pH (see fig.14).

2.7.4 ADDITION OF CBZ AND METABOLITES TO BLOOD

CBZ, CBZ-E and H_2CBZ were added to blood in an attempt to reproduce the effects of CBZ treatment on erythrocyte ALA-D.

Experiments were performed on fresh heparinised blood in 3 situations:

(a) untreated (b) sonicated and (c) washed red cells. In those experiments which involved whole blood (untreated or sonicated),CBZ, CBZ-E or H_2CBZ were added in 10µl acetone or methanol to 2ml of blood to give concentrations of up to 50mg/l. Appropriate controls to which acetone or methanol above had been added were also prepared. The samples were incubated for 1 hour at 37°C or for 5 minutes at 60°C. ALA-D activity was then determined immediately, after 16 hours and again after 40 hours. The preparations were stored at 4°C between assays. Washed red cells were resuspended (50% v/v) in sodium phosphate buffer (0.15M,pH7.4) to which drugs had been added as described for whole blood to give concentration of up to 50mg/l and then treated as described above.

Similar experiments were performed on the rat liver extract in which ALA-D was assayed (see 2.2.2) and on purified bovine hepatic ALA-D (see 2.9.1).

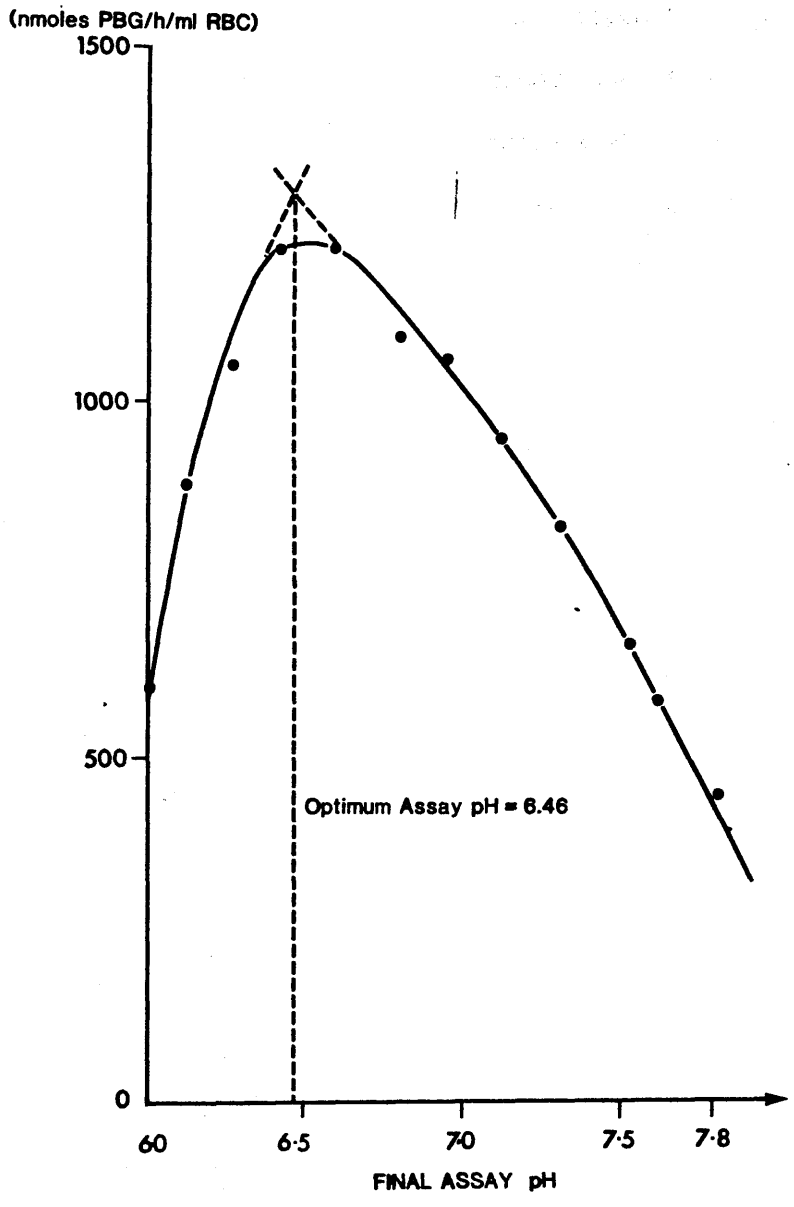


FIG. (14)

CALCULATION OF OPTIMUM ASSAY pH FOR ALA-D

2.8 IN VITRO DRUG METABOLISM STUDIES

Attempts to reproduce the effect of CBZ treatment on erytrocyte ALA-D by direct addition of CBZ have been described (2.7.4). The studies described in this section investigated the possibility that the observed effect was linked to metabolism of the drug. This was achieved by incubating purified enzyme with CBZ in the presence of microsomes containing a cytochrome P-450 dependent mixed function oxidase system. The experiments consisted of 3 steps:

(i) preparation of microsomes

(ii) preparation of ALA-D

(iii) incubation of microsomes and CBZ with ALA-D.

2.8.1 PREPARATION OF MICROSOMES

Microsomes were prepared essentially by the method described by Hajek, Cook and Novak (1982) from the livers of untreated rats, rats treated with 75mg/Kg CBZ b.d. for 4 days (as described in 2.5.2) and rats treated with 0.1% (w/v) phenobarbitone in drinking water for 5 days. The animals were sacrificed and the livers excised and perfused as described in 2.5.3. All subsequent manipulations were performed at $4^{o}C$ using ice-cold solutions. Livers were diced and homogenized in 3 volumes of TRIS-acetate buffer (0.1M,pH7.5) containing potassium chloride (0.1M) and disodium EDTA (1mM) using a Potter-Elvehjam homogenizer. The homogenate was centrifuged at 2,500g for 15 minutes and the supernatant removed and spun at 8,000g for 30 minutes. The resultant supernatant was filtered through glass wool and re-centrifuged at 10,000g for 30 minutes. The pellet was discarded and the supernatant filtered as before then spun at 105,000g

for 90 minutes. The supernatant from this step was discarded and the pellet was resuspended in sodium phosphate buffer (0.1M,pH7.4) containing Na_2EDTA(1mM) then repelletted by centrifugation at 144,000g for 60 minutes. The resultant pellet which contained the microsomes was resuspended in TRIS-acetate buffer (50mM,pH7.4) containing Na_2EDTA (1mM) and 20% glycerol to give 20-50mg protein/ml then stored at -80°C. The cytochrome P-450 content was determined as described in section 2.11.1.

2.8.2 PURIFICATION OF ALA-D

ALA-D purified for the biosynthetic preparation of porphobilinogen was used. The procedure was as detailed in section 2.9.1.

2.8.3 IN VITRO METABOLISM OF CARBAMAZEPINE

The incubation conditions for the *in vitro* metabolism of CBZ were adapted from Koizumi *et al* (1984) and Fujita *et al* (1984). The total incubation volume was 1.5ml and typically contained 0.2ml microsomes (5 nmoles cyt P-450), 0.88ml potassium phosphate buffer (incubation buffer: 100mM,pH7.4) containing DTT to give a final DTT concentration of 2mM, 0.5 units of ALA-D in 0.1ml of incubation buffer and 20 µl of a 10mg/ml solution of CBZ in acetone. The reaction was started by the addition of 0.3ml of incubation buffer containing 5mM NADPH. The mixture was incubated at 37°C in 5ml conical flasks with agitation. At various time intervals after initiation of the reaction 0.1ml was removed and assayed for ALA-D activity as described in 2.2.2 (iv). In some experiments, a further 0.05ml was removed and diluted to 1ml with incubation buffer for analysis of CBZ and metabolites by the HPLC

method described in section 2.4.2. At each time point, assays were performed in duplicate and two measurements of the Ehrlichs-PBG colour salt made for each of these duplicates. Control incubations were also performed in which NADPH, CBZ or the microsomes were replaced by incubation buffer.

The results were expressed as the percentage of ALA-D activity present at the start of the incubation which remained after a given time.

2.9 BIOSYNTHESIS OF PORPHOBILINOGEN

Porphobilinogen (PBG) was required for a number of assays (see section 2.2.3 and 2.2.4). Due to the high cost of the commercially available preparation, it was decided to synthesize PBG from ALA using immobilized ALA-D. The procedure consisted of 3 stages:

(i) purification of ALA-D from bovine liver

(ii) biosynthesis of PBG using immobilized ALA-D and

(iii) isolation of synthesized PBG

The overall synthesis was generally as described by Anderson and Desnick, (1982). The purification of ALA-D was taken from Graham (1978) and the subsequent steps in the procedure modified from March, Parikh and Cuatrecasas (1974) and Gurne and Shemin (1973).

2.9.1 PURIFICATION OF 5-AMINOLAEVULINIC ACID DEHYDRATASE

ALA-D was purified from bovine liver obtained fresh from the slaughter-house. Unless indicated, all procedures were carried out at 4°C. Portions of liver were diced and homogenized in 3 volumes of ice cold potassium phosphate buffer (0.02M,pH6.8) containing potassium chloride (0.15M) and 2-mercaptoethanol (1mM). The homogenate was spun at 2,500g for 45 minutes and the resultant supernatant heated in a water bath to 60°C, held there with constant stirring for 5 minutes then placed in crushed ice to cool. The extract was centrifuged at 2,500g for 20 minutes and the pellet discarded. 24.7g of ammonium sulphate (NH_4 SO_4) was added per 100ml of supernatant with constant stirring, and the solution left for 1 hour after total solution of NH_4SO_4. The precipitate was removed by spinning at 2,500g and discarded. A further 10.6g of NH_4SO_4 per 100ml was added as

previously described and the resultant precipitate containing the bulk of the remaining ALA-D activity pelletted at 2,500g and stored overnight at -25°C.

The following day, the precipitate was dissolved in the minimum amount of potassium phosphate buffer (0.05M,pH6.8) containing 2-mercaptoethanol (1mM) to give a protein concentration of 20-30mg/ml). The solution was then heated to 72-74°C in a water bath and held there for 3 minutes followed by rapid cooling in crushed ice. The supernatant obtained following centrifugation of the solution was placed in dialysis tubing (Visking Size 5-24,32") and dialysed overnight against 2 liters of the buffer in which the pellet was dissolved. The next day precipitated protein was removed by centrifugation.

The final yield of ALA-D was 7.1% and the specific activity was 50.7 umol PBG formed/hour/mg protein, a purification factor of 23.8. ALA-D was precipitated by adding NH_4SO_4 to 40g/100ml, spun down and stored at -80°C. Following this treatment, it was necessary to add DTT to the enzyme before use to restore activity. This is described in the relevant sections.

2.9.2 IMMOBILIZATION OF ALA-D AND BIOSYNTHESIS OF PBG

50ml of cyanogen-bromide Sepharose CL-4B (Sigma) was activated by washing with hydrochloric acid (1mM), water, suspending in sodium hydrogen carbonate for 3 hours and washing with potassium phosphate buffer (0.1M,pH7.0). It was then mixed with 600mg of protein from 2.9.1 dissolved in 20ml of potassium phosphate buffer (0.1M,pH7.0) containing DTT (0.3mM). Potassium chloride (KCl) was added to 0.4M

and the mixture stirred overnight at 4°C, then washed with 1 litre of potassium phosphate buffer (0.1M,pH7.0) containing KCl(0.7M). The gel-protein complex was resuspended in 500ml of TRIS-HCl buffer (0.2M,pH8.0) containing DTT (5mM) and left overnight at 4°C. The next day the buffer was decanted off and the complex washed with 500ml of potassium phosphate buffer (0.1M,pH7.0) containing DTT (2mM) then poured into a column (10 x 2.5cm).

PBG was produced by pumping a solution of ALA (2mM) in potassium phosphate buffer (20mM,pH6.7) with DTT(0.3mM) at a flow rate of 1.0ml/minute. The column was wrapped in aluminium foil to protect PBG produced from light and was maintained at 25°C, at which temperature 80-85% of the ALA was converted to PBG.

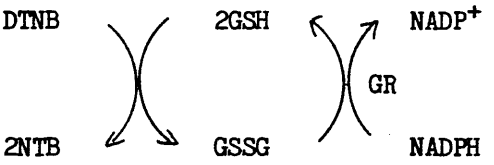
2.9.3 ISOLATION OF PORPHOBILINOGEN

PBG was separated from ALA and DTT by retention on a 1 x 30cm column of Dowex anion exchange resin (in the acetate form) placed in series with the column described in 2.9.2 and kept at 4°C in the dark. When the effluent gave a positive test with Ehrlichs reagent, (described in 2.3.1), the column was disconnected, washed with 2 volumes of ice-cold distilled water and PBG eluted with acetic acid (4M). The PBG containing solution was freeze-dried, but in the latter stages of the process the frozen solution turned to a brown liquid. This should not have occurred and may be a consequence of the acidic pH. This material was dissolved in water and the pH adjusted to 6.0 with ammonium hydroxide (2M) and the solution lyophilized to a pale brown powder.

3g of material were prepared in this way, but analysis revealed that only 30% (by weight) was PBG. The nature of the remaining 70% was uncertain, but it was not porphyrin (there was less than 0.05% uroporphyrin by weight). However, use of this PBG (after correcting for the fact that only 30% was PBG when preparing solution) in the assays described in 2.2.3 and 2.2.4 gave identical results when compared with those obtained from commercially available PBG.

2.10 MEASUREMENT OF GLUTATHIONE

Analysis of total glutathione (reduced + oxidized) in human blood and rat liver was performed by modifications of the methods of Brehe and Burch (1976) and Sunderman et al (1984). The principle of the method is that reduced glutathione (GSH) can react with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to release the highly coloured 2-nitro-5-thiobenzoic acid (NTB). In the presence of glutathione reductase (GR) and NADPH, a cycle is formed (Tietze,1969) which can be schematically illustrated as shown:



This method has the advantage of being selective for glutathione because although DTNB will react with almost any thiol, after the initial stage of the assay when the cycle is established, the rate of production of NTB is completely dependant on the concentration of glutathione present.

2.10.1 GLUTATHIONE IN HUMAN BLOOD

Method

10µl of fresh, heparinised, venous blood was hemolysed by adding 1.99ml of sodium phosphate buffer (0.01M, pH7.4) containing Na_2EDTA (5mM). 0.3ml of this solution was added to two 3ml cuvettes, each holding 2.7ml of sodium phosphate buffer (0.1M, pH7.4) containing Na_2EDTA (5mM), DTNB (0.25mg/ml) and NADPH (0.11mM). Both cuvettes were incubated at 30°C in a double beam spectrophotometer for 5 minutes, after which 10µl of glutathione reductase (Type III from

Yeast, Sigma, 220 U/ml) was added to the test cuvette and the rate of change in absorbance of the test cuvette with respect to the blank at 412nm was monitored for 5 minutes. The concentration of glutathione in the haemolysate was calculated by referring to a standard curve prepared by assaying 300µl of a solution of glutathione in haemolysis buffer with concentration ranging from 0-10 µM. After correcting for dilution, glutathione concentration was expressed as µmoles/ml whole blood.

2.10.1 GLUTATHIONE IN RAT LIVER

Method

Rats were killed and the livers excised and prepared as described in 2.5.2. 2.0g of liver were homogenised in 8ml of ice-cold Tris-HCl buffer (0.15M, pH7.4) using 5 strokes of a motor-driven Potter Elvehjem homogenizer. 4ml of perchloric acid (3M) containing Na_2EDTA (6mM) were added and the homogenate centrifuged at 18,000g at 4°C for 15 minutes. 0.1ml of the supernatant was diluted to 25ml sodium phosphate buffer (0.1M, pH7.4) containing Na_2EDTA (5mM). 0.3ml of this solution was then assayed exactly as described for the blood haemolysate. The pellet of denatured protein was dried at 100°C for 6 hours then weighed. After correcting for dilution, glutathione concentration was expressed as µmoles/g of liver.

2.11 ADDITIONAL METHODS

2.11.1 MICROSOMAL CYTOCHROME P-450

Cytochrome P-450 (cyt P450) was measured in purified rat liver microsomes.

(i) Preparation of microsomes

Livers were removed from rats and perfused as described in 2.5.2. All subsequent procedures were performed at 4°C. Typically 4g of liver were homogenized in 10ml ice-cold potassium phosphate buffer (0.15M,pH7.0) using a Polytron ultrasonic mechanical homogenizer. After an initial spin (1,500g for 15 minutes), to remove major cell debris, the sample was centrifuged at 17,500g for 20 minutes to remove mitochondria, nuclei, etc. The post-mitochondrial supernatant was spun at 105,000g for 1 hour and the resultant supernatant discarded. The pellet which contained the microsomes, was resuspended in the buffer described above to give a protein concentration of around 2mg/ml.

(ii) Measurement of cytochrome P450

The cyt P450 content of the microsomes was determined by the method of Omura and Sato (1964). Briefly, the solution of resuspended microsomes was split between two 1cm path length glass cuvettes and carbon monoxide (CO) was bubbled through one of the cells (designated the test cell). Sodium dithionite was added to both cuvettes and CO once again bubbled through the test cell, which was then scanned against the reference cell to produce a difference spectrum covering the range 400 to 500 nm at 20°C.

Cyt P450 concentration was calculated from the difference spectrum using the extinction coefficient ϵ =91mM for the difference in extinction between 450 and 490nm and expressed as nmoles cyt P450/mg microsomal protein.

2.11.2 DETERMINATION OF PROTEIN CONCENTRATION

Protein concentrations in samples were determined by the method of Lowry et al (19851) using crystalline bovine serum albumin as the standard.

Briefly, a range of standard solutions was prepared ranging from 0 to 200µg protein/ml. Unknown samples were diluted with distilled water to lie within this range. Standards and samples were then treated in tandem as described in the aforementioned paper and the extinction at 750nm measured. A standard curve of protein concentration versus A_{750} was prepared and used to determine the protein concentration in the unknown samples. A correction factor for the initial dilution was applied and results were expressed as mg/ml.

2.11.3 DETERMINATION OF BLOOD LEAD CONCENTRATIONS

Blood lead concentrations were determined by flameless atomic absoprtion spectrophotometry. Blood was collected in potassium EDTA tubes, then 100ul was diluted with 1 ml of 0.1% Triton X-100. Blood lead concentrations were then determined using the standard conditions (for the apparatus described in section 2.1.2) listed in Perkin-Elmer Analytical Methods for Furnace Atomic Absorption Spectroscopy publication No.332-A1.

2.11.4 DETERMINATION OF URINARY 6-β-HYDROXYCORTISOL EXCRETION

Measurements of urinary 6-β-hydroxycortisol were kindly performed by Dr. B.K. Park of the Department of Pharmacology and Therapeutics, Liverpool University by a direct radio-immunoassay (Park, 1978).

2.12 STATISTICAL ANALYSES

Groups of associated results were expressed as the mean value ± 1 standard deviation.

The significance of differences between sets of data were assessed using Students t-test.

Linear and curvilinear relationships were determined using linear and curvilinear regression analyses.

All of the above tests and the relevant calculations were performed on an Apple IIe computer using the Biostats III statistics package (R.C.Targett, 1982).

Results from all statistical analyses were taken to be statistically significant when the probability of an event occurring on the basis of a formulated null hypothesis was less than 5% ($P<0.05$).

CHAPTER 3

CHAPTER 3

THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON HAEM BIOSYNTHESIS IN MAN

3.1 INTRODUCTION

As explained in the final section of Chapter 1, there is some controversy over which drugs are safe to use in seizure management in epileptic porphyria patients. This chapter aimed to resolve this problem by examining the effects of three anticonvulsant drugs on selected aspects of haem biosynthesis in epileptic patients receiving long-term anticonvulsant therapy. The three drugs studied were carbamazepine (CBZ), sodium valproate (VPA) and phenytoin (DPH). The long-term affects of CBZ were of particular interest because as described in the final section of Chapter 1, Rapeport *et al*, (1984) and Yeung *et al* (1983) found changes in the haem biosynthetic pathway in individuals treated with CBZ which were representative of the biochemical features of the latent or remission phase of acute intermittent porphyria (AIP). These changes included elevation of ALA-S activity, depression of PBG-D activity, increased urinary ALA excretion (and in one case increased PBG) and an increase in the % URO in the urinary porphyrin fraction. These studies examined only subjects who had taken the drug for periods of less than three weeks. Therefore, a major portion of the investigations described in this chapter were devoted to the long-term effects of CBZ on haem biosynthesis in man in an attempt to explain these previous findings and to evaluate the potential of this treatment as a model for

studying AIP. Four groups were investigated (see Appendix I for details).

(1) a group of epileptic patients on CBZ monotherapy

(2) a group of epileptic patients on sodium valproate (VPA) monotherapy

(3) a group of epileptic patients on phenytoin (DPH) monotherapy

(4) a group of healthy control subjects age and sex matched to each of the above groups

In addition to this, some analyses were carried out on samples from a few epileptic patients taking phenobarbitone. Only a selection of the analyses applied to the other groups were performed and the numbers involved were not large enough for accurate statistical analysis. These figures therefore are not presented in the tables of results but will be referred to in the text where appropriate.

In the light of the earlier studies of Rapeport et al (1984) and Yeung et al (1983) it was decided that it was not necessary to assay all of the enzymes in the haem biosynthetic pathway. COPRO-O, PROTO-O and FERRO-C were omitted because they are not relevant to the potential porphyrogenicity of CBZ, VPA or DPH and also because they did not appear to be involved in the syndrome described by Rapeport et al and Yeung et al, for example (a) the two enzymes affected by CBZ are in the first part of the pathway (b) they have no bearing on the increased ALA excretion and (c) the increase in the URO fraction of the urinary porphyrins could have occurred in one of two ways, neither of which involve COPRO-O, PROTO-O or FERRO-C. The first of these is through a depression of PBG-D activity as is found in AIP, leading to a build up of PBG which would undergo non-enzymatic condensation and

cyclization to produce URO-genI. This would be excreted in the urine as URO-I, (the oxidized product of URO-genI), because it is a poor substrate for URO-D compared with the enzymatically produced UROgenIII (see section 1.2.4). The second way in which the URO fraction could be increased is through a reduction in URO-D activity as occurs in PCT (see 1.2.10) leading to accumulation of UROgenIII which would subsequently be excreted in the urine as URO-III. In any case, a change in the activities of these enzymes would be reflected in the porphyrin excretion pattern.

The chapter is divided into five sections, this introduction being the first. The second section discusses the effects of chronic anticonvulsant therapy on the activities of the enzymes in the pathway and the third looks at the effects of the same on the urinary excretion of porphyrins and precursors. The fourth section examines the effects of chronic treatment on 6β-hydroxycortisol excretion (an index of mixed function oxidase activity) and the fifth summarises the chapter.

Plasma drug levels were also measured where applicable. Not all of the patients studied were able to provide both blood samples and 24 hour urine collections, nor was it always possible to assay all of the required enzymes in a given blood sample. For these reasons the number of results for different analyses may vary within a group.

3.2 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON THE ENZYMES OF HAEM BIOSYNTHESIS IN BLOOD CELLS

As stated in the introduction to this chapter, the activities of some of the enzymes of haem biosynthesis were measured in epileptic patients undergoing long-term anticonvulsant therapy. The following enzyme analyses were performed:

Blood enzyme analyses

5-aminolaevulinic acid synthase (ALA-S)

5-aminolaevulinic acid dehydrase (ALA-D)

porphobilinogen deaminase (PBG-D)

uroporphyrinogen decarboxylase (URO-D)

The results of these analyses are presented in table (2). Each enzyme will be discussed in turn with reference to this table.

3.2.1 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON ALA-S ACTIVITY

The mean activities of ALA-S in both CBZ and VPA treated patients were raised significantly with respect to controls ($P<0.001$ for both groups), but there was no significant difference in those patients on chronic DPH therapy. Possible explanations for the lack of effect with DPH will be discussed later in this sub-section. In none of the groups studied was there any correlation between plasma drug levels and ALA-S activity. Why is ALA-S derepressed in CBZ and VPA treated patients? There are three possible mechanisms by which this could occur. These are (a) through an increased haem requirement (b) through a blockage at a specific enzyme site in the pathway with consequent reduction in haem out-put and (c) through direct derepression of ALA-S. The mechanisms whereby these situations may arise and cause an increase in ALA-S activity are discussed in detail in the introductory chapter (see 1.2.9), but it is likely that in the

TABLE (2)

SUMMARY OF THE RESULTS OF BLOOD ENZYME ANALYSES IN CONTROLS AND IN ANTICONVULSANT TREATED PATIENTS

	ALA-S[a]	ALA-D[b]	PBG-D[c]	URO-D[c]
CONTROLS	154 ± 57 (85 - 303) n=26	1224 ± 216 (684 - 1602) n=36	30.8 ± 4.7 (21.9 - 39.6) n=31	17.9 ± 2.4 (13.2 - 21.8) n=12
CBZ	336 ± 273* (61 - 934) n=23	747 ± 384* (78 - 1752) n=50	25.4 ± 6.6* (9.7 - 39.7) n=43	17.3 ± 3.8 (8.1 - 23.5) n=25
VPA	475 ± 188* (88 - 619) n=8	1299 ± 312 (768 - 1647) n=12	30.4 ± 4.9 (23.9 - 38.5 n=12	17.6 ± 2.4 (13.8 - 21.7) n=8
DPH	162 ± 105 (43 - 360) n=8	1185 ± 366 (561 - 1689) n=11	32.6 ± 8.2 (23.5 - 38.6) n=8	17.8 ± 3.1 (12.6 - 22.7) n=8

* $P<0.001$ as compared to controls

Values presented are mean ± 1 S.D.

Figures in brackets represent ranges

a units are pmoles ALA produced/h/mg protein

b units are nmoles PBG produced/h/ml erythrocytes

c units are nmoles URO produced/h/ml erythrocytes

d units are nmoles decarboxylations/h/ml erythrocytes

case of both CBZ and VPA, derepression occurred through an increased haem requirement. CBZ is metabolized by the hepatic mixed function oxidase (m.f.o) system (Frigerio, Cavo-Briones and Belvedere, 1976) and is also a potent inducer of this system in man (Eichelbaum et al, 1985). As cyt P450, the functional component of the hepatic m.f.o. system, requires haem as a prosthetic goup, then it follows that increased levels of haem will be required to meet the demand generated by increased cytochrome P450 apoprotein synthesis. In the case of CBZ, it is also possible that derepression of ALA-S occurred through mechanism (b) since as will be shown, CBZ causes a reduction in ALA-D activity. Derepression of ALA-S due to a deficiency of ALA-D activity occurs in lead poisoning and plumboporphyria (see 1.2.10). Although, as will be discussed later in this chapter, this may play some part in the derepression of ALA-S in some of the CBZ treated patients, this is not likely to be the principal means by which this was accomplished in the majority of patients. How could VPA cause an increased haem requirement? VPA is not primarily metabolized by the hepatic m.f.o. system, but the system must have some potential for metabolizing the drug since drugs which do induce hepatic m.f.o. activity accelerate VPA metabolism and clearance (Mihaly et al, 1979; Reunanen et al, 1980). VPA has also been shown to have inhibitory effects on the hepatic m.f.o. system (Kapetanovic et al, 1980) and it is possible that in this way it reduces the amount of functional cyt P450 and thus increases the demand for haem, leading to derepression of ALA-S. As part of a separate study, the short term effects of VPA treatment on leucocyte ALA-S activity were investigated. 8 healthy male volunteer subjects ingested either VPA (500mg t.d.s.) or

a placebo for a period of 7 days. ALA-S activities were measured after 1, 3 and 5 days. The results, shown in table (3), reveal a highly significant mean increased in ALA-S activity of 128% ($P<0.001$) after 3 days treatment with VPA. The mechanism whereby this occurs is probably as just discussed. Further discussion of the reasons why ALA-S should be derepressed in VPA and CBZ treated patients is presented in section 3.4.

The reasons why CBZ and VPA should have caused chronic elevation of ALA-S activity but DPH did not is uncertain. DPH has been shown to increase ALA-S activity during short-term treatment (McColl et al, 1980) in the same way as CBZ (Rapeport et al, 1983). However CBZ-treatment resulted in sustained elevation of activity (albeit at lower levels than in the initial stages of treatment) and it would appear that DPH did not. Measurement of the plasma DPH concentrations in the 8 patients whose ALA-S activities were measured revealed that in four of these patients, plasma drug concentrations were almost undetectable and were very low in another two of the patients. In view of the doses supposedly taken by the patients, this is suspicious of non-compliance. This non-compliance renders any significant deviations from the control group inconclusive in the case of DPH.

3.2.2 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON ALA-D ACTIVITY

Carbamazepine was the only drug to affect ALA-D activity: the mean ALA-D activity of the CBZ-treated group was 61% of the mean activity of the control group ($P<0.001$). In the 23 patients for which ALA-S data was also available the mean activity was 71% that of the control group. It is possible that this reduction in ALA-D activity is partly responsible for the increased ALA-S activity in some

TABLE (3)

THE EFFECTS OF SHORT TERM VPA TREATMENT ON

LEUCOCYTE ALA-S ACTIVITY IN MAN

DAYS OF TREATMENT	ALA-S ACTIVITY[a]	
	PLACEBO	VPA
1	153.4 ± 40.9	164.1 ± 65.9
3	157.5 ± 41.1	373.5 ± 115.6*
5	161.5 ± 32.3	335.5 ± 140.8*

* $P<0.001$ as compared to placebo phase.

8 healthy males ingested VPA (500mg,tds) or a placebo.

Leucocyte ALA-S activities were determined after treatment for 1, 3 and 5 days.

[a] units are pmoles ALA produced/h/mg/protein

patients because Meredith et al (1978) showed that below ALA-D activities of550nmol PBG/h/ml erythroctes, (48% of the mean control value in this study) haem biosynthesis was impaired to such an extent that ALA-S was derepressed. 19 of the patients studied fell into this category. However, the findings of Meredith et al are not completely applicable to this situation since that study was concerned with the effects of lead on the pathway. Lead affects enzymes other than ALA-D (see 1.3.10). Therefore in the majority of CBZ treated patients in this study, ALA-S was not derepressed as a consequence of the reductions in the levels of ALA-D activity. In support of this, no correlation was found between ALA-D and ALA-S activities. A correlation did exist however, between total plasma CBZ and ALA-D activity. For 47 patients in which both these parameters were measured there was a negative linear correlation between the two ($r=-0.45, P<0.01$), fig.(15). Interestingly, as regards fig.(15), extrapolation to zero concentration of CBZ gives an ALA-D activity of 1167 nmolPBG/h/ml erythrocytes. This compares well with the mean ALA-D activity found in controls (1124 ± 216 nmolPBG/h/ml erythrocytes, n = 36). There are three possible explanations as to why ALA-D activity is depressed:

(a) CBZ or a metabolite directly reduces enzyme activity

(b) CBZ or a metabolite indirectly reduces enzyme activity

(c) the derepression of ALA-D activity is somehow related to the increase in ALA-S activity

At this stage only option (c) can be eliminated since no correlation was found between the two and many cases of increased ALA-S activity have been reported without any depression of ALA-D. Further

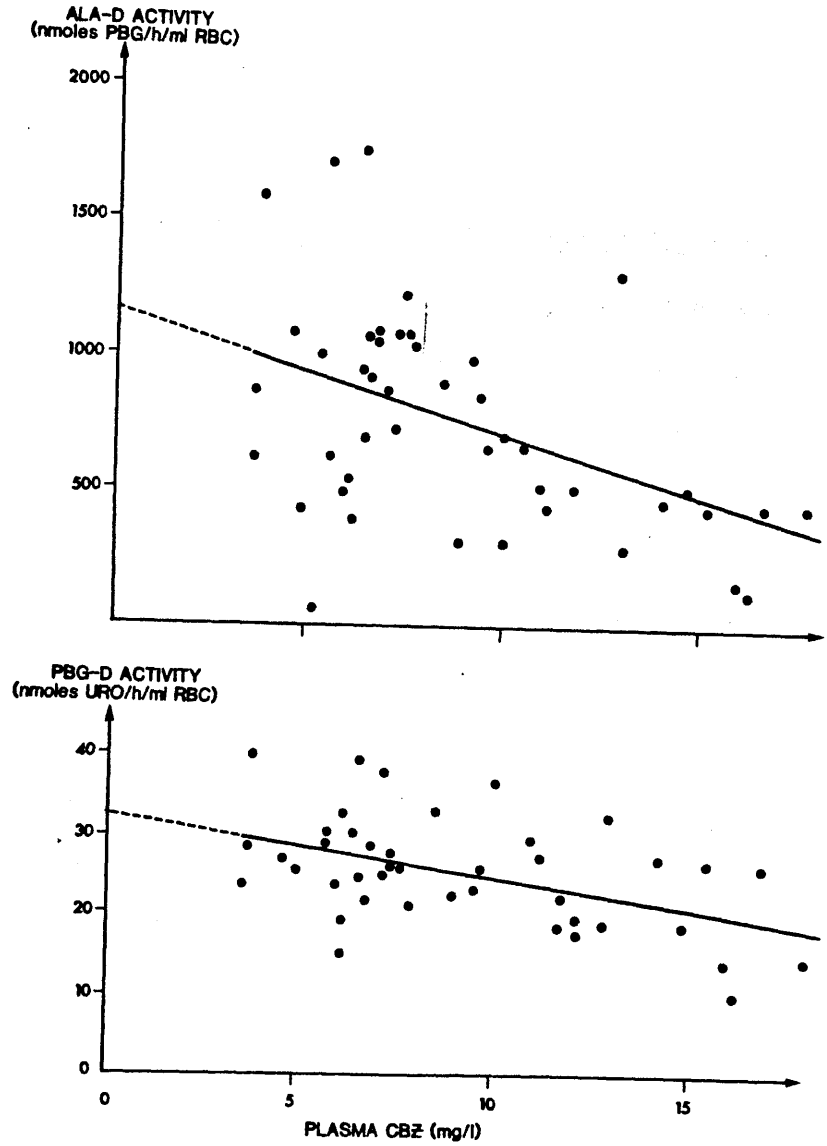


FIG. (15)

ERYTHROCYTE ALA-D AND PBG-D ACTIVITIES: RELATIONSHIPS WITH PLASMA CBZ

Statistically significant relationships were established between erythrocyte ALA-D and PBG-D activities and plasma CBZ concentrations in samples from epileptic patients on CBZ monotherapy.

Enzyme	n	Linear Regression Correlation Coefficient
ALA-D	47	- 0.45 , $p<0.01$
PBG-D	42	- 0.48 , $p<0.01$

investigations into the nature of the effect are described in Chapter 4.

ALA-D activity was also determined in 3 subjects on phenobarbitone monotherapy, but was not significantly different from controls (mean activity = 1128 ± 150 nmolPBG/h/ml erythrocytes).

3.2.3 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON PBG-D ACTIVITY

PBG-D activity was determined using the method designated the URO-1-S assay (see 2.3.2). The results show that CBZ, but neither VPA nor DPH, significantly reduced PBG-D activity. The mean PBG-D activity of the CBZ treated group was 82% of the mean control value ($P<0.002$). This reduction in PBG-D activity is in agreement with the findings of Yeung *et al*,(1983) and Rapeport *et al*, (1984) but does not match the findings of Rideout *et al*, (1983) or Doss and Schafer (1984), both of whom reported normal PBG-D activities during CBZ treatment. Reasons for this difference will be discussed presently. A negative linear correlation was found between total plasma CBZ and erythrocyte PBG-D in 42 patients ($r = -0.48$, $P<0.01$), fig.(15). Using the data from these patients in fig.(15), extrapolation to a theoretical zero concentration of CBZ gives a PBG-D activity of 32.5 nmolesURO/h/ml erythrocytes, which compares very well with the mean value of 30.8 nmolesURO/h/ml erythrocytes found in 31 controls. This suggests that CBZ does indeed cause a depression of PBG-D activity. If this were the case then the mechanisms whereby this could occur would be as for ALA-D. The fact remains, however, that other workers could not repeat these results. The answer to these discrepancies may lie in the methodologies used by the different groups. The assay from which these results were obtained utilized

ALA as a substrate and was dependent on ALA-D activity to generate PBG, the true substrate for the assay. The same assay was used by Yeung et al,(1983) and Rapeport et al, (1984) for their findings. The assays adopted by the other groups used PBG as a substrate. As ALA-D activity is reduced in patients undergoing CBZ therapy (see previous sub-section), a fact also noted by Rideout and Doss, it is possible that there is insufficient active enzyme to generate saturating quantities of PBG. The rate of PBG synthesis from ALA may be reduced to such an extent that the rate of UROgenI production is not dependent on the levels of PBG-D activity but on the rate of PBG production by ALA-D. This possibility is fully explored in later chapters.

PBG-D activity was also determined in 3 subjects on phenobarbitone monotherapy, but was not significantly different from controls (mean activity 32.8 ± 6.1 nmolURO/h/ml erythrocytes).

3.2.4 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON URO-D ACTIVITY

No changes were found in URO-D activity in any of the groups studied with respect to controls. This enzyme is not, as far as is known, inducible or repressible. Given that a transient effect of CBZ on the enzyme is improbable, then it is unlikely that URO-D activity would be reduced in individuals undergoing short-term treatment. This refutes the suggestion made earlier in this chapter that a reduced URO-D activity may be the reason for the increase in the % URO in the porphyrin fraction of urine observed by Rapeport et al, (1984).

3.3 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON THE URINARY EXCRETION OF PORPHYRINS AND PRECURSORS IN MAN

As stated in the introduction to this chapter, the urinary excretion of porphyrins and precursors were measured in epileptic patients undergoing long term anticonvulsant therapy. The following urinary analyses were performed:

24 hour 5-aminolaevulinic acid excretion

24 hour porphobilinogen excretion

24 hour total porphyrin excretion

HPLC analysis of porphyrin composition

The results of these analyses are presented in table (4). Each of these analyses will now be discussed with reference to this table.

3.3.1 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON URINARY ALA EXCRETION

None of the drugs studied produced mean 24 hour urinary ALA excretion values which differed significantly from the control group, though both CBZ and VPA treated groups did display slight increases. This indicates, at least in the 22 patients studied for which ALA-S data was also available, that CBZ does not inhibit ALA-D to such an extent that the flow of intermediates through the pathway is disturbed *in vivo*, as this would produce an accumulation of ALA. This reinforces the statement made earlier in this chapter that ALA-S in these patients was not elevated as a consequence of a blockage in the pathway.

No correlation was found between 24 hour ALA excretion and ALA-S or ALA-D activities for any of the groups studied.

TABLE (4)

SUMMARY OF THE RESULTS OF URINE ANALYSES IN CONTROLS AND IN ANTICONVULSANT TREATED PATIENTS

	ALA[a]	PBG[a]	TOTAL PORPHYRIN[b]	% PORPHYRIN COMPOSITION				
				%COPRO	%5-COOH	%6-COOH	%7-COOH	%URO
CONTROL n=16	13.5 ± 6.5	1.8 ± 2.2	64.6 ± 34.1	76.1 ± 6.7	4.7 ± 1.2	2.0 ± 0.6	4.7 ± 2.6	12.3 ± 3.9
CBZ n=22	17.9 ± 10.0	2.5 ± 2.1	98.4 ± 76.3	74.5 ± 9.6	5.2 ± 2.2	2.5 ± 1.3	6.0 ± 2.6	12.1 ± 4.7
VPA n=8	17.9 ± 11.2	9.7±11.4*	150.9±109.9*	84.8 ± 8.3*	2.4 ± 1.9**	0.7 ± 0.8**	2.9 ± 1.1	9.1 ± 7.3
DPH n=5	14.4 ± 6.9	2.8 ± 2.6	71.2 ± 19.9	64.1 ± 7.2*	8.0 ± 3.9	3.7 ± 1.4**	9.3 ± 2.9**	14.8 ± 4.3

* $P<0.01$, ** $P<0.001$ as compared to controls

Values presented are mean ± 1 S.D.

(a) = µmol/24 hours

(b) = µg/24 hours

3.3.2 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON URINARY PBG EXCRETION

The VPA treated group was the only one to exhibit significantly different excretion values for PBG as compared to controls, though both CBZ and DPH treated subjects showed slight increases. For 8 subjects on VPA, the mean PBG excretion over 24 hours was 539% that of controls ($P<0.01$). The increased PBG excretion during VPA treatment may have been due to the elevated ALA-S activity. The same may not have occurred in the case of CBZ because (a) in the patients whose urines were studied, ALA-S was not derepressed to the same extent as in those patients on VPA or (b) because less PBG was produced due to reduced ALA-D activity. This latter suggestion would imply that a reduced level of PBG synthesis did not have adverse effects on haem biosynthesis because a greater percentage of the PBG produced would be utilized i.e. there would be less wastage. There was no correlation between PBG excretion and ALA-S, ALA-D or PBG-D activities in any of the groups studied. Interestingly, a relationship was found between urinary ALA and PBG excretion in all groups with the exception of the CBZ-treated group. Combining the data from the control, DPH and VPA groups revealed a positive linear correlation between these two parameters, ($n=29, r=0.57, P<0.01$). No such relationship was found in the CBZ treated group ($n=22, r=0.041$, $P>0.1$) table (4). This disparity between the two sets of data is probably due to the reduction in ALA-D activity (caused by CBZ treatment), leading to subtle differences in the ALA/PBG ratio.

3.3.3 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON URINARY TOTAL PORPHYRIN EXCRETION

No significant differences were found between the CBZ and DPH treated groups and the control group, although in the case of CBZ there was a trend towards higher values. Patients treated with VPA showed a significant mean increase of 232% over controls which is, as for PBG, a reflection of the increased ALA-S activity. The reasons why a higher level of porphyrin excretion was not observed in CBZ-treated patients are probably the same as just discussed in the case of PBG excretion, namely ALA-S activities were not as high and also because the reduced ALA-D activity may mask the effect to some extent. No correlations were found between total urinary porphyrin and ALA-S, ALA-D, PBG-D or URO-D activities nor was there any correlation with the values for 24 hour ALA or PBG excretion.

3.3.4 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON URINARY PORPHYRIN COMPOSITION

Analysis by the HPLC method described in sub-section 2.3.4 of the percentage composition in urine of each of the porphyrins produced in the transformation of URO to COPRO revealed little change with respect to controls. The mean excretory pattern obtained with CBZ was virtually identical to that observed in controls. This contrasts with the findings of Yeung *et al* (1983) and Rapeport *et al* (1984), both of whom reported large increases in the URO fraction. The reasons for this difference are uncertain, but the most likely explanation is that the level of ALA-S activity plays an important role. Rapeport *et al* (1983, 1984) demonstrated that treatment with CBZ over a period of two weeks produced peak increases in ALA-S of

657% and 1145% of controls (figures are for 400mg CBZ/day and 600mg CBZ/day respectively), during which time the % fraction of URO in urine was also elevated. This was followed by a decline in ALA-S levels. The results presented here show that, although ALA-S activity does not remain at the very high levels observed in the initial stages of treatment, it does not return to normal levels and remains slightly derepressed. Moreover, at these moderately increased levels of ALA-S activity, no abnormality was found in the urinary porphyrin excretion pattern, implying that the transiently very high activities produced in the initial stages of treatment are the cause of the altered porphyrin profile. This could occur as follows. In the initial stages of treatment when ALA-S activities are very high, so much PBG is produced that PBG-D activity is saturated, and the excess PBG will be excreted in the urine. This is possible because after ALA-S, PBG-D has the lowest endogenous activity in the haem biosynthetic pathway (Elder,1982). Thus the situation is analogous to that described in AIP (see 1.2.10): urinary PBG will accumulate and will cause an elevation of the urinary URO fraction through non-enzymatic polymerisation and cyclization. In support of this theory, a slight but not statistically significant increase in PBG excretion was found by Rapeport et al,(1984). When ALA-S activity falls, PBG-D can cope because there is not such excessive production of PBG. The fact that ALA-D activity is depressed may also have a role to play at this stage since in the previous section it was shown that ALA-D activity was sufficiently reduced to alter the ALA : PBG ratio. An additional factor which may have some bearing on these results is that the method used here

contains certain refinements which greatly improved the resolution of the individual porphyrins. For example, Rapeport et al, (1984) found that COPRO accounted for almost 100% of the urinary porphyrin in subjects with only slight traces of the other porphyrins. In the results presented in this chapter, COPRO accounted for around 76% of the total urinary porphyrin excretion in controls and URO, 7-COOH, 6-COOH and 5-COOH accounted for around 12, 5, 2 and 5% respectively. This is in good agreement with the findings of Ostrowski et al (1984) who reported COPRO values of 58-87% and URO values of 13-31%, and also agrees well with Doss (1978).

VPA had some significant effects on the urinary porphyrin profile, predominantly an increase in the COPRO fraction with consequent reduction in each of the other fractions. The mechanism whereby this occured is uncertain, but it was not due to increased activity of URO-D (see 3.2.1). It could have arisen through a slight inhibition of COPRO-O, though this was not further investigated. A more likely explanation is that the increased flow through the pathway as a result of derepression of ALA-S provides more substrate for URO-D and allows an increased rate of COPRO formation which may lead to a greater excretion of COPRO. This proposal assumes that there is an endogenously higher level of URO-D activity than COPRO-O activity, which is the case in rat liver (Bishop and Desnick, 1982). In human liver, however, the activities of the two enzymes are almost equivalent (Elder, Lee and Tovey, 1978), though the above proposed is still valid because URO-D has a higher km value and could therefore cope with more substrate (Elder, 1982).

DPH also affected the urinary porphyrin excretion profile, with a significant reduction in the COPRO fraction and corresponding increases in the remaining fractions. However, for the reasons stated earlier in this chapter, the results obtained from the studies described here must be treated with caution due to the low drug concentrations found in this group of patients. An additional complicating factor may be the small number of urines studied from this group (n=5). For conclusive results, a larger number of urine samples would be required from patients complying with their therapeutic drug regime.

3.4 THE EFFECTS OF CHRONIC ANTICONVULSANT THERAPY ON URINARY 6-β-HYDROXYCORTISOL EXCRETION

6β-hydroxycortisol (6β-OHC) is a minor metabolite of cortisol formed primarily in the endoplasmic reticulum of hepatocytes by the mixed function oxidase (m.f.o.) system. It is excreted in the urine without conjugation and has been used as an *in vivo* index of m.f.o. activity. (Park, 1981). Measurement of the levels of this compound in the urine of epileptic patients receiving chronic anticonvulsant therapy therefore allows assessment of the state of m.f.o. activity in these patients as compared to age and sex-matched controls. The analyses were performed on 24 hour urine collections from the following groups:

(i) a group of epileptic patients receiving CBZ monotherapy

(ii) a group of epileptic patients receiving VPA monotherapy

(iii) a group of controls, age and sex matched with the above

The results are presented in table (5) and show that chronic treatment with either CBZ or VPA produced a highly significant increase in 6β-OHC excretion. Increases of 147% ($P<0.005$) and 104% ($P<0.05$) compared to the control group were found for CBZ and VPA treated groups respectively. This result was not unexpected in the case of CBZ since this compound is a well-known inducer of m.f.o. activity and has previously been reported to cause increased 6β-OHC excretion (Roots, Ley and Hildebrandt, 1977). This provides support for the proposal that ALA-S is chronically derepressed in these patients to produce sufficient haem for increased cyt P450 synthesis. The result with VPA, however was unexpected. Since VPA is not primarily metabolized by this system, it would not be expected to induce it. There may be increased m.f.o. activity in order to

TABLE (5)

THE EFFECTS OF CHRONIC CBZ AND VPA MONOTHERAPY

ON URINARY 6β-HYDROXYCORTISOL EXCRETION IN EPILEPTIC PATIENTS

TREATMENT	6β-OHC EXCRETION
Control (n=16)	291 ± 102
CBZ (n=19)	720 ± 526**
VPA (n=8)	595 ± 552*

* P<0.05, ** P<0.005

6β-OHC was measured in 24 urine collections by radioimmunoassay. The units used are μg 6BOHC excreted/24 hours.

compensate for the inhibitory effects of VPA on the system (Kapetanovic et al, 1980). However, regardless of the mechanism by which m.f.o. activity is increased (as evidenced by the rise in 6β-OHC excretion), the increase is the likely cause of the chronic derepression of ALA-S found in these patients.

There was no correlation between 6β-OHC excretion and ALA-S activity or total porphyrin excretion in any of the groups studied.

Conclusions of Section 3.4

CBZ and VPA produced increases in 6β-OHC excretion in patients treated chronically with either drug. These increases reflect a rise in hepatic m.f.o. activity, which is probably the underlying cause of the derepression of ALA-S which is found in these patients.

3.5 SUMMARY OF CHAPTER 3

The conclusions from each of the groups studied are summarised separately.

3.5.1 THE EFFECTS OF CHRONIC CARBAMAZEPINE TREATMENT ON HAEM BIOSYNTHESIS

The long term effects of CBZ on the enzymes studied were a sustained increase in ALA-S activity (which did not correlate with plasma drug concentration) and a depression of both ALA-D and PBG-D activities (both of which correlated with plasma drug concentration). ALA-S was probably derepressed as a result of an increase in hepatic cytochrome P450 synthesis. This was supported by the finding of an increased 6B-OHC excretion in these patients. The mechanism of the depression of ALA-D and PBG-D activities is uncertain. URO-D activity was unaffected by CBZ. Urinary ALA, PBG and total porphyrin excretions were slightly elevated in comparison to controls, but not to a statistically significant extent and there was no correlation between any of these parameters and plasma CBZ concentration. The depression of ALA-D activity, however, produced a change in the relationship of urinary ALA to PBG. The results of the analyses of urinary porphyrin excretion profiles during prolonged CBZ therapy were no different from controls. This finding is in conflict with Rapeport *et al*, (1984) but the difference is probably attributable to the very high levels of ALA-S activity produced in the initial stages of CBZ treatment in that study.

3.5.2 THE EFFECTS OF CHRONIC VALPROIC ACID TREATMENT ON HAEM BIOSYNTHESIS

The only enzyme shown to be affected by VPA was ALA-S. This was most probably derepressed as a result of increased hepatic cytochrome

P450 synthesis, required to maintain the high levels of m.f.o. activity found in these patients. This increase in m.f.o. activity is probably the result of over compensating for the inhibitory effect of VPA on the system. There was no correlation between plasma drug concentration and ALA-S activity. VPA also produced an increase in the urinary excretion of ALA, PBG and total porphyrin, though only the latter two were affected to a statistically significant degree. These increases were probably due to the increased ALA-S activity. Again, there was no correlation between any of these parameters and plasma VPA concentration. There was also a significant change in the urinary porphyrin excretion profile, which may be attributed to increased availability of pathway intermediates.

3.5.3 THE EFFECTS OF CHRONIC PHENYTOIN TREATMENT ON HAEM BIOSYNTHESIS

In the patients studied, DPH did not affect any of the enzymes or the urinary excretion of ALA, PBG and total porphyrin. These results cannot be considered to be conclusive, primarily because of the very low plasma drug concentrations in the patients studied.

3.5.4 FURTHER EXPERIMENTS

On the basis of these results, it was decided to further investigate the effects of CBZ on haem biosynthesis with particular reference to ALA-D, PBG-D and the urinary excretion of porphyrins and precursors. These investigations constitute the remainder of this thesis.

CHAPTER 4

4. CARBAMAZEPINE AND HAEM BIOSYNTHESIS IN MAN

This chapter investigates in greater detail the effects of CBZ on certain aspects of haem biosynthesis in man. It is divided into four sections. The first of these is concerned with ALA-D, the second with PBG-D and the third with the urinary porphyrin excretion profiles. The fourth summarises the results of this chapter. At this point it is appropriate to recall two aspects of CBZ therapy which may have some bearing on the effects which will be discussed. The first of these is carbamazepine-10,11-epoxide (CBZ-E), the principal metabolite of CBZ. This accounts for 10-20% of the total circulating levels of the drug in man. (Brodie, Forrest and Rapeport, 1983). There is a relationship between plasma CBZ-E (as determined by HPLC) and total plasma CBZ (as determined by EMIT). This is demonstrated in fig. (16) using data from 47 epileptic patients on CBZ monotherapy. The curvature of the line at high plasma CBZ concentrations means that the % CBZ-E increases with increasing drug levels. This is due to auto-induction: CBZ stimulates its own metabolism (Eichelbaum *et al*, 1975). The second aspect of CBZ which is of interest is free CBZ. CBZ is normally about 80% bound to serum proteins. The remaining percentage is termed "free CBZ". Free CBZ is in equilibrium with CBZ inside cells and is therefore considered to be the fraction with pharmacological activity. Free CBZ is also related to total CBZ (as determined by EMIT). This is illustrated in fig (17) using data from 33 epileptic patients on CBZ monotherapy. Both CBZ-E and free CBZ are discussed in more detail in the introductory chapter (1.2.3).

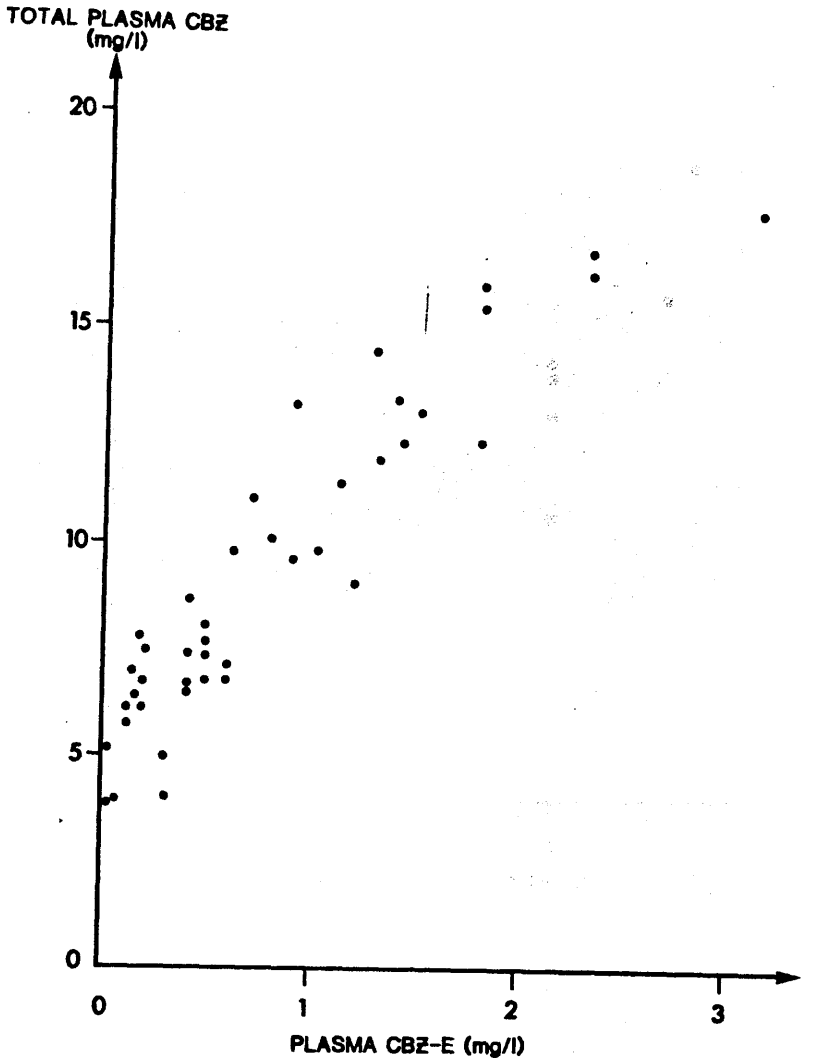


FIG. (16)

THE RELATIONSHIP BETWEEN TOTAL CBZ AND CBZ-E IN HUMAN PLASMA

Samples were taken from 43 epileptic patients on chronic CBZ monotherapy.

"Total CBZ" refers to CBZ and all metabolites thereof as determined by the EMIT system (see sub-section 2.4.1). CBZ-E was determined by the HPLC method described in sub-section 2.4.2.

Linear Regression Correlation Coefficient = 0.93, $p<0.001$

Curvilinear Regression Correlation Coefficient = 0.94, $P<0.001$

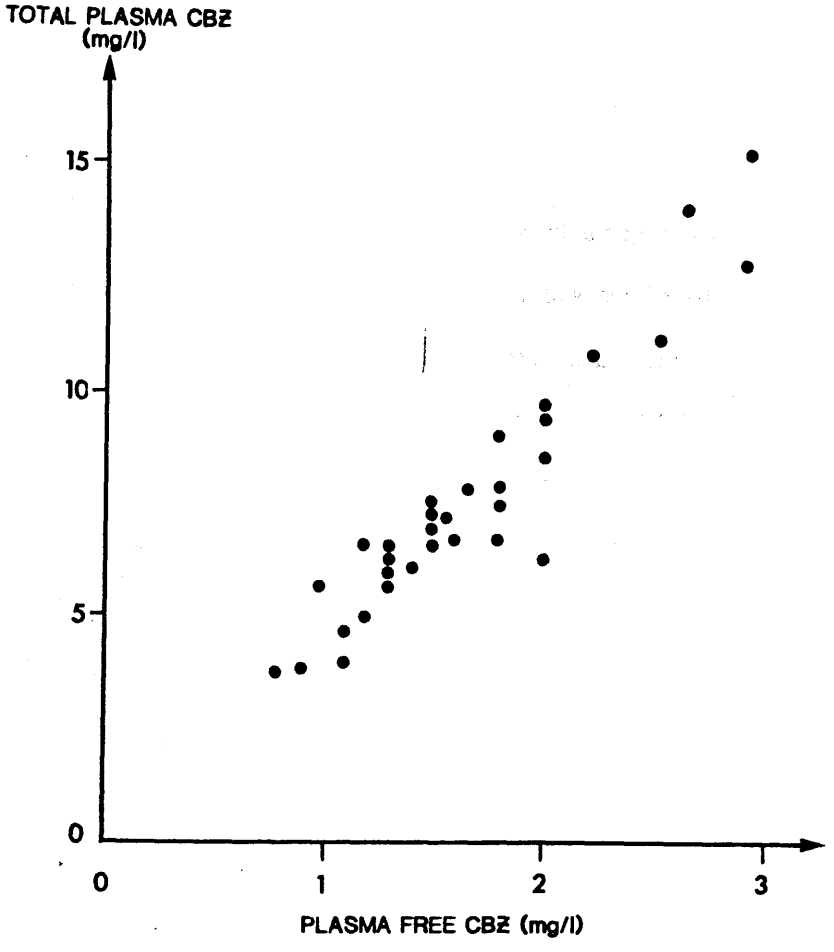


FIG. (17)

THE RELATIONSHIP BETWEEN TOTAL CBZ AND FREE CBZ IN HUMAN PLASMA

Samples were taken from 33 epileptic patients on chronic CBZ monotherapy.

"Total CBZ" refers to CBZ and all metabolites thereof as determined by the EMIT system (see sub-section 2.4.1). Free CBZ was determined by equilibrium dialysis as described in sub-section 2.4.3

Linear Regression Correlation Coefficient = 0.92, $p<0.001$.

All of the analyses described in this chapter were performed on fresh blood or urine samples as described in the appropriate methods section.

4.1 CARBAMAZEPINE AND 5-AMINOLAEVULINIC ACID DEHYDRATASE

Results in the previous chapter revealed that treatment with CBZ caused a reduction in the activity of erythrocyte ALA-D in man, the extent of which was related to the total plasma levels of CBZ. This section examines the nature of the relationship between CBZ and ALA-D in greater detail. There are several ways in which the reduction in ALA-D activity observed could have occurred, each of which could be due to a direct or indirect effect of CBZ. These are (a) a reduced rate of synthesis of the enzyme (b) partial inactivation (or modification) of all molecules of enzyme or (c) total inactivation of a percentage of the enzyme. Option (a) is not very likely because ALA-S is the only enzyme in the pathway so far shown to be inducible or repressible. The remaining possibilities are more probable and are also easier to test. Each of the sub-sections which now follow discusses various aspects of the relationship between CBZ and ALA-D with possibilities (b) and (c) in mind.

4.1.1 ATTEMPTS TO REVERSE THE CBZ-MEDIATED REDUCTION IN ALA-D ACTIVITY

One of the first steps in understanding the nature of any enzyme inhibition is to establish if it is reversible or not. Therefore the experiments described in this sub-section were designed to resolve this question. Two different techniques were used in an attempt to restore ALA-D activity. The first attempted to achieve this through treatment with activators and the second by dialysis. Each of these is discussed separately.

(i) Treatment of ALA-D with activators

ALA-D from all tissues is very susceptible to loss of activity through oxidation of essential thiol groups or removal of essential zinc ions. Reduction of these oxidised thiols with sulphydryl reagents such as dithiothreitol (DTT), cysteine or mercaptoethanol, and the addition of zinc ions generally restores enzyme activity to what it was previously. These compounds are therefore referred to as activators of the enzyme. Even in fresh blood from healthy subjects, not all of the ALA-D present in the erythrocytes is catalytically active. Treatment with these activators however, realises the full potential activity and will be referred to as "restored activity" whereas activity in untreated blood will be termed "circulating activity". A good example of the effectiveness of these activators is in reversing the effects of lead - a potent inhibitor of ALA-D (Mauras and Allain, 1982). This experiment investigated if the reduction in erythrocyte ALA-D activity which occurs during CBZ therapy was reversible by treatment with DTT and Zn^{2+} using the methodology described in section 2.2.2.

The analyses were performed on blood from the same groups of patients as described in Chapter 3. DPH and VPA treated patients were also included for completeness. For each subject studied a value was obtained for circulating activity and restored activity. The results are presented in graphic form in fig (18). Table (6) summarises the data.

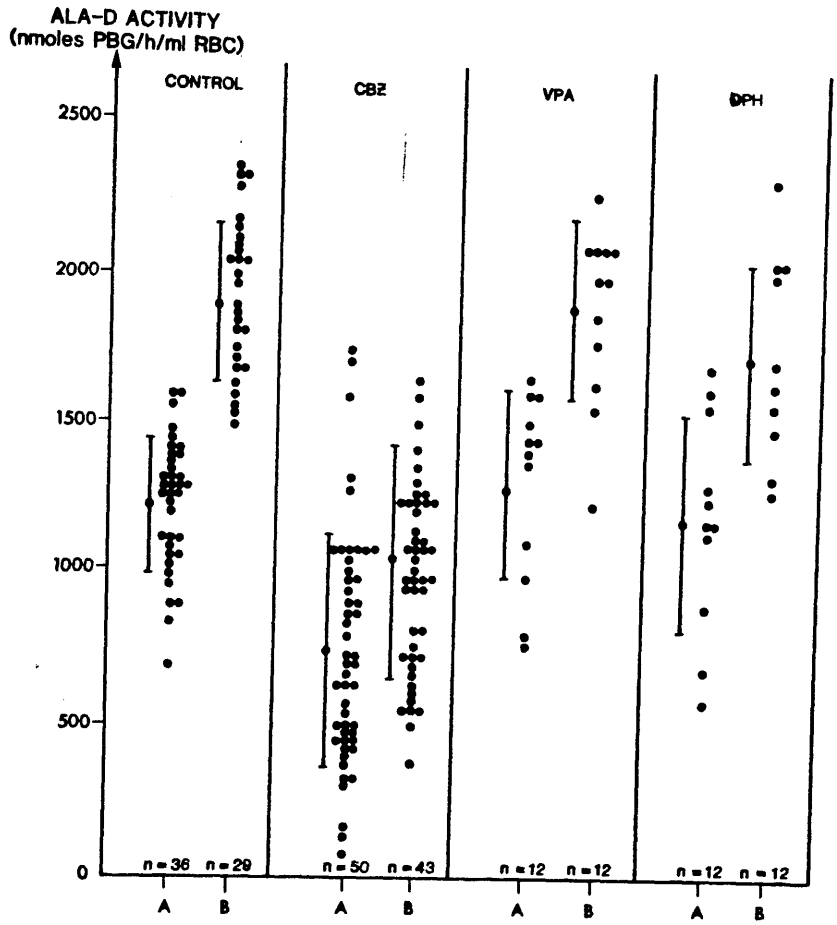


FIG. (18)

CIRCULATING AND RESTORED ERYTHROCYTE ALA-D ACTIVITIES IN CONTROL SUBJECTS AND IN EPILEPTIC PATIENTS RECEIVING ANTICONVULSANT MONOTHERAPIES

In each compartment, A = circulating activities and B = restored activities. Only CBZ treated subjects were significantly different from controls on the basis of students t-test. ($p<0.001$ for both A and B).

The data is presented in tabular form in table (6).

TABLE (6)

CIRCULATING AND RESTORED ALA-D IN CONTROLS AND IN CBZ,VPA AND DPH TREATED PATIENTS

	Circulating ALA-D activity	Restored ALA-D activity
CONTROL	1224 ± 216 ,n=36	1902 ± 264 , n=29
CBZ	747 ± 384* ,n=50	1050 ± 393* , n=50
VPA	1299 ± 312 ,n=12	1881 ± 291 , n=12
DPH	1185 ± 366 ,n=11	1728 ± 333 , n=11

* P<0.001 as compared to controls

Values presented are mean ± 1 S.D.

Units are nmoles PBG produced/h/ml erythrocytes

The circulating activities in all 4 groups were increased following treatment with activators. The activity in CBZ-treated samples, although showing an increase, did not return to normal levels of activity. This contrasts with the effect of these activators on ALA-D which has been inactivated by binding lead. In the process of restoring activity, the mean ALA-D activity of the CBZ-treated group fell from 61% of the mean circulating activity of the controls to 55% of the mean restored activity, suggesting that less activation occurs in CBZ-treated erythrocytes. This was confirmed by calculating the mean % increase in activity for each patient. Control ALA-D activities were increased by a mean value of 56.8 ± 28.6% (n=29) whereas CBZ treated activities increased by a mean value of 41.2 ± 32.7% (n=44; $P<0.02$). This latter figure excludes values where the circulating ALA-D activity was less than 300nmol/PBG/h/ml erythrocytes since on activation these produced an artificially high percentage increase. This observation can be interpretated as showing that in CBZ-treated patients a greater percentage of the enzyme is present in the catalytically active state. No correlation was found between the percentage activation and plasma CBZ concentration. As expected VPA and DPH behaved exactly as the controls, being activated by mean values of 51.6 ± 40.5% (n=12) and 54.3 ± 33.7% (n=11) respectively.

(ii) Dialysis of ALA-D

If activity was lost through the action of a non-covalently bound inhibitor, it should be possible to remove this inhibitor by dialysis, thus restoring activity. This experiment investigated if depressed ALA-D activity could be restored in this way and was performed as described in section 2.7.1.

ALA-D activity was assayed in washed erythrocytes and sonicated erythrocytes, both before and after dialysis as described in section 2.7.1, in 6 CBZ-treated individuals with reduced ALA-D activities and in 6 age and sex matched controls. The results are presented in table (7).

From the results, it is clear that dialysis did not restore activity in either washed or sonicated cells.

Conclusions about the reversibility of CBZ-mediated inhibition of ALA-D

In contrast to lead-mediated inhibition of ALA-D, treatment of the enzyme from individuals with reduced ALA-D activity due to CBZ treatment with the activators DTT and Zn^{2+} did not restore activity. This suggests that the inhibition does not occur through interaction of the drug with essential thiol groups. Further evidence for this will be presented in section 4.1.5. CBZ treated patients also had a greater percentage of the ALA-D present in erythrocytes in the active state. There are two possible explanations for this. The first is that inactive and active ALA-D are in equilibrium and CBZ treatment can only affect the inactive enzyme, reducing the ratio between the two species. However, as a consequence of the equilibrium, activity would also be reduced in the active fraction. The second possibility

TABLE (7)

THE EFFECTS OF DIALYSIS ON ERYTHROCYTE ALA-D ACTIVITY IN CONTROLS AND CBZ-TREATED SUBJECTS

	CONTROL		CBZ	
	WASHED ERYTHROCYTES	SONICATED ERYTHROCYTES	WASHED ERYTHROCYTES	SONICATED ERYTHROCYTES
NON-DIALYSED CONTROL	867 ± 186	843 ± 174	246 ± 237	219 ± 210
DIALYSED	909 ± 162	825 ± 147	219 ± 195	201 ± 189

Values presented are mean ± 1 S.D.

Units are nmoles PBG produced/h/ml erythrocytes

is that CBZ treatment affects all of the enzyme molecules equally but the cell increases its reserve of zinc ions and reduced glutathione (a tripeptide responsible for maintaining protein cysteine residues in the reduced state), thus leading to a reduced total activity but with a higher percentage of enzyme in the active state. The inhibition may be a covalent interaction between the inhibitor species and ALA-D, as it was not reversible by dialysis. The nature of this interaction is open to speculation.

4.1.2 TIME COURSE OF THE CBZ-MEDIATED REDUCTION IN ALA-D ACTIVITY

Knowledge of the time course of the reduction in erythrocyte ALA-D activity caused (either directly or indirectly) by CBZ would be useful in the consideration of the nature of the effect. Numbers of patients in this section are limited because it was only infrequently that new epileptic patients were started on long-term CBZ monotherapy. However, ALA-D activities were determined in 4 such individuals before starting on CBZ and then after 1 week and after 4 weeks of treatment. The effects of a single large dose of CBZ on ALA-D activity in 9 volunteers over a period of 42 hours were also measured. These results are presented first.

(i) The effects of a single dose of CBZ on erythrocyte ALA-D activity

ALA-D activities were measured in 9 healthy adults (5 females, 4 males) after administration of a single dose of CBZ equivalent to 10mg/Kg body weight. Activities were determined 10,14,18,34 and 42 hours after ingestion of the drug, and in the same subjects at the same time intervals after taking a placebo. The results are presented in table (8).

TABLE (8)

THE EFFECTS OF A SINGLE DOSE OF CBZ ON ERYTHROCYTE ALA-D ACTIVITY OVER TWO DAYS

Time after drug/placebo (hrs)	CONTROL PHASE		CBZ-TREATED PHASE		
	Circulating activity[a]	Restored activity[a]	Circulating activity[a]	Restored activity[a]	[CBZ][b]
10	1182 ± 348	1902 ± 372	1125 ± 156	1926 ± 267	9.1 ± 0.8
14	1149 ± 330	1854 ± 399	1158 ± 195	1941 ± 249	8.6 ± 1.8
18	1146 ± 309	1854 ± 378	1110 ± 174	1863 ± 285	8.0 ± 1.4
34	1161 ± 336	1902 ± 375	1158 ± 192	1896 ± 273	5.9 ± 1.4
42	1161 ± 333	1827 ± 330	1155 ± 195	1863 ± 300	4.4 ± 0.8

Values presented are mean ± 1 S.D.

(a) units are nmoles PBG produced/h/ml erythrocytes

(b) units are mg/l plasma

The results show that even in the presence of high plasma CBZ levels (9mg/l) there was no effect on ALA-D activity. Furthermore, there was no significant change in either circulating or restored activities with respect to control values at any time point in the experiment and both activities remained essentially constant in both the control and drug-tested phases.

(ii) The effects of CBZ-treatment on erythrocyte ALA-D activity over four weeks

4 epileptic patients, over a period of a year, were initiated onto a regime of 200mg CBZ twice daily. Table (9) shows ALA-D activities both before and after taking the drug. The results are expressed as percentages of control values. No results are presented after 4 weeks because in three of the subjects the dosage was increased after this time preventing further analysis of the time course of the reduction.

The table shows that there was a reduction in the mean activity of both circulating and restored ALA-D after 1 week (13.5% and 20.3% respectively). Further reductions of 20% and 17.5% respectively occurred after a further 3 weeks treatment with CBZ.

Conclusions about the time course of CBZ-mediated reduction in ALA-D activity

The reduction of ALA-D activity by CBZ is a slow process: maximal depression of enzyme activity does not occur after 1 week of exposure to the drug as further reductions were observed after a another 3 weeks. However it is not certain if these effects were

TABLE (9)

THE EFFECTS OF CBZ TREATMENT ON ERYTHROCYTE ALA-D ACTIVITY OVER FOUR WEEKS

Subject	Circulating activity			Restored activity		
	0	1 Week	4 Weeks	0	1 Week	4 Weeks
1	100	93.9	58.8	100	84.4	56.7
2	100	91.9	64.0	100	78.9	50.4
3	100	79.9	76.3	100	78.6	73.0
4	100	80.2	66.8	100	76.7	68.5
Mean ± 1 S.D.	100	86.5 ± 7.5	66.5 ± 7.3	100	79.7 ± 3.3	62.2 ± 10.4

Results are expressed as percentages of initial values

Patients were given 200mg CBZ b.d.

maximal for the dosage of CBZ used. Most activity was lost from the inactive pool of ALA-D confirming the observation made in section 4.1.1 namely that CBZ-treated patients have a greater percentage of their potential ALA-D activity realised. Exposure of untreated subjects to levels of CBZ which were shown to cause reduction in ALA-D activity during prolonged treatment (see figs. (15) and (19)) for almost two days produced no changes in enzyme activity.

There are a number of possible explanations as to why CBZ treatment takes so long to produce the observed depression of ALA-D activity. If the reduction in activity is the result of a direct action of CBZ or CBZ-E on the enzyme then either the reaction is slow or the drugs take a long time to penetrate the erythrocyte. This latter proposal is improbable as both compounds are highly lipid soluble and should easily pass through cell membranes. Alternatively, if ALA-D activity is indirectly reduced by CBZ treatment, it may be that it takes time for CBZ and CBZ-E to reduce the levels of some compound or protein essential for the maintenance of ALA-D activity. One possible example of this, glutathione, is investigated in 4.1.5. A further possibility is that some other metabolite of CBZ, present only at very low concentrations, or unstable, is the species responsible for the observed effect. If this was the case it would be very difficult to prove.

4.1.3 THE RELATIONSHIP BETWEEN CBZ AND ALA-D ACTIVITY

This section examines in greater detail the relationship established in section 3.1 between plasma CBZ concentration and ALA-D activity. There are several variables to be examined. In the case of CBZ these are total plasma levels of the drug, free plasma levels

of the drug (i.e. drug which is not protein bound) and the concentration of CBZ-E, the principle metabolite (there are many other metabolites but these are present in such small amounts it was not possible to detect them in the samples studied). These variables were plotted against circulating ALA-D and restored ALA-D activity and a correlation sought using linear regression analysis. The samples were obtained from epileptic patients receiving CBZ monotherapy. Free drug was determined using equilibrium dialysis (section 2.4.3) CBZ and CBZ-E were measured by an HPLC method (section 2.4.2).

The results consist of 3 sections (i) total CBZ (ii) free CBZ and (iii) CBZ-E. The numbers for each section varied and therefore to correctly evaluate if consideration of a particular variable improved the correlation coefficient obtained between total CBZ and ALA-D activity it was necessary to determine a correlation coefficient for the relationship between total CBZ levels and ALA-D activity for each group. All results are presented in table (10). When considering these results it is important to remember that a correlation between two variables does not always mean they are directly related: there may be some intermediary factor.

(i) Total CBZ and ALA-D activity

As was stated in section 3.1, a negative linear correlation was found to exist between total plasma CBZ and circulating ALA-D activity. When total CBZ was plotted against restored ALA-D activity for the same samples, the correlation coefficient improved from -0.45 to -0.51 ($n=47, P<0.001$). This is shown in fig. (19). As will be seen, better correlations were found when restored levels of ALA-D activity were considered in all groups studied.

TABLE (10)

CORRELATION COEFFICIENTS FOR PLOTS OF TOTAL CBZ, FREE CBZ AND CBZ-E AGAINST CIRCULATING AND RESTORED ALA-D ACTIVITY

	Circulating ALA-D activity	Restored ALA-D activity	Number of points
Total CBZ	- 0.4**	- 0.51***	47
Total CBZ	- 0.33	- 0.41 *	33
Free CBZ	- 0.26	- 0.29	33
Total CBZ	- 0.42**	- 0.50***	40
CBZ alone	- 0.51***	- 0.57***	40
CBZ-E alone	- 0.43**	- 0.52***	40

* P<0.05 ** P<0.01 *** P<0.001

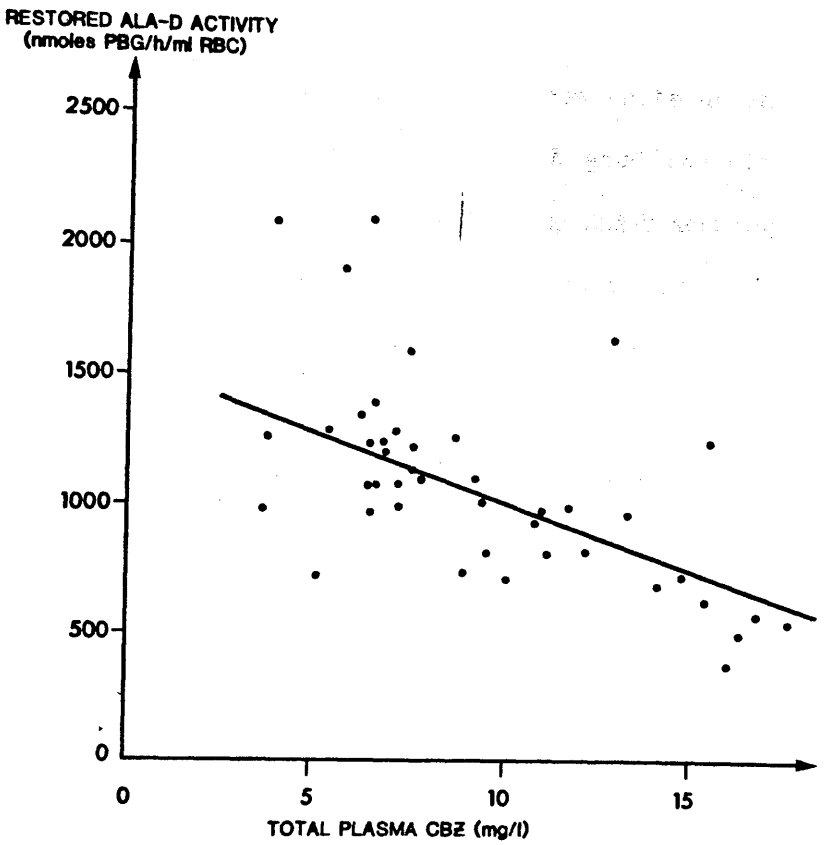


FIG. (19)

THE RELATIONSHIP BETWEEN RESTORED ERYTHROCYTE ALA-D ACTIVITIES AND PLASMA CBZ

The figure shows a significant relationship between restored erythrocyte ALA-D and plasma CBZ in samples from 47 epileptic patients receiving chronic CBZ monotherapy.

Linear regression correlation coefficient = -0.51 , $p<0.001$
Curvilinear regression correlation coefficient = -0.60, $p<0.001$

(ii) Free CBZ and ALA-D activity

Free CBZ concentrations were determined because it is the free fraction of the drug which is available for binding to receptors, diffusing into cells and producing its pharmacological effects as opposed to the remainder of the drug which is bound to serum proteins and is therefore unable to enter cells. A good correlation might be expected between free CBZ and ALA-D activity, but none was found. These results, however, are not conclusive because, in contrast to the other groups, there was not a significant correlation between total CBZ levels and circulating ALA-D activity and although there was a statistically significant correlation between total CBZ and restored activity, this was much weaker than that obtained in other groups ($P<0.05$ as opposed to $P<0.001$). The results therefore do not necessarily imply that there is not a correlation because the controls, which should have displayed significance, either failed to do so, or did not do so to the same extent that they ought. The reason that these results were not as significant as those obtained earlier is due to the fact that fewer data points were available and the scatter was greater, thus weakening the correlation coefficients. However, in the cases of both circulating and restored activity, consideration of the free fraction weakened the correlation coefficient obtained from total drug levels and therefore the free fraction is probably not of significance in the relationship between CBZ and ALA-D.

(iii) CBZ-E and ALA-D activity

This sub-section investigated the relationship between CBZ-E and ALA-D activity. This would be a likely candidate for the effects observed because most epoxides are chemically active. The original results were confirmed by the finding of a significant correlation between total CBZ values and both circulating and restored ALA-D activities (the correlation in the case of restored activity being the stronger) in the 40 subjects studied in this section. When total CBZ values were broken down into CBZ and CBZ-E concentrations (See final section of table (10)), significant negative linear correlations were found between both of these parameters and ALA-D activity in the circulating and restored states, (0.43 and-0.52 respectively for CBZ-E, $P<0.001$). For both CBZ and CBZ-E, the correlation was strongest when considering restored activities. This would be expected in the case of CBZ because it accounts for up to 90% of the total drug and in the case of CBZ-E because there is a very strong relationship between the concentrations this compound and total CBZ levels (see fig.16). However, in considering CBZ alone, there was a large increase in the correlation coefficient in both ALA-D states whereas the increase found with the epoxide was slight.

Conclusions about the relationship between CBZ and ALA-D activity

There was a negative linear relationship between plasma CBZ and both circulating and restored ALA-D activity. In each of the

situations studied the correlation was best when considering restored rather than circulating activity. The concentration of free drug is probably not of importance although this is uncertain for the reasons stated. Individually, CBZ and CBZ-E both appeared to be related to ALA-D activity, although CBZ seemed to be of greater importance. However, this is only to be expected because there was so much more CBZ present. The correlation found with the epoxide is of uncertain significance as it may merely be a result of the fact that epoxide concentrations are directly related to the total drug levels. Lastly, all of the correlations described are weakened by several factors the most important of which are (a) the large fluctuations in drug levels which occur throughout the day and (b) the large inter-individual variations in ALA-D activity. The principle point made in this sub-section is that a relationship does exist between the plasma concentration of CBZ and the activity of ALA-D in the erythrocytes.

4.1.4 THE EFFECTS OF CBZ TREATMENT ON THE APPARENT Km AND OPTIMUM ASSAY PH OF ERYTHROCYTE ALA-D

In the introduction of section 4.1, it was stated that there were two possible explanations for the mean reduction of approximately 40% in ALA-D activity: (a) total inactivation of 40% of the enzyme present (i.e. a reduced amount of normal enzyme is present) or (b) modification of all enzyme present such that the catalytic activity of each molecule is reduced by 40%. If (a) was the case then the Km and the pH optimum of the enzyme would be unaltered as activity present would come from unaffected enzyme. If however (b) was the case then the Km of the enzyme for its substrate would be altered and there is a possibility that the optimum assay pH of the

enzyme would also be changed (Chiba, Tashiro and Kikuchi, 1977; Wigfield and Farant, 1979; Farant and Wigfield, 1984). Measurement of these two parameters, therefore, should allow a distinction to be made between the two possibilities outlined above.

The results from each of these experiments are now presented.

(i) The Km of ALA-D in control and CBZ-treated erythrocytes

True Km values were not determined as this would have involved lengthy purifications of ALA-D from large volumes of blood from each of the individuals studied. Instead apparent Km values for ALA-D in whole blood were calculated by measuring the rate of reaction in the presence of different concentrations of substrate as described in section 2.7.2. Two groups were studied: (a) a group of 7 CBZ treated patients (4 males and 3 females; mean circulating ALA-D activity = 729 ± 369 nmol PBG/h/ml erythrocytes and (b) a group of 7 age and sex matched controls (mean circulating ALA-D activity = 1296 ± 372 nmol PBG/h/ml erythrocytes). The results are presented in table (11).

This method of determining Km values in whole blood is not as precise as a similar experiment using purified enzyme would be, hence the spread of results (control; 0.257-0.310mM: CBZ; 0.233-0.334mM). There was however no significant difference between the two groups, the mean values being absolutely identical. The mean Km value of 0.273mM obtained from this experiment compares excellently with the figure of 0.270mM reported by Anderson and Desnick (1979) for purified erythrocyte ALA-D.

TABLE (11)

APPARENT Km VALUES FOR ERYTHROCYTE ALA-D IN CONTROLS AND CBZ-TREATED PATIENTS

Sample No.	Control	CBZ-treated
1	0.257	0.272
2	0.318	0.300
3	0.238	0.310
4	0.252	0.287
5	0.281	0.257
6	0.334	0.285
7	0.233	0.203
Mean ± 1 S.D.	0.273 ± 0.039	0.273 ± 0.036

The values in the table are ALA concentrations in mmoles/l

(ii) The pH optimum of ALA-D in control and CBZ-treated erythrocytes

A range of buffers of varying pH was prepared as described in section 2.7.3 and ALA-D activity was determined in the usual way. Two groups were studied: (a) a group of 6 CBZ treated patients (mean circulating ALA-D activity = 669 ± 225 nmol PBG/h/ml erythrocytes) and (b) a group of 6 age and sex-matched controls (mean circulating ALA-D activity = 1251 ± 243 nmol PBG/h/ml erythrocytes). Fig (14) in sub-section 2.7.3 shows a typical pH activity profile and the manner in which the optimum assay pH was determined. The results are presented in table (12). Note that the pH values quoted are the final pH values in the assay, not the pH of the buffer solutions used.

A spread of values was found in each case, but there was no statistically significant difference in the optimum assay pH between the two groups. The mean figure of pH6.5 compares excellently with the results of Farant and Wigfield, (1984).

Conclusions about the effects of CBZ treatment on the Km and optimum assay pH of erythrocyte ALA-D

The mean Km and optimum assay pH of ALA-D in CBZ-treated subjects showed no differences with respect to controls and the mean values calculated for each of these parameters was in good agreement with the literature. This suggests that option (a) as discussed in the introduction to this sub-section is the most valid, namely that ALA-D activity is depressed because there is a reduced amount of normal enzyme present.

TABLE (12)

OPTIMUM ASSAY PH VALUES FOR ERYTHROCYTE ALA-D ACTIVITY IN CONTROLS AND IN CBZ-TREATED PATIENTS

Sample No.	CONTROL	CBZ
1	6.54	6.42
2	6.44	6.58
3	6.53	6.42
4	6.54	6.51
5	6.46	6.55
6	6.51	6.35
Mean ± 1 S.D.	6.50 ± 0.04	6.43 ± 0.09

The difference in the mean optimum assay pH in the two groups is not significant.

4.1.5 THE EFFECTS OF CBZ TREATMENT ON ERYTHROCYTE GLUTATHIONE

In the preceding sub-sections it has been established that ALA-D activity is irreversibly reduced during CBZ treatment and that the depression of activity is related to the plasma concentration of the drug. As was stated in the introductory chapter, ALA-D contains a number of reduced thiol groups, the oxidation of which results in a loss of enzyme activity. These groups are normally maintained, at least in a large percentage of ALA-D molecules, in a reduced state by reduced glutathione (GSH), a tripeptide found in large quantities in erythrocytes responsible for maintaining the cysteine residues in cellular proteins in a reduced state. The amino acid sequence of GSH is shown below

N-glutamylcysteinylglycine

GSH itself contains a thiol group and can become oxidized resulting in the union of two GSH molecules through a disulphide bond to produce the oxidized form, GSSG. This can be converted back to GSH by the action of glutathione reductase (GR). If CBZ or one of its metabolites caused a loss of ALA-D activity through indiscriminate reaction with essential thiol groups, it should also reduce the erythrocyte concentration of GSH and consequently the concentration of GSSG. CBZ-E is a likely candidate for such a reaction due to the reactive nature of the epoxide moiety. This sub-section examines therefore the effect of CBZ on total erythrocyte glutathione (i.e. GSH + GSSG).

Total glutathione levels were determined in blood samples from the four groups of subjects shown:

(i) a group of 10 CBZ-treated patients

(ii) a group of 10 control subjects, age and sex attached with the above

(iii) a group of 9 VPA treated patients

(iv) a group of 10 DPH treated patients

Interest was primarily directed at the first two groups to find out if glutathione levels were affected by CBZ. The VPA and DPH treated groups were included for completeness. The method used to measure glutathione was as described in section 2.10.1. The results of the study are presented in table (13). The mean ALA-D activities for each group are also shown.

No significant differences were found between the CBZ-treated group and controls, even although ALA-D activities varied almost two-fold, nor was there any correlation between CBZ levels and blood glutathione concentrations. Thus CBZ treatment does not affect the intracellular GSH concentration. This implies two things: (a) neither CBZ nor any of its metabolites react indiscriminately with thiol groups and (b) ALA-D activity is not reduced due to a deficiency of intracellular GSH. These results however presume that GSH-CBZ or GSH-CBZ metabolite complexes (the proposed products of reaction of CBZ or a metabolite with GSH in the hypothesis put forward at the start of this section) are not substrates for GR. The assumption is probably valid because such a structure is quite different to that of GSSG, glutathione being small and highly polar whereas CBZ is bulky and hydrophobic. These differences would adversely affect binding to the reductase. Also other adducts of GSH and drugs e.g. acetaminophen, are not metabolized by GR and do not interfere with the assay (Adams, Lauterburg and Mitchell, 1983).

TABLE (13)

ERYTHROCYTE ALA-D ACTIVITIES AND BLOOD GLUTATHIONE CONCENTRATIONS IN CONTROLS AND IN CBZ, VPA AND DPH-TREATED PATIENTS

	BLOOD GLUTATHIONE[a]	ERYTHROCYTE ALA-D ACTIVITY[b]
CONTROLS n=10	1.15 ± 0.21	1248 ± 198
CBZ n=10	1.10 ± 0.12	762 ± 402*
VPA n=9	1.11 ± 0.11	1260 ± 354
DPH n=10	1.12 ± 0.19	1236 ± 363

* $P<0.001$ as compared to controls

Values presented are mean ± 1 S.D.

a units are mmoles glutathione/l blood

b units are mmoles PBG produced/h/ml erythrocytes

As expected no significant changes with respect to controls were found with the VPA and DPH treated groups, nor was there any correlation between drug levels and blood gluthatione concentrations.

4.1.6 BLOOD LEAD CONCENTRATIONS IN CBZ-TREATED PATIENTS

Erythrocyte ALA-D is susceptible to inhibition by lead in the blood. Due to the number of lead water pipes in the Glasgow area, many individuals have sufficiently high blood levels to significantly reduce the activity of ALA-D. Since all of the patients studied live in this area, it was therefore necessary to check their blood lead levels to affirm that the observed depression of ALA-D activity was due to CBZ treatment and not to a higher blood lead concentration than the other groups studied. Blood lead concentrations were therefore determined in 24 controls and 28 CBZ treated patients by the method described in section 2.11.3. ALA-D activities for the two groups were also measured. The results are summarised in table (14).

The results show that mean lead concentrations were not higher in the CBZ treated group than they were in control group, even although ALA-D activities in the former were greatly reduced. Indeed the mean blood lead concentration of the CBZ treated group was actually lower than the mean control value. Thus the observed reduction of ALA-D activity in these patients is not due to lead inhibition of the enzyme.

TABLE (14)

ERYTHROCYTE ALA-D ACTIVITIES AND BLOOD LEAD LEVELS

IN CONTROLS AND IN CBZ-TREATED PATIENTS

	CONTROL n=24	CBZ-TREATED n=28
ALA-D[a]	1230 ± 231	618 ± 276*
Blood lead[b]	0.45 ± 0.21	0.38 ± 0.25

* $P<0.001$

Values presented are mean ± 1 S.D.

a units are nmoles PBG produced/h/ml erythrocytes

b units are μmoles/l

4.1.7 SUMMARY AND GENERAL DISCUSSION OF SECTION 4.1

This section has investigated several aspects of the depression of ALA-D activity which occurs during treatment with CBZ. A summary and a discussion on the implications of the results now follows.

Chapter 3 established that CBZ, or a metabolite thereof, reduced ALA-D activity. In contrast to lead inhibition, activity could not be restored by treating the enzyme with activators nor was the loss of activity reversed by dialysis. Thus the effect is irreversible and possibly due to a covalent interaction between the inhibitory substance and the enzyme. The depression in activity takes some time to become manifest: no changes were observed after two days treatment, but effects were detected after treatment for 1 week. This indicates one of three things: (a) the proposed inhibitory substance reacts slowly with ALA-D; (b) it takes time for the proposed inhibitor to accumulate to levels which significantly affect ALA-D; (c) it takes time for some compound essential for the maintenance of ALA-D activity to be depleted to an extent at which activity would be adversely affected. In the case of the latter suggestion, glutathione, a likely candidate, was not affected. A negative linear correlation was found between total CBZ (as determined by EMIT) and both circulating and restored ALA-D activities. This correlation was not improved by consideration of the concentration of free CBZ, but both CBZ alone and CBZ-E showed an improved correlation with ALA-D activity with respect to those obtained using total CBZ levels. In all cases considered, the correlation of drug concentration with restored ALA-D activity was much better than that obtained with values for circulating activities. No changes were found in the apparent Km or

pH optimum of ALA-D from subjects with greatly reduced activities indicating that the low activity observed was due to a reduced amount of normal enzyme rather than a normal level of modified enzyme, i.e. the effect is all or nothing. The reduction of ALA-D activity in this group was not due to a higher than normal blood lead level.

Throughout this section it has been assumed that ALA-D activity was reduced due to enzyme inhibition, modification or destruction. However, none of the experiments described exclude the possibility of activity being depressed as a consequence of a reduction in the rate of enzyme synthesis. Indeed this could explain several aspects of the effect, for example, why it takes so long to occur. The synthesis of ALA-D could only be affected in actively transcribing cells, in this case, the erythroid cells. It would take some time before the affected cells matured and made up a significant part of the erythrocyte population. However, as was stated in the introductory section, ALA-D synthesis has not been shown to be inducible or repressible. Furthermore, results presented later in this thesis confirm that the assumptions made here, namely, that CBZ or a metabolite are the causative factors of the reduction in ALA-D activity and that the effect was not due to a reduction in synthesis, are valid. The results from this section do not confirm if activity was depressed as a consequence of a direct or indirect effect of CBZ treatment. This is explored further in later sections.

In the majority of patients, ALA-D activity was not depressed to an extent where it presented a serious threat to the flow of intermediates through the pathway, although it did cause some perturbations of the ALA/PBG relationship (see 3.3.2). Although the

mean activity of the CBZ treated group was 61% of that of controls, 26% of these individuals had activities less than 30% of mean control values and 3 subjects had activities less than 15% of mean control activities. If ALA-D activity was already impaired by, for example, a high blood lead concentration or high alcohol intake, further depression of ALA-D activity by treatment with CBZ of the magnitude observed in these individuals may produce a significant impairment in the flow of intermediates through the pathway. This would lead to further derepression of ALA-S with consequent over production of ALA and the potential clinical problems associated with this. This is discussed further at the end of this chapter.

4.2 CARBAMAZEPINE AND PORPHOBILINOGEN DEAMINASE

In chapter 3 it was shown that long-term treatment with CBZ resulted in a depression of PBG-D activity, the extent of which was related to CBZ concentration (see fig. (15)). This confirmed the findings of both Rapeport et al, (1984) and Yeung et al, (1983), but was in conflict with those of Doss and Schafer, (1984) and Rideout et al, (1983). Both of these groups obtained their results with an assay which utilized PBG as a substrate whereas the results from this study and those of Rapeport et al, (1984) and Yeung et al, (1983) used ALA as substrate and depended on ALA-D to act as a coupling enzyme, converting ALA to PBG. In support of the data presented in the previous section, both Doss and Schafer, (1984) and Rideout et al, (1983) noted that ALA-D activity was depressed by CBZ. Both suggested that since the assay used by Yeung et al, (1983) depended on ALA-D to produce PBG, reduced activity of this enzyme would mean insufficient substrate was produced for the PBG-D assay and consequently an artificially low result would be obtained. This would therefore also apply to the results of Rapeport et al, (1984) and those presented in Chapter 3. This may seem unlikely when the relative activities of these two enzymes in peripheral blood are considered (from the controls in chapter 3, mean ALA-D activity was 1224 nmolPBG/h/ml erythrocytes and the mean PBG-D activity was 39.8 nmolURO/h/ml erythrocytes). Even in the case of CBZ treated patients there is still an apparent excess of ALA-D activity. However when activities are expressed in ALA equivalents, the relative activities of ALA-D and PBG-D become 2448 nmoles ALA/h/ml erythrocytes (in the case of CBZ,1494 nmoles ALA/h/ml erythrocytes) and 318[illegible] nmoles

ALA/h/ml erythrocytes respectively. A further point to consider when comparing relative activities of the two enzymes is that the aforementioned ALA-D values were measured at an assay pH of 6.95, but the coupled assay system for determination of PBG-D activity operated at pH 7.5. Using the results from the study described in section 4.1.4, it was calculated that ALA-D activity at this pH was reduced by a mean value of 34.0 ± 2.9% with respect to activity at pH 6.95. Taking this into account further reduces the mean relative activities of ALA-D and PBG-D to 1616 nmoles ALA/h/ml erythrocytes (984 nmoles ALA/h/ml erythrocytes in the case of CBZ) and 318.4 nmoles ALA/h/ml erythrocytes respectively. Thus there may not be as large an excess of ALA-D as appears at first sight. In support of the proposal put forward by Doss and Shafer, (1984) and Rideout et al (1983), plotting PBG-D results from preceding chapters (determined by the URO-1-S assay) against ALA-D activities in the same subjects (24 controls, 40 CBZ, 12 VPA and 8 DPH) demonstrates that the two parameters are, in fact related, fig. (20).

The objective of this section was to ascertain whether or not CBZ affects PBG-D activity and if so, why. Two methods of determining activity were used: (a) utilizing ALA as substrate and (b) utilizing PBG as substrate. To differentiate between the two methods, results from the first will be termed URO-1-S activity whereas those from the second will be referred to as PBG-D activity. The methodologies are described in section 2.2.3.

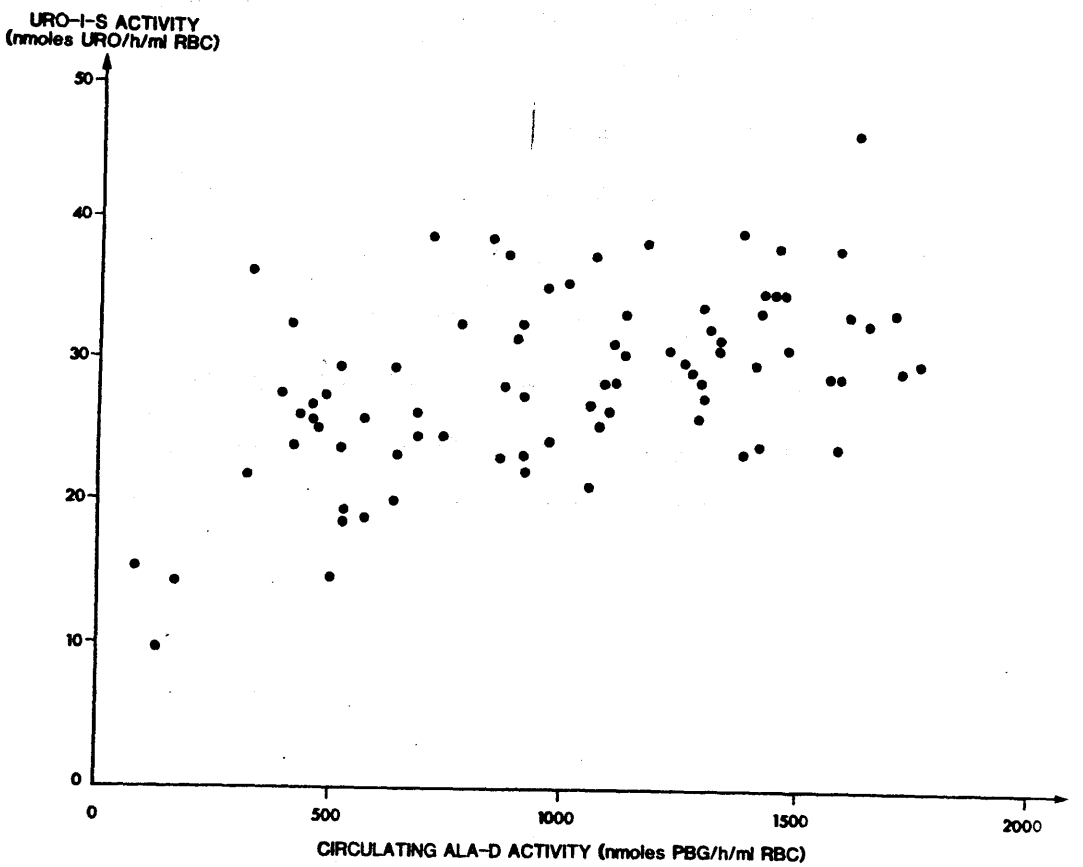


FIG. (20)

THE RELATIONSHIP BETWEEN ALA-D AND PBG-D (AS DETERMINED BY THE URO-I-S METHOD) ACTIVITIES IN HUMAN ERYTHROCYTES

The graph shows a significant relationship between circulating levels of erythrocyte ALA-D and PBG-D activity (determined by the URO-1-S method) in 84 subjects when analysed using either linear or curvilinear regression. This relationship may exist because the URO-1-S assay relies on ALA-D present in the erythrocytes to act as a coupling enzyme to generate PBG - the substrate for the assay

Linear regression correleation coefficient = 0.56, $P<0.001$
Curvilinear regression correlation coefficient = 0.68, $P<0.001$

4.2.1 THE EFFECTS OF CBZ TREATMENT ON ERYTHROCYTE URO-1-S AND PBG-D ACTIVITIES

As stated in the foregoing introduction PBG-D activity was measured by two methods, one using ALA as substrate (URO-1-S assay) and the other utilizing PBG as substrate (PBG-D assay). Enzyme activity was determined by both methods in the 4 groups of subjects listed below:

(i) a group of 43 CBZ treated patients

(ii) a group of 23 controls age and sex-matched with (i)

(iii) a group of 12 VPA treated patients

(iv) a group of 8 DPH treated patients

The results are presented in table (15).

The results show that, as expected, when the URO-1-S method was used, mean CBZ activities were 84% of mean control activities, but VPA and DPH treated subjects did not differ significantly from controls. However, when PBG-D activities were determined (using PBG as substrate), none of the groups studied, including CBZ, showed any significant differences with respect to controls. The mean activities for each group were all very close. Mean PBG-D activities were 90.1%, 90.1% and 91.0% of mean URO-1-S activities for the control, VPA treated and DPH treated groups respectively, but were 117.4% for the CBZ treated group. This indicates that the relationship between these two activities was upset by CBZ treatment.

Conclusions on the effects of CBZ-treatment on erythrocyte URO-1-S and PBG-D Activities

CBZ treatment caused a reduction in URO-1-S activity with respect to controls, but did not affect PBG-D activity. Whether or not this difference is attributable to the effect of CBZ treatment on ALA-D is investigated in the following sub-sections.

TABLE (15)

COMPARISON OF TWO METHODS, URO-1-S AND PBG-D, FOR THE DETERMINATION OF ERYTHROCYTE PBG-D ACTIVITY IN CONTROLS AND IN CBZ, VPA AND DPH TREATED PATIENTS

	URO-1-S ACTIVITY[a]	PBG-D ACTIVITY[a]	PBG-D ACTIVITY AS % OF URO-1-S ACTIVITY
CONTROL n=23	30.2 ± 5.0	27.3 ± 3.2	90.1 ± 9.8
CBZ n=43	25.4 ± 6.6*	28.8 ± 5.5	117.4 ± 41.8*
VPA n=12	30.4 ± 4.9	27.2 ± 3.6	90.1 ± 7.1
DPH n=8	32.6 ± 8.2	29.3 ± 5.8	91.0 ± 7.5

* $P<0.002$ as compared to controls

Values presented are mean ± 1 S.D.

a units are nmoles URO produced/h/ml erythrocytes

4.2.2 THE RELATIONSHIP BETWEEN APPARENT URO-1-S AND ALA-D ACTIVITIES DURING CBZ TREATMENT

The preceding sub-section established that URO-1-S activity appeared to be reduced during CBZ treatment whereas PBG-D was unaffected. This sub-section investigates the possibility of a relationship between URO-1-S activity and reduced ALA-D activity. When considering this relationship, values for restored ALA-D activity (whole blood treated with $ZnCl_2$ and DTT as described in section 2.2.2.) were used. This was because when ALA-D was used as a coupling enzyme in the URO-1-S assay, it was activated prior to assay by pre-incubation with zinc sulphate and DTT.

For each of the samples in table (15), the ratio of URO-1-S activity to PBG-D activity was plotted against ALA-D activity (n=75, as restored activities were not available for all samples). The resultant graph is shown in fig. (21), and demonstrates that the two parameters are in fact related. As restored ALA-D activity increases the ratio of URO-1-S activity to PBG-D activity approaches a constant value. The mean ratio of URO-1-S activity to PBG-D activity was 1.10 ± 0.13 (n=23), 1.11 ± 0.09 (n=12) and 1.10 ± 0.09 (n=8) for control, VPA treated and DPH treated groups respectively but 0.90 ± 0.22 (n=43) for CBZ (not all of these are plotted for reasons just described). Control, VPA and DPH values fall in the upper horizontal part of the graph, where the ratio of URO-1-S to PBG is independent of restored ALA-D activity. CBZ values fall in the lower, left hand side of the graph where the ratio appears to be dependent on restored ALA-D activity. The cut-off point appears to be a restored ALA-D activity of 1500 nmol PBG/h/ml erythrocytes.

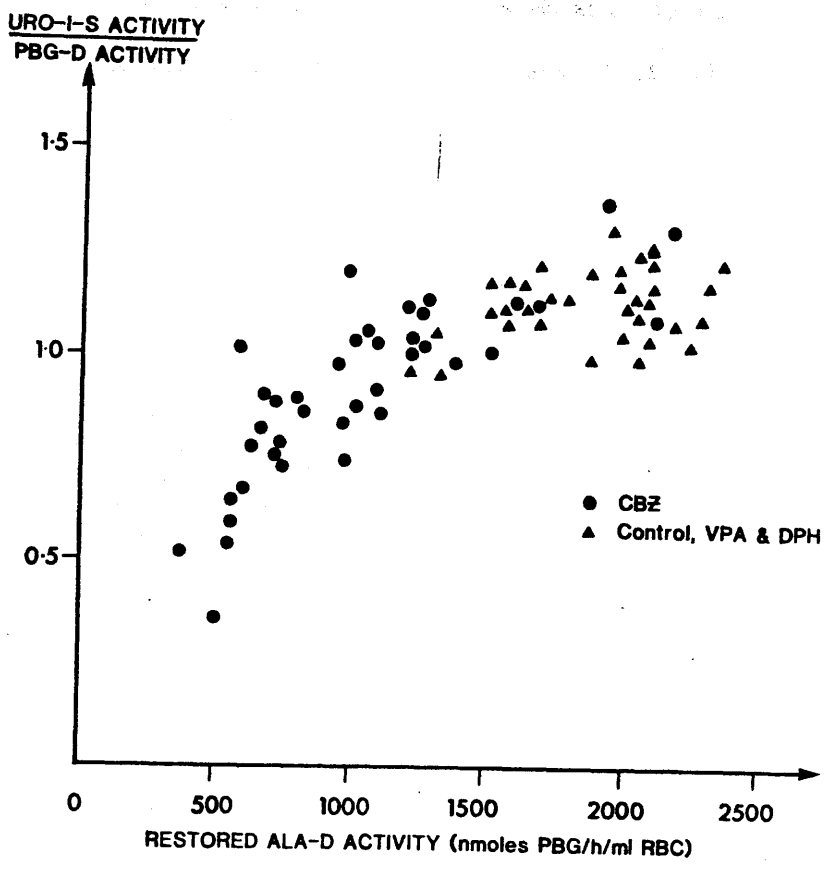


FIG. (21)

THE RELATIONSHIP BETWEEN RESTORED ERYTHROCYTE ALA-D ACTIVITY AND PBG-D ACTIVITY DETERMINED BY TWO DIFFERENT METHODS

PBG-D activities were determined by two different methods: utilizing PBG as substrate (PBG-D assay) or using ALA as pre-substrate (URO-1-S assay). The ratio of these measurements was then plotted against the restored ALA-D activities of the samples. This ratio was independent of restored ALA-D activities in controls, sodium valproate (VPA) and phenytoin (DPH) treated patients. However, below restored ALA-D activities of 1500 nmoles PBG/h/ml RBC (which includes most CBZ treated patients), the ratio of PBG-D to URO-1-S was related to restored ALA-D activity. This proves that in the majority of CBZ treated patients, determination of PBG-D activities by the URO-1-S method does not give reliable results.

Below this level of activity, URO-1-S results seemed to be lower than they actually were as evidenced by the departure from the mean control ratio of URO-1-S/PBG-D activity of 1.10. Thus if there is not a minimum restored ALA-D activity of 1500 nmolPBG/h/ml erythrocytes, an artificially low URO-1-S activity will result. This is not a problem for normal patients as 96.5% of all controls studied had restored ALA-D activities in excess of this. In contrast, only 10% of the CBZ treated patients studied had restored activities greater than 1500 nmolPBG/h/ml erythrocytes. This means that for the CBZ treated group, 90% were giving a falsely low URO-1-S result, the extent of the deviation from the true activity being dependent on the extent of reduction in ALA-D activity.

Conclusions on the relationship between apparent URO-1-S and restored ALA-D activities

The activity of URO-1-S was dependent on the level of restored ALA-D activity up to 1500 nmol PBG/h/ml erythrocytes above which it became independent. This was shown by examining the ratio of URO-1-S and PBG-D activity, the latter being entirely independent of ALA-D activity. Almost all control subjects studied had restored ALA-D activities exceeding this minimum value and therefore the assay would normally give true results. This is so even for lead-poisoned individuals because although the circulating ALA-D activities are low in these subjects, treatment with activators results in a higher than normal level of restored activity. In the case of CBZ however, almost all of the patients studied had a falsely low URO-1-S activity, apparently due to the irreversibly reduced levels of restored ALA-D activity.

This sub-section has shown that URO-1-S activity is, up to a point, related to restored ALA-D activity. The results, however, do not prove that the apparent reduction in URO-1-S activity is a direct result of a low restored ALA-D activity in the sample. This is investigated in the following sub-section.

4.2.3 THE EFFECTS OF SUPPLEMENTARY ALA-D ACTIVITY ON ERYTHROCYTE URO-1-S ACTIVITY

If, as suggested, URO-1-S activities in CBZ treated patients are low as a direct consequence of reduced ALA-D activity, increasing the ALA-D activity in these samples should produce an increase in URO-1-S activity. This was done by adding purified ALA-D to blood samples from both CBZ-treated patients and controls as described in section 2.2.3. Two experiments were performed:

(i) investigated the effects of adding a sigle, measured amount of ALA-D to a set of controls and CBZ-treated patients with a wide range of ALA-D activities

(ii) investigated the effects of adding increasing amounts of purified ALA-D to a single control blood sample and a single CBZ treated blood sample.

(i) The effects of adding a constant amount of ALA-D activity on erythrocyte URO-1-S activities

11 CBZ-treated patients and 7 controls were studied. URO-1-S, PBG-D, circulating and restored ALA-D activities, and URO-1-S activity supplemented with 6.8×10^{-4} units of ALA-D (as described in section 2.2.3) were measured in each of the subjects. The results are summarised in table (16).

TABLE (16)

THE EFFECTS OF ADDING SUPPLEMENTARY ALA-D ON ERYTHROCYTE URO-1-S ACTIVITY IN WHOLE BLOOD FROM CONTROLS AND CBZ-TREATED PATIENTS

	URO-1-S ACTIVITY[a]	PBG-D ACTIVITY[a]	URO-1-S + ALA-D ACTIVITY	INCREASE IN URO-1-S FOLLOWING ADDITION OF ALA-D[a]	ALA-D ACTIVITY[b]	RESTORED ALA-D ACTIVITY[b]
CONTROL n=7	32.8 ± 4.1	28.4 ± 2.9	38.1 ± 4.4	5.3 ± 1.3	1182 ± 243	1842 ± 210
CBZ n=11	24.9 ± 4.6*	29.0 ± 4.8	35.0 ± 4.6	10.1 ± 3.5*	525 ± 246**	912 ± 321**

* $P<0.002$, ** $P<0.001$ as compared to controls

Values presented are mean ± 1 S.D.

a units are nmoles URO produced/h/ml erythrocytes

b units are nmoles PBG produced/h/ml erythrocytes

There was, as expected, a significant difference in URO-1-S activity between the controls and CBZ-treated patients but no difference in PBG-D activities between the two groups. Mean ALA-D activities of the CBZ group were 44.4% and 49.5% of the mean circulating and restored control activities respectively. When ALA-D activity was supplemented by the addition of purified enzyme, URO-1-S activities increased in both groups, but the increase was greater in the case of the CBZ treated group, the mean increase in activity being 10.1nmol/hr/ml erythrocytes as opposed to 5.3 nmol/hr/ml erythrocytes. This was reflected by mean CBZ-treated URO-1-S activities rising from 75.9% of control URO-1-S activites to 91.9% of control values following addition of ALA-D. There was a relationship between the initial level of ALA-D activity and the increase in URO-1-S activity, fig. (22). The lower the initial activity of ALA-D, the greater was the increase following addition of purified ALA-D. However, the increase in URO-1-S activity became constant above an initial restored ALA-D activity of 1500 nmolPBG/h/ml erythrocytes. This finding confirmed the results found in the previous subsection: addition of supplementary ALA-D only affected activity to a greater extent than controls if the initial level of restored activity was less than 1500 nmolPBG/h/ml erythrocytes. The reasons for the increase in URO-1-S activity in controls following addition of ALA-D will be discussed in the second part of this subsection.

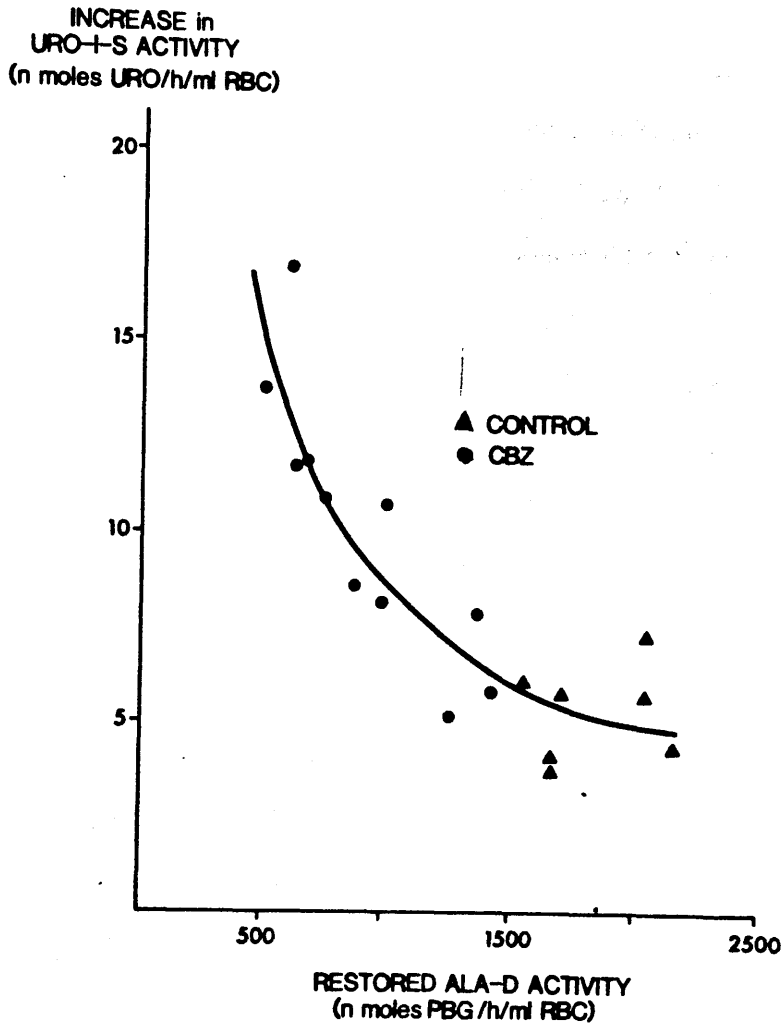


FIG. (22)

THE EFFECTS OF SUPPLEMENTARY ALA-D ON ERYTHROCYTE URO-1-S ACTIVITY: RELATIONSHIP TO INITIAL RESTORED ERYTHROCYTE ALA-D ACTIVITY

The figure shows that the extent of the increase in URO-1-S activity following the addition of a fixed quantity of ALA-D to the assay was related to the initial restored ALA-D activity up to a level of 1500 nmoles PBG/h/ml RBC. Thereafter the increase was rapidly becoming independent of ALA-D and attributable to contaminating PBG-D in the purified ALA-D as shown in fig. (23). This proves that URO-1-S activities appear low due to a deficiency of ALA-D.

Geometric regression correlation coefficient = 0.88, $P<0.001$, $(n=18)$

(ii) The effects of adding increasing amounts of ALA-D activity on erythrocyte URO-1-S activity

Increasing amounts of purified ALA-D were added to a series of URO-1-S assays in blood from 1 control subject (circulating and restored ALA-D activities were 1290 and 1605nmol PBG/h/ml erythrocytes respectively) and from 1 CBZ treated subject (circulating and restored ALA-D activities were 33 and 516 nmolPBG/h/ml erythrocytes respectively). The number of units added and the effects of each addition on erythrocyte URO-1-S activity are shown in table (17). Fig. (23) presents these results in graphic form by plotting the magnitude of the increase over the initial activity against the number of units added. This reveals that in the control, there is a linear relationship between the increase in URO-1-S activity and the number of units added i.e. for each unit added there is a constant increase in URO-1-S activity. This was not the case with the CBZ treated subject. In this patient the initial additions produced very large increases in URO-1-S activity. These increments became smaller as ALA-D activity was increased and from the figure, would appear to approximate the rate of increase in URO-1-S activity observed with the control after 6.8×10^{-4} units were added, at which time the restored ALA-D activity of the sample would have been 1236 nmolPBG/h/ml erythrocytes. This is not too far removed from the figure of 1500 nmolPBG/h/ml erythrocytes calculated as the minimal ALA-D activity required for URO-1-S activity to be independent of ALA-D activity. It is clear from the graph that this occurs because the slope approximates the slope of the control curve which is independent of ALA-D

TABLE (17)

THE EFFECTS OF ADDING INCREASING AMOUNTS OF ALA-D TO URO-1-S ACTIVITY IN WHOLE BLOOD FROM A CONTROL SUBJECT AND FROM A CBZ TREATED PATIENT

	UNITS OF ALA-D ADDED	URO-1-S ACTIVITY[a]	INCREASE IN ACTIVITY[a]
CONTROL			
1	0	27.8	0
2	1.4×10^{-4}	28.6	0.8
3	3.4×10^{-4}	29.7	1.9
4	6.8×10^{-4}	31.6	3.8
5	10.2×10^{-4}	34.1	6.3
6	13.6×10^{-4}	35.3	7.5
CBZ			
1	0	17.3	0
2	1.4×10^{-4}	23.1	5.8
3	3.4×10^{-4}	27.1	9.8
4	6.8×10^{-4}	31.0	13.7
5	10.2×10^{-4}	32.9	15.6
6	13.6×10^{-4}	34.6	17.3

a units are nmoles URO produced/h/ml erythrocytes

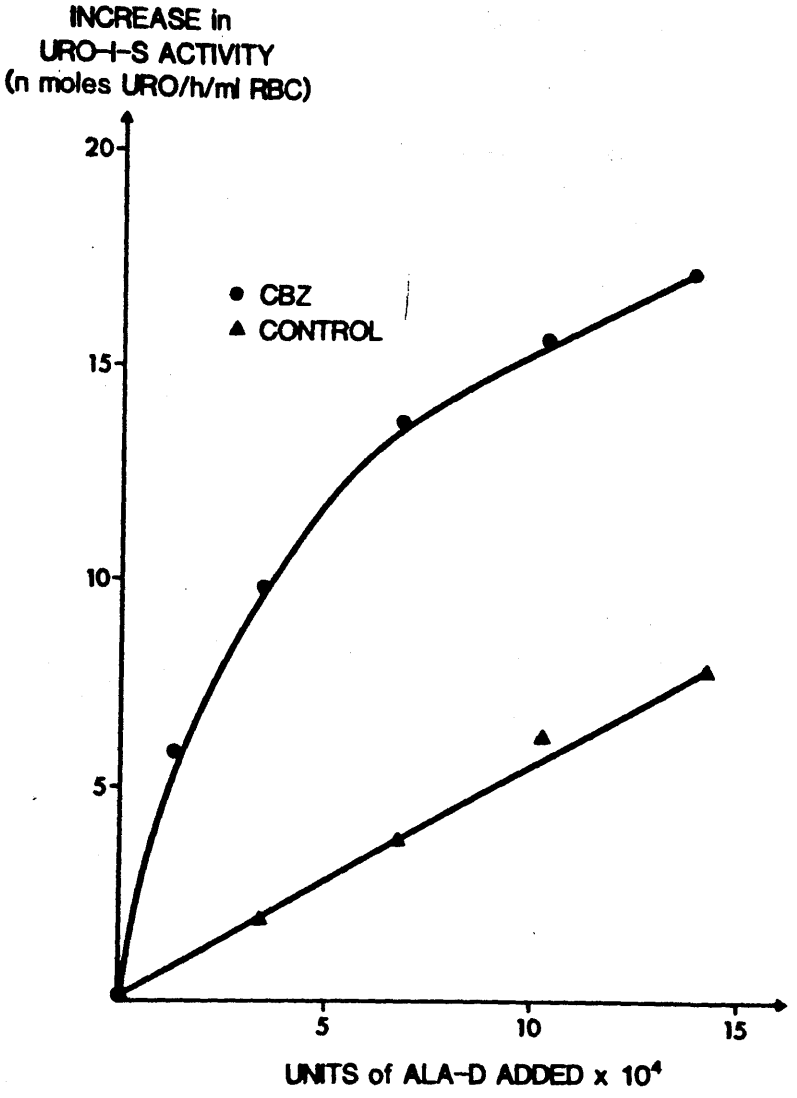


FIG. (23)

THE EFFECTS OF SUPPLEMENTARY ALA-D ON ERYTHROCYTE URO-1-S ACTIVITY IN A CONTROL SUBJECT AND IN A CBZ TREATED PATIENT

The figure shows the effects of supplementary purified ALA-D on erythrocyte URO-1-S activity in a control and in a CBZ treated patient. In the control subject, supplementary ALA-D caused a linear increase in URO-1-S activity per unit added. This was due to the presence of some contaminating PBG-D in the preparation. In the CBZ treated subject the initial increases were much greater, but after 6.8×10^{-4} units had been added, the rate of increase became the same as in the control, i.e. ALA-D had ceased to be rate-limiting. At this point, the total ALA-D activity in the assay was equivalent to 1236 nmoles PBG/h/ml RBC, which compares excellently with the results from figs. (19) and (22).

activity, having a restored ALA-D activity of 1605 nmolPBG/h/ml erythrocytes. After the largest addition of ALA-D, the URO-1-S activity in the CBZ sample was 98% of the URO-1-S activity in the control sample compared to 62% of the activity in the control sample before any additions had been made. It seems therefore that the addition of supplementary ALA-D corrects falsely reduced URO-1-S activities implying that URO-1-S activity was indeed low as a direct consequence of a low ALA-D activity.

The reason for the increase in control URO-1-S activity following the addition of purified ALA_D observed both here and in the first part of this subsection is uncertain, but the fact that it is constant for each unit of ALA-D added and does not appear to reach a plateau suggests that the purified ALA-D used contains some URO-1-S activity. However as described in section 2.2.3, purified ALA-D was checked for URO-1-S activity and although some activity was found, the levels were very low. According to the figures obtained, the largest amount of ALA-D added would only have increased the average URO-1-S activity by 1.2 nmol/h/ml erythrocytes. This could alternatively be expressed as an increase of 882 nmol/h/ml erythrocytes per unit of ALA-D activity added. This falls far short of the mean increase of 5750 nmol/h/ml erythrocytes per unit of ALA-D activity added which was found in the controls. One possible explanation for this discrepancy is that the URO-1-S activity determined in the purified ALA-D was reduced because the enzyme was unstable in the very dilute protein solution in which URO-1-S activity was measured. The activity would not be affected in

this way in assays of whole blood since this contains sufficient protein to stabilize the enzyme.

Conclusions on the effects of supplementary ALA-D activity on erythrocyte URO-1-S activity

These experiments have shown that PBG-D activity (when measured by the method designated URO-1-S) is reduced in patients taking CBZ as a direct consequence of the low levels of ALA-D activity produced by this drug. This was proven by showing that addition of supplementary ALA-D activity produced increases in PBG-D activity determined by the URO-1-S method which were inversely related to the initial restored ALA-D activity i.e. the lower ALA-D activity was, the greater was the increase in URO-1-S activity following addition of ALA-D. Furthermore, subsequent additions of ALA-D caused CBZ treated URO-1-S activities to approach the values obtained in control samples. Thus the apparent reduction in PBG-D activity was reversed by addition of ALA-D and was therefore due to the low ALA-D activities found in these patients.

4.2.4 SUMMARY AND GENERAL DISCUSSION OF SECTION 4.2

In this section, PBG-D activity was determined by two different methods in a series of blood samples from control and CBZ-treated subjects to investigate if the reduction in PBG-D activity which was found during CBZ treatment was a consequence of the reduction in ALA-D activity produced by CBZ in these patients. The two different methods used (a) ALA as substrate (URO-1-S assay) and (b) PBG as substrate (PBG-D assay). Using method (a), significant reductions in activity were found in CBZ treated patients compared to controls, but when method (b) was used to determine activity in the same samples, the results from the two groups were not significantly different.

Examination of the results demonstrated that there was a relationship between restored ALA-D activity and PBG-D activity as determined by the URO-1-S assay. Subsequent experiments revealed that this was directly attributable to the CBZ mediated depression of activity and that the effect could be reversed by the addition of supplementary purified ALA-D. In the case of CBZ treated patients, therefore, the rate of PBG synthesis was the factor governing the rate of URO production rather than the amount of PBG-D present, and consequently the activity measured did not reflect the true activity of this enzyme but the activity of restored ALA-D. This limitation only became effective at restored ALA-D activities of less than 1500 nmolPBG/h/ml erythrocytes and therefore would not affect the determination of PBG-D activity by the URO-1-S method in normal blood samples.

These results indicate that there is in fact no effect of CBZ on PBG-D activity during long-term treatment and also that the reductions in activity found by Rapeport et al, (1984) and Yeung et al, (1983) were artefacts. This shows that a misdiagnosis of AIP because of a reduced PBG-D activity is possible if this assay is used without first checking the restored level of ALA-D activity. It would be advisable when using this assay to screen for latent AIP to determine the restored levels of ALA-D whenever a positive result is obtained to check that ALA-D activity was sufficiently high to guarantee a satisfactory supply of PBG.

4.3 CARBAMAZEPINE AND URINARY PORPHYRIN EXCRETION

This section is concerned with the effects of CBZ treatment on the urinary porphyrin excretion profile. This profile defines the relative proportions of each of the 5 porphyrins, from URO through to COPRO, in a 24 hour urine collection as described in 2.3.4. The reason for investigating the effects of CBZ on this profile was that Rapeport *et al* (1984) reported that the profile was altered by CBZ treatment yet it was shown in sub-section 3.3.4 of this thesis that chronic CBZ therapy had no effect. This disparity was investigated in the first part of this section. In the second part the urinary porphyrin excretion profiles from a number of porphyric patients with acute intermittent porphyria (AIP) or porphyria cutanea tarda (PCT) were determined. Both of these conditions (described in detail in 1.3.10) produce characteristic profiles which differ greatly from normals. These were investigated to see if a statistically significant comparison could be made between either of these conditions and the changes reported by Rapeport *et al* (1984) following administration of CBZ.

Since this section is primarily devoted to explaining the findings of Rapeport *et al*, (1984), the methods used for determining the profile were exactly as described in that paper. Although this method gave poorer resolution of the individual porphyrins than the procedure outlined in 2.3.4, this was the only way in which the questions raised could be answered.

4.3.1 THE EFFECTS OF CBZ ON URINARY PORPHYRIN EXCRETION

Rapeport et al, (1984) reported large increases in the % URO in the urinary porphyrin excretion profiles of volunteer subjects who took CBZ, but it was shown in sub-section 3.3.4 of this thesis that in epileptic patients receiving chronic CBZ monotherapy, there was no change in the profile compared to controls. Slightly different methods were used. This raised the question: was the difference in the findings a consequence of the different methodologies or was there a temporary change in the urinary porphyrin excretion profile in the initial stages of CBZ treatment? To answer this question, urinary porphyrin excretion profiles were examined in four epileptic patients prior to starting CBZ therapy and at intervals thereafter. All of the patients began treatment on a dosage of 200mg CBZ b.d. This dosage was maintained in each of the patients except patient No.1, whose dose was increased to 200mg t.d. after six weeks. The results for all 4 patients are shown in table (18). Before starting on CBZ, the profile in each patient was identical to that found in controls (see table (19) for the mean results of 10 control urines) and consisted of 96% or more coproporphyrin (COPRO) with traces of the others. Of these remaining porphyrins, URO was the most significant, accounting for 1-4% of the total. After starting to take CBZ, there were changes in the profile. The % COPRO fell and the relative proportions of the other fractions, principally URO, increased. However, the extent and the timing of the maximal change from the baseline profile varied from patient to patient. Following this, 3 of the patients showed a return towards a normal excretion pattern. The return to normal in patient No.1 was slower, possibly due to his

TABLE (18)

THE EFFECTS OF CBZ TREATMENT ON THE URINARY PORPHYRIN EXCRETION PROFILE IN FOUR EPILEPTIC PATIENTS

PATIENT NO.	DAYS OF TREATMENT	% PORPHYRIN COMPOSITION				
		4-COOH	5-COOH	6-COOH	7-COOH	8-COOH
1	0	97	trace	-	-	2
	3	95	trace	trace	trace	3
	7	52	trace	1	21	26
	14	50	trace	-	trace	50
	28	60	2	1	10	27
	56	77	trace	trace	5	18
2	0	95	trace	trace	trace	4
	3	94	trace	-	trace	4
	7	29	8	5	trace	57
	14	81	trace	3	4	12
	28	90	trace	-	3	5
3	0	95	trace	-	-	4
	3	96	trace	-	1	2
	7	82	trace	trace	2	16
	14	83	trace	trace	10	7
	28	90	trace	2	2	5
4	0	100	-	-	-	-
	7	97	trace	-	trace	2
	14	52	3	trace	trace	44
	28	57	-	-	11	31
	56	96	trace	-	trace	2

The porphyrin composition was determined in aliquots of 24 hour urine collections from 4 epileptic patients prior to and after commencement of CBZ therapy (200mg b.d.). This dosage was maintained in all patients over the period of the collections except patient No.1 whose dose was increased to 200mg t.d. after six weeks.

increased dose. Thus CBZ treatment does cause a change in the profile, but the change is not permanent. An explanation for this would be that in the initial stages of treatment, very high levels of ALA-S activity were attained due to derepression of the enzyme. When ALA-S activities are so high, much more ALA than normal would be produced. This would be converted to PBG quite easily since ALA-D is present in excess. However PBG-D could not cope with the sudden increase in PBG and the excess would be excreted in the urine. This is likely because after ALA-S, PBG-D has the lowest endogenous activity in the pathway (Elder, 1982). The excess PBG in the urine could undergo non-enzymatic polymerisation and cyclization. When ALA-S activity falls, less PBG will be produced, PBG-D will be able to cope and consequently less PBG will be excreted in the urine. This would lead to a decrease in the quantities of URO detectable in the urine. Unfortunately urinary PBG estimations were not performed in these patients, and so the theory outlined has not been proven. There are however a number of facts to support it. The first of these is that in AIP, even in the latent phase, an abnormal urinary porphyrin profile is found, characterized by a large increase in the % URO (see 4.3.2). This change is brought about by a 50% decrease in PBG-D activity, even though ALA-S may only be slightly elevated. The situation here, where PBG-D is constant but ALA-S activity is greatly increased, should produce a similar result. A further fact supportive of these arguments is that in the paper of Rapeport et al (1984) as regards the effects of CBZ on haem biosynthesis, a slight (but not statistically significant) increase in PBG excretion was detected. Bearing these facts in mind, the proposed explanation seems quite valid.

Conclusions on the effects of CBZ on urinary porphyrin excretion

Treatment of individuals with CBZ produced changes in the urinary porphyrin excretion profile. In particular the % URO was increased. These changes however were not permanent and, despite continued treatment, the profile gradually returned to normal. There was no consistency in the extent or the timing of the changes. It seemed likely that the changes found in the profile were linked to the derepression of ALA-S.

4.3.2 CBZ AND URINARY PORPHYRIN EXCRETION: COMPARISONS WITH THE URINARY PORPHYRIN EXCRETION PROFILES IN PORPHYRIA

The aim of this section was to determine if the changes in the urinary porphyrin excretion profiles produced by CBZ treatment could be matched with the distinctive altered profiles found in the porphyrias. The only porphyric patients available in sufficient numbers to investigate this were 20 patients with acute intermittent porphyria (AIP) and 10 patients with porphyria cutanea tarda (PCT). All of these patients had previously been classified by thorough enzymatic investigation in the Porphyria Research Laboratory, Gardiner Institute, Western Infirmary, Glasgow. Those patients with AIP were divided into patients experiencing an acute attack of the disease (n=10) and those who were either latent or in remission. 10 control subjects were also studied. The results are presented in table (19). Unfortunately, as the results in the preceding sub-section show, the changes in the urinary porphyrin profile following CBZ treatment cannot be said to be representative of any of the conditions shown because the changes produced by CBZ were not consistent. However it could be argued that at certain time points after the initiation of

TABLE (19)

URINARY PORPHYRIN EXCRETION PROFILES IN CONTROLS AND IN PATIENTS SUFFERING FROM ACUTE INTERMITTENT PORPHYRIA (AIP) AND PORPHYRIA CUTANEA TARDA (PCT)

	% PORPHYRIN COMPOSITION					MEAN TOTAL PORPHYRIN EXCRETION (nmoles/ 24 hours)
	4-COOH	5-COOH	6-COOH	7-COOH	8-COOH	
CONTROL (n=10)	96.4 ± 1.9	trace	trace	trace	2.2 ± 1.5	75.6
AIP ATTACK (n=10)	38.2 ± 23.3	8.5 ± 7.6	5.8 ± 8.4	5.8 ± 9.0	41.7 ± 24.0	707.7
AIP LATENT OR IN REMISSION (n=10)	39.0 ± 21.8	6.4 ± 11.3	9.0 ± 9.6	10.1 ± 6.3	35.5 ± 13.1	57.9
PCT (n=10)	6.3 ± 4.5	3.6 ± 3.4	8.7 ± 8.5	25.6 ± 13.9	55.3 ± 23.4	1332

The analyses were performed on aliquots of urine from 24 hour urine collections. The designation of porphyria was based on thorough enzymatic characterization.

treatment in some individuals the profile could be confused with that found in AIP, but never with that found in PCT. This is important because of recent interest in screening for porphyria by the analysis of urinary porphyrin composition. The relevance of this is discussed in 4.3.3.

Conclusions

No real comparison can be made between the changes in the profile produced by CBZ treatment and those changes found in AIP and PCT. However at times the profile produced by CBZ in some individuals did resemble that found in AIP.

4.3.3 SUMMARY OF SECTION 4.3

This section investigated the effects of CBZ treatment on the urinary porphyrin excretion profile. Shortly after commencing treatment there was a major change in the profile, characterized by a large increase in the %URO. The extent of this change varied from individual to individual and in the time at which the maximal departure from normal was observed. The altered profile gradually returned to normal. These findings suggested that the changes in the excretion profile were a consequence of the very high levels of ALA-S activity found in the initial stages of treatment. This is of interest because it implies that any drug which causes derepression of ALA-S to the same degree as CBZ does, would result in an altered urinary porphyrin excretion profile. At times this altered profile may resemble the characteristic profiles of AIP and presumably those of other porphyrias (with the exception of PCT). This is of great importance, particularly in the light of recent attempts to screen for porphyria by HPLC analysis of urinary porphyrins (Doss, 1978;

Schreiber, Raisys and Labbe, 1983; Ostrowski et al, 1984; Francis and Smith, 1985), as it could result in a misdiagnosis of porphyria if comprehensive enzymatic screening tests were not performed. Therefore before attempting to screen for porphyria in this way it must be affirmed that the subject has not taken any drugs known to cause derepression of ALA-S.

4.4 SUMMARY OF CHAPTER 4

The studies described in this chapter were prompted by the findings of Chapter 3 and were devoted to investigating the effects of CBZ on erythrocyte ALA-D, erythrocyte PBG-D and the urinary porphyrin excretion profile.

As regards the reduction in the levels of ALA-D activity during CBZ treatment, a number of findings were made. These were that the effect on enzyme activity was irreversible, was not immediately apparent, was related to plasma "total" CBZ and CBZ-E concentrations, and was not due to an altered Km or a shift in the optimum assay pH. Thus it seems that the low activity in CBZ treated subjects arises through a reduced amount of normal enzyme. It was also ascertained that activity was not lost as a result of reduced erythrocyte glutathione levels or higher than normal blood lead concentrations. However, the results did not confirm if the effect occurs through a direct or indirect action of CBZ or a metabolite thereof.

This effect of CBZ on ALA-D was the underlying cause of the apparent loss of erythrocyte PBG-D activity in the subjects studied in Chapter 3, where the assay relied on ALA-D to act as a coupling enzyme. This chapter demonstrated that CBZ treatment does not affect PBG-D activity. This was shown by adopting a PBG-D assay which used PBG itself as a substrate, obviating the need for a coupling enzyme. Further experiments proved that the apparent reduction of PBG-D activity observed in Chapter 3 was due to a deficiency of ALA-D coupling enzyme. The minimum ALA-D activity required for it not to be rate-limiting was also calculated.

CBZ was shown to produce temporary disturbances in the urinary porphyrin excretion profile which were characterized by large

increases in the %URO. These changes were maximal shortly after commencing treatment and then gradually returned to normal. The changes in the profile were probably related to derepression of ALA-S. These results explain why Rapeport et al (1984) found changes in the urinary porphyrin excretion profiles of volunteer subjects taking CBZ for a short period of time but no changes were found in epileptic subjects on chronic CBZ therapy.

This chapter has answered some of the questions raised by the results of Chapter 3. The remainder of this thesis investigates the effects of CBZ on haem biosynthesis in other systems and is particularly concerned with elucidating the mechanism whereby ALA-D activity is reduced.

CHAPTER 5

CHAPTER 5

CARBAMAZEPINE AND HAEM BIOSYNTHESIS IN THE RAT

In Chapters 3 and 4, the effects of CBZ on the first part of the haem biosynthetic pathway in man were investigated. These studies, considered in conjunction with those of Yeung *et al* (1983) and Rapeport *et al* (1984), revealed that in the early stages of treatment CBZ caused derepression of ALA-S, resulting in very high levels of activity. Although activity fell from these initially high values, it did not return to baseline but remained significantly higher than controls. It was suggested that this derepression of ALA-S was a consequence of increased cytochrome P450 requirement. ALA-D activity was chronically depressed during CBZ treatment, and there was a significant correlation between ALA-D activity and plasma CBZ concentration. The reduction in the levels of ALA-D activity produced by CBZ is a slow process, suggesting that it may not be the result of a direct effect of CBZ on the enzyme. PBG-D and URO-D activities were unaffected by CBZ treatment. There was also no effect of CBZ on the remaining three enzymes of the pathway as judged by quantitative and qualitative analysis of porphyrins excreted in the urine. However, the urinary porphyrin excretion profile was perturbed in the initial stages of CBZ treatment. This effect was transient and probably produced by the very high levels of ALA-S activity found during this period. CBZ also altered the relative amounts of the porphyrin precursors ALA and PBG excreted in the urine. In this chapter, studies of the effects of CBZ on haem biosynthesis in the rat are described. The aim of these studies was to determine if the rat would provide a suitable model for further investigation into

the effects of CBZ described above page. The use of rats for such studies has obvious advantages over the use of human subjects, not the least of which is that hepatic enzyme activities can be directly measured. There are also several draw-backs, the principle ones being the difficulty of obtaining sufficient quantities of unclotted blood from which to prepare plasma for the various drug analyses, and the small amounts of urine produced which limits the number of urinary analyses which can be performed. Owing to the difficulties in obtaining uncoagulated blood, drug concentrations were determined in serum rather than plasma. For most analyses, "total" CBZ concentrations were determined using the EMIT system but where sufficient volumes were available, HPLC analysis of CBZ-E was also performed. No measurements of free CBZ concentration were made. This chapter also investigates the effects of CBZ treatment on hepatic cytochrome P450, the terminal component of the mixed function oxidase system and the proposed intermediary in the derepression of ALA-S shown to occur in man. To complement the studies performed in man, the effect of CBZ on hepatic glutathione concentrations was investigated.

This chapter is therefore composed of 6 sections which are as follows:

(1) the metabolism of CBZ in the rat

(2) the effects of CBZ on the enzymes of haem biosynthesis

(3) the effects of CBZ on the urinary excretion of porphyrins and precursors

(4) the effects of CBZ on hepatic cytochrome P450

(5) the effects of CBZ treatment on hepatic glutathione concentrations

(6) summary

In all of the experiments, the animals used were male Sprague-Dawley rats, weighing between 150-200g at the start of each study. As described in the methods section, CBZ was administered orally as a suspension in cottonseed oil. Cottonseed oil alone was given to controls. Doses were typically administered twice daily (b.d.) at 8.30 a.m. and 8.30 p.m. Animals were starved for 24 hours prior to sacrifice, which was performed 2 hours after the last dose. All animals taking part in the experiments were weighed each day. Animals in which treatment caused a loss of appetite with subsequent loss of weight or failure to gain weight at the same rate as controls were excluded.

5.1 THE METABOLISM OF CBZ IN THE RAT

This section examines the metabolism of CBZ in the rat. These experiments were performed to determine if metabolism of CBZ in the rat was comparable to metabolism in man and to investigate what doses could be tolerated. Doses in excess of 100mg CBZ/kg body weight b.d. were found to produce a loss of appetite followed by weight loss and general poor health and appearance. Consequently, the doses employed in these experiments did not exceed this. Also, the doses used were relatively larger than those used in man, (a typical starting dose in man would be about 6mg/kg). This was necessary because of the faster rate of drug metabolism and excretion found in the rat.

The section is divided into two sub-sections, the first of which examines serum concentrations of CBZ and the second of which investigates which metabolites of CBZ exist in rat serum.

5.1.1 SERUM CONCENTRATIONS OF CBZ IN THE RAT

All CBZ determinations in this subsection were made using the EMIT system (see 2.4.1). CBZ concentrations were measured in serum samples from groups of 6 rats 1.5, 2, 3, 4, 5.5, 7, 9 and 11 hours after a single dose of CBZ of 50mg CBZ/kg body weight. The rats were starved for 24 hours before administration of the drug. The results are presented in fig. (24). In the first 1.5 hours after dosage there was a rapid increase in serum CBZ. This was followed by a slow decline in drug levels. After 4 hours this decrease was interrupted by a secondary, slower rise in serum CBZ concentration which again started to fall after 7 hours. The entire experiment was repeated and gave the same pattern: a rapid rise in serum drug levels followed

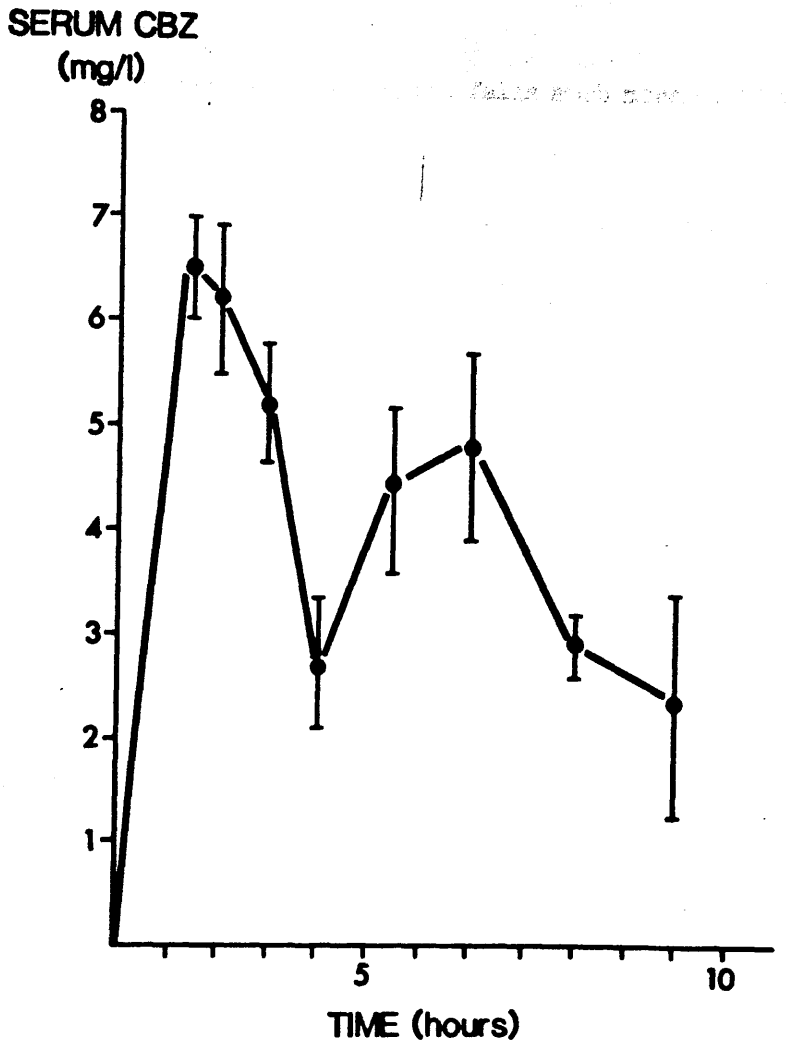


FIG. (24)

CONCENTRATIONS OF CBZ IN RAT SERUM FOLLOWING ORAL ADMINISTRATION OF THE DRUG

48 male Sprague-Dawley rats were starved for 24 hours then given a single dose of 50mg CBZ per kg body weight. At the time indicated, groups of 6 rats were sacrificed and blood was collected for determination of serum CBZ concentrations by EMIT. The figure shows the mean ± 1 S.D. concentrations at these times. The secondary rise in serum CBZ levels is probably due to reabsorption of drug and metabolites excreted in bile.

by a slower decrease which was interrupted by a secondary increase in serum CBZ. This secondary peak in serum CBZ was probably due to reabsorbtion of CBZ which had been excreted into bile. This enterohepatic cycling of CBZ in rats has been noted by Morselli (1975).

The serum concentration of CBZ falls much more rapidly in the rat than in man. Bearing in mind that the results just discussed were from un-induced rats (that is rats which had not been exposed to drugs which may have caused an increase in the activity of the hepatic mixed function oxidase system), the half-life of CBZ ranged from 2-4 hours. This compares with a half-life of 26 hours for a non-induced human and 12 hours for an induced human (Eichelbaum et al, 1985). This greatly accelerated rate of clearance may partly explain why rats can tolerate such high doses of CBZ compared to man. As is the case with man, the mixed function oxidase system can become induced, resulting in a more rapid elimination of CBZ. Table (20) shows the effect of regular treatment with CBZ (60mg CBZ/kg body weight b.d.) on serum concentrations of the drug. Two hours after the last of ten doses, (5 days treatment), the mean total CBZ concentration in serum was only 27% of the mean concentration 2 hours after the second dose. The rate of clearance is probably maximal at this time as evidenced by the results of a separate experiment in which one group of 6 rats was treated with 75mg CBZ/kg body weight b.d. for 5 days and a second group treated with the same dose for 12 days. (see table (21)). The mean serum concentrations of CBZ, (2 hours after the last dose in both groups), were 5.3 ± 0.7 mg/l after 5 days and 5.3 ± 1.0 mg/l after 12 days. This shows that CBZ is not cleared any faster after

TABLE (20)

SERUM CBZ CONCENTRATIONS IN THE RAT

DURING CBZ TREATMENT

Duration of Treatment (h)	Serum [CBZ] (mg/l)
14	13.2 ± 4.9
38	8.1 ± 3.9
62	4.0 ± 2.3
86	-
110	3.6 ± 0.2

Each result represents the mean ± 1 S.D. for 4 animals.

60mg/Kg CBZ was administered at time 0 and thereafter at 12 hourly intervals.

Animals were starved for 24 hours prior to sacrifice, 2 hours after the last dose of drug.

12 days treatment than it is after 5 days treatment. This is probably related to induction of cytochrome P450, which is covered in Section 5.4.

5.1.2 METABOLITES OF CBZ IN THE RAT

As in man, the major metabolite of CBZ in the rat was found tp be carbamazepine-10-11-epoxide (CBZ-E). In this sub-section CBZ-E concentrations in rat serum were determined by the HPLC method described in sub-section 2.4.2. Analysis of the amounts of epoxide present in serum samples from 24 rats treated with CBZ revealed that the relative proportions of CBZ-E and the parent drug were greatly different from those in man. In these 24 rats, CBZ-E accounted for 87.8 + 5.6% of "total" serum CBZ as determined by EMIT. In man, for 39 individual subjects, CBZ-E accounted for only 9.2 + 4.6% of total plasma CBZ. The figures for CBZ-E levels in the rat are analysed in greater detail in table (21). This shows that the percentage of epoxide in the rat was remarkably constant and independent of both dose and time. This much higher percentage of CBZ-E in the rat is a reflection of the greater metabolizing capacity of rat liver and may explain (a) why rats can tolerate greater doses of CBZ and (b) why the drug has a shorter half-life in rats. This second suggestion is based on the fact that CBZ-E is cleared faster than CBZ (Patel, Levy and Trager, 1978).

TABLE (21)

SERUM CBZ AND CBZ-E CONCENTRATIONS IN THE RAT DURING CARBAMAZEPINE TREATMENT

DOSE	DURATION	n	MEAN TOTAL CBZ[a]	MEAN % CBZ-E
75mg/kg b.d.	5 days	6	5.3 ± 0.7	85.3 ± 4.8
75mg/kg b.d.	12 days	6	5.3 ± 1.0	87.8 ± 5.6
100mg/kg b.d.	7 days	6	8.1 ± 1.9	89.6 ± 5.8
50mg/kg b.d.	7 days	6	4.9 ± 1.0	88.6 ± 6.0

Overall, CBZ-E concentrations were 87.8 ± 5.6% of total CBZ concentrations (n=24).

[a] serum concentration determined by EMIT. Units are mg/l.

5.1.3 SUMMARY OF SECTION 5.1

Metabolism of CBZ in the rat differs from metabolism of the drug in man. Although the principle metabolite was CBZ-E in both cases, the epoxide accounted for a much greater percentage of total CBZ in the rat than in man. This is a consequence of the greater activity of the hepatic mixed function oxidase system in rat. This also accounts for the fact that CBZ has a much shorter half-life in the rat, since the more rapid and extensive the metabolism of CBZ to the epoxide, the more quickly will the levels of the drug be reduced. This section also demonstrated that there was a secondary peak in the plot of serum CBZ concentration following a single dose versus time. This was probably due to enterohepatic cycling of CBZ. As is the case in man, the rate of clearance of CBZ increased after several days treatment and was maximal after 5 days. This was probably due to induction of drug metabolizing systems and is more fully investigated in later sections.

5.2 CBZ AND THE ENZYMES OF HAEM BIOSYNTHESIS IN RAT LIVER

The experiments described in this section examined the effects of CBZ treatment on ALA-S, ALA-D, PBG-D and URO-D activities in rat liver. These were the same enzymes that were studied in peripheral blood cells of man. The activities of these enzymes in rat liver during CBZ treatment were measured to evaluate how useful the rat would be as a model for further investigating the effects of the drug documented in Chapters 3 and 4. All enzyme activities were measured in liver using the methods described in the relevant sub-sections of section 2.2. Both dose response and time course studies of the effects of CBZ on the enzymes mentioned above were performed. The results of these studies are presented in the following sub-sections, each of which describes the effects of the drug on a particular enzyme. Enzyme results are presented in the order in which the enzymes occur in the pathway.

5.2.1 THE EFFECTS OF CBZ TREATMENT ON RAT LIVER ALA-S ACTIVITY

In Section 5.1, it was shown that CBZ-E is the major metabolite of CBZ in the rat. This compound is produced by the action of the hepatic mixed function oxidase system. The fact that CBZ is metabolized in this way in the rat would suggest that ALA-S activity may become derepressed during CBZ treatment through the mechanisms described in sub-section 1.3.9. The results of measuring ALA-S activity over 5 days treatment with 60mg CBZ/kg body weight b.d. are presented in table (22). These show that ALA-S activity was indeed derepressed in rats treated with CBZ, but at no time did ALA-S activity correlate with serum CBZ concentration. This was probably because concentrations were all very similar due to all animals being

TABLE (22)

THE EFFECTS OF CBZ TREATMENT ON HEPATIC HAEM ENZYMES OVER FIVE DAYS

TREATMENT(h)	ALA-S[a]		ALA-D[b]		PBG-D[c]		SERUM
	CONTROL	CBZ	CONTROL	CBZ	CONTROL	CBZ	[CBZ][d]
14	71.2 ± 4.9	73.6 ± 6.0	14.4 ± 1.2	13.6 ± 1.4	50.0 ± 8.7	51.9 ± 5.3	13.2 ± 4.9
38	73.8 ± 8.1	128.3 ± 20.3**	12.4 ± 2.2	12.0 ± 0.6	52.6 ± 4.4	55.2 ± 2.6	8.1 ± 3.9
62	70.5 ± 9.2	338.2 ± 52.9***	13.0 ± 2.4	13.0 ± 1.4	50.6 ± 1.5	53.6 ± 5.4	4.0 ± 2.3
86	68.9 ± 12.1	296.2 ± 63.1***	12.4 ± 1.8	10.2 ± 0.6*	54.7 ± 16.5	54.2 ± 1.8	-
110	75.0 ± 7.9	192.9 ± 40.6**	13.2 ± 0.6	10.4 ± 0.6**	48.5 ± 5.2	50.5 ± 4.2	3.6 ± 0.2

* $P<0.01$, ** $P<0.005$, *** $P<0.001$

Each result presented in the table is the mean ± 1 S.D. for results from 4 animals.
60mg/kg CBZ was administered at time 0 and thereafter at 12 hourly intervals.
Animals were starved for 24 hours prior to sacrifice.

(a) units are nmoles ALA produced/h/g protein

(b) units are μmoles PBG produced/h/g protein

(c) units are nmoles URO produced/h/g protein

(d) units are mg/l

given the same dose. However, further experiments demonstrated that the extent of derepression was dependent on the dose of CBZ given (see table (23)). Returning to the results in table (22), the observed increase in activity was highly significant ($P<0.001$) and was maximal after 3 days treatment, at which time the mean CBZ-treated ALA-S activity was 480% of the mean control activity. Whereas the increase in activity was rapid (even after 2 days treatment mean treated activities were 174% of mean control activities), when the levels of activity began to fall, (after 3 days), the rate of fall was relatively slow. This pattern of a rapid increase in activity followed by a slower decrease is the classical picture of derepression of ALA-S in rat liver linked to induction of cytochrome P450 synthesis (De Matteis and Gibbs,1972; Rajamanickam et al, 1975). The relationship between cytochrome P450 and ALA-S will be discussed in Section 5.4. Further experiments revealed that, in contrast with the results from human subjects, described in Chapter 3, ALA-S activity in the rat does not remain elevated during prolonged CBZ therapy. Table (24) shows that after treatment with a higher dosage of CBZ (75mg/kg b.d.), ALA-S activities were increased with respect to controls after 5 days, but after a further 7 days treatment had returned to control levels (in fact activities were significantly lower than controls, $P<0.05$). Studies on the effects of chronic CBZ therapy in man described in Chapter 3 revealed that leucocyte ALA-S activities (which are believed to mirror hepatic ALA-S activities) were elevated, the mean activity of treated patients being 218% of the mean activity of age and sex matched controls. Table (25) shows that, in the rat, treatment with even greater doses of CBZ does not

TABLE (23)

THE EFFECTS OF THREE DIFFERENT DOSES OF CBZ

ON RAT LIVER ALA-S ACTIVITY AFTER 5 DAYS TREATMENT

TREATMENT	ALA-S ACTIVITY[a]
CONTROL	67.4 ± 7.9
CBZ 25mg/Kg b.d.	103.2 ± 32.6*
CBZ 50mg/Kg b.d.	180.5 ± 90.4**
CBZ 75mg/Kg b.d.	279.5 ± 120.1***

* P<0.02, ** P<0.01, *** P<0.001 compared to controls.

Each result represents the mean ± 1 S.D. for 6 animals.

Animals were starved for 24 hours prior to sacrifice.

a units are nmoles ALA produced/h/g protein.

TABLE (24)

THE EFFECTS OF CBZ ON HEPATIC HAEM ENZYMES

AFTER TREATMENT FOR 5 AND 12 DAYS

	CONTROL	GROUP 1	GROUP 2
ALA-S[a]	88.4 ± 3.0	311.1 ± 177.2***	74.4 ± 18.2*
ALA-D[b]	10.5 ± 1.4	7.3 ± 1.8**	7.0 ± 1.6**
PBG-D[c]	60.1 ± 12.2	62.6 ± 7.3	63.1 ± 8.6
URO-D[d]	4.9 ± 2.1	4.6 ± 0.9	4.8 ± 1.2
SERUM [CBZ][e]	-	5.3 ± 0.7	5.3 ± 1.0

* P<0.05, ** P<0.005, *** P<0.001 as compared to controls.

Each result presented in the table is the mean ± 1 S.D. for 6 animals. Group 1 was given 75mg/kg CBZ b.d. for 5 days - Group 2 was given the same dosage for 12 days.

a units are nmoles ALA produced/h/g protein

b units are μmoles PBG produced/h/g protein

c units are nmoles URO produced/h/g protein

d units are μmoles decarboxylations/h/g protein

e units are mg/l

TABLE (25)

THE EFFECTS OF THREE DIFFERENT DOSES OF CBZ ON HEPATIC HAEM ENZYMES AFTER TREATMENT FOR 14 DAYS

	CONTROL	GROUP 1	GROUP 2	GROUP 3
ALA-S[a]	52.0 ± 7.2	54.3 ± 8.1	55.2 ± 9.6	55.6 ± 12.3
ALA-D[b]	7.8 ± 0.9	7.1 ± 0.3	6.4 ± 0.5*	5.4 ± 0.4**
PBG-D[c]	63.5 ± 8.9	62.9 ± 12.9	65.8 ± 5.1	61.7 ± 13.2
URO-D[d]	6.8 ± 2.1	6.9 ± 3.0	7.1 ± 2.3	6.8 ± 1.8
SERUM[CBZ][e]	-	3.7 ± 0.5	5.3 ± 0.6	8.1 ± 1.9

* $P<0.005$, ** $P<0.001$ as compared to controls.

Each result presented in the table is the mean ± 1 S.D. for results from 6 animals. All treated animals were given 75mg/kg CBZ b.d. for 7 days. The following doses were then administered for the next 7 days: Group 1, 50 mg/kg; Group 2, 75mg/kg b.d., Group 3, 150mg/kg b.d. Animals were starved for 24 hours prior to sacrifice.

a units are nmoles ALA produced/h/g protein

b units are μmoles PBG produced/h/g protein

c units are nmoles URO produced/h/g protein

d units are μmoles decarboxylations/h/g protein

e units are mg/l.

result in sustained derepression. In this case, after a fortnight's treatment with 3 different doses of CBZ, there was no depression of activity as was found above, indicating that the seemingly significant decrease in activity noted in table (24) was probably an artefact caused by the small numbers of rats used in the experiment. Possible explanations for the difference in the response of ALA-S to CBZ treatment in man and rat will be discussed in sub-section 5.2.5 which concludes this section.

Conclusions on the effects of CBZ treatment on rat liver ALA-S

As was the case in man, CBZ caused a derepression of ALA-S in rat liver. The derepression of activity was probably linked to cytochrome P450 and this will be considered in Section 5.4. The extent of derepression was related to the dose of CBZ given. However, in contrast to man, the elevation of ALA-S activity was not sustained but had returned to baseline after treatment for 12 days. This will also be further discussed in Section 5.4.

5.2.2 THE EFFECTS OF CBZ TREATMENT ON RAT LIVER ALA-D ACTIVITY

The effects of CBZ treatment on ALA-D activity in the rat were investigated. The results of measuring ALA-D activity over 5 days treatment with 60mg CBZ/kg body weight b.d. are presented in Table (22). The results show that there was a significant reduction of ALA-D activity in the liver after 4 and 5 days of treatment with the drug, ($P<0.01$ and 0.005 respectively). At this time, there was no correlation between activity and serum CBZ concentration. This was probably because there were few points to correlate and all of the animals had similar CBZ concentrations. The relationship between ALA-D activity and serum CBZ will be discussed later in this sub-

section. Activity was not immediately affected, the first changes onlu becoming apparent after 4 days treatment. This is comparable to the situation found in man where it was demonstrated that ALA-D activity was not affected up to two days after a single dose but was reduced after treatment for 1 week. In order to verify that the reduction in activity observed was not produced simply by the use of small numbers of animals, a further experiment was conducted with a group of 8 control rats and a group of 8 rats treated with 100mg CBZ/kg body weight b.d. The drug was administered for 6 days, after which the mean hepatic ALA-D activity of the CBZ treated group was 80% of the mean activity of the control group, a highly significant difference ($P<0.001$). The results of this experiment are shown in fig. (25). As a further control, PBG-D activities were measured in the same animals and showed no change. As part of the same experiment activities of both these enzymes were measured 2 hours after a single dose and were no different from controls.

In all of the preceding investigations into the effects of CBZ treatment on ALA-D activity, the enzyme was assayed in the presence of dithiothreitol (DTT) and $ZnCl_2$. This shows that, as was the case in man, the reduction in levels of enzyme cannot be reversed by treatment with these activators. To investigate if perhaps ALA-D activity was affected at an earlier stage than was shown in table (22), but the effect was masked by the inclusion of activators in the assay, a further study, identical to the first described in this sub-section, was performed. In this study ALA-D activity was determined with and without added activators. In the presence of DTT and $ZnCl_2$, the same pattern as was shown in this previous study was found, namely reduced ALA-D activity after 4 and 5 days treatment. However no

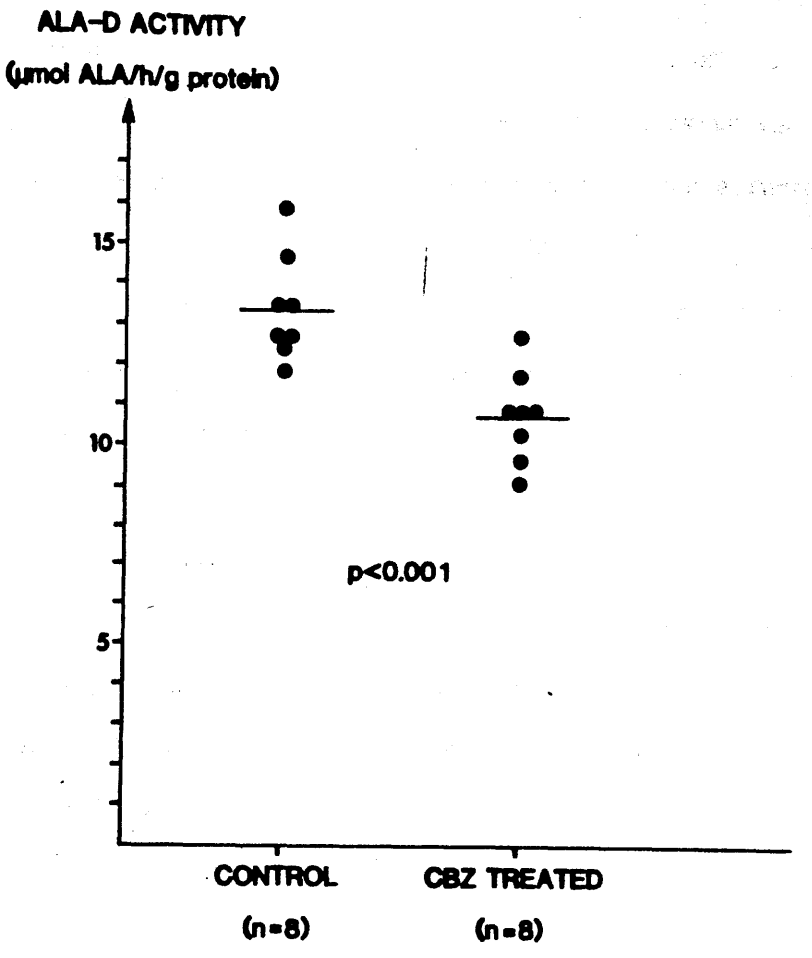


FIG. (25)

THE EFFECTS OF CARBAMAZEPINE TREATMENT ON ALA-D ACTIVITY IN RAT LIVER

8 male Sprague-Dawley rats were treated with 100mg CBZ in cottonseed oil per kg body weight b.d. for 6 days then sacrificed and hepatic ALA-D activities measured. 8 control rats were treated as above except that cottonseed oil alone was given. The results show that CBZ caused a 20% reduction in the mean ALA-D activity in rat liver, $P<0.001$.

satisfactory results were obtained in the absence of the activators because activities were very variable (activities ranged from zero to 80% of the activities found in the presence of activators).

The effects of CBZ on ALA-D activity after 5 and 12 days treatment were also determined. The results of this study are presented in table (24) and reveal that ALA-D activity was maximally depressed after 5 days treatment, as treatment for a further 7 days produced no further depression of activity. This contrasts with the effects of CBZ treatment on ALA-D activity in human erythrocytes. In that system, as shown in sub-section 4.1.2, activity was not maximally reduced after treatment for 1 week. Why should activity remain unaffected for 3 days then over the next 2 days suddenly fall and remain at a constantly reduced level? It may be that in the study from which the results in table (22) were obtained, the effect was gradual, but in the earlier stages of treatment was too slight to detect: the maximum extent of the reduction in activity as compared to controls was only 21%. An additional complicating factor was the small numbers used in this study. Regardless of this, however, subsequent studies with larger numbers of animals have shown that the effect is maximal after 5 days treatment. Why should this be the case in rat liver and not in human erythrocytes? There are several reasons, the most relevant being (a) rats are metabolically more active, (b) rats are receiving larger doses of drug, (c) there is a greater rate of drug turn-over in the rat, and (d) there is a greater rate of protein turn-over in the rat. These points could explain the faster attainment of reduced activities in the following ways. If the epoxide is the compound responsible for the diminuition of ALA-D activity, the higher concentrations produced in the rat would cause

the levels of enzyme activity to be more rapidly affected. Alternatively if the effect was caused by some other metabolite of CBZ produced only in small amounts, the faster turn-over of the drug in the rat would mean that greater quantities of this compound were produced and would exert their effects more rapidly on ALA-D. Additionally, ALA-D was measured in the liver of rats, but in the erythrocytes in man and due to the proximity of the ALA-D in liver to the drug metabolizing systems, there would be higher total concentrations of all metabolites of CBZ. If CBZ or a metabolite was acting at the level of ALA-D synthesis then the effect would be more rapidly manifest than in man owing to the greater rate of protein turn-over found in rat liver.

The depression of ALA-D activity in rat liver produced by CBZ treatment also exhibited dose dependency. The results of a study in which rats were given 3 different doses of CBZ over a two week period are shown in table (25). This reveals that ALA-D activity (during treatment) was related to serum CBZ concentrations, mean CBZ treated ALA-D activities were 91%, 82% and 69% of mean control values for mean serum CBZ concentrations of 3.7, 5.3 and 8.1 mg/l respectively. Note that this does not mean that CBZ is directly responsible for the actual reduction in the levels of ALA-D activity.

Conclusions on the effects of CBZ treatment on rat liver ALA-D

CBZ treatment irreversibly reduced the levels of ALA-D activity in rat liver, presumably by the same mechanism by which it did so in man. As was found in man, the extent of the reduction in enzyme activity was related to the plasma CBZ concentration. A further parallel between the depression of ALA-D activity in rat liver and the depression of erythrocyte ALA-D in man was that the effect took some

time to manifest itself. However, in the rat, the maximal reduction in activity was attained much more quickly than in man.

5.2.3 THE EFFECTS OF CBZ TREATMENT ON RAT LIVER PBG-D ACTIVITY

The effects of CBZ treatment on rat liver PBG-D activity were investigated. The results of these investigations, which constituted dose response, effects of CBZ on enzyme activity over 5 days treatment and the effects of 12 days treatment, are presented in tables (22) (24) and (25). These show that CBZ had no effect on PBG-D activity, confirming the findings of Chapter 4.

5.2.4 THE EFFECTS OF CBZ TREATMENT ON RAT LIVER URO-D ACTIVITY

The effects of CBZ treatment on rat liver URO-D activity were investigated. The results of measurements of URO-D activity after 5 days and 12 days treatment with CBZ and after 2 weeks of different doses of CBZ are presented in tables (24) and (25). Unfortunately activities of this enzyme over the first 5 days of drug treatment were not measured. However, given that no effects were found after this time and that a transient effect of CBZ on the enzyme is improbable, it is likely that the activity of URO-D in rat liver was not affected by CBZ. This confirms the findings made in human erythrocytes in Chapter 3.

5.2.5 SUMMARY AND GENERAL DISCUSSION OF SECTION 5.2

In general, the effects of CBZ on the enzymes of haem biosynthesis were the same in rat liver as in human peripheral blood cells. Of the enzymes studied, ALA-S and ALA-D were the only ones affected by treatment and although the general effects on these enzymes were the same as in man, there were some differences.

In the initial stages of treatment, as was the case in man, there was a rapid dose-related derepression of ALA-S followed by a slower decline in the levels of activity. This suggested that it occurred as a consequence of induction of the hepatic mixed function oxidase system, in particular, the cytP450 component. This proposal was confirmed as will be shown in section 5.4. However unlike man, in whom ALA-S remained derepressed (see Section 3.1), activities in rat liver returned to normal. This was perhaps due to the overall greater metabolic rate which exists in rats as regards protein turnover and drug metabolism. The best explanation would be that ALA-S aside, the remaining enzymes of the pathway have endogenously greater activities than the corresponding enzymes in man. This would mean that the increased haem requirement could be met more readily. In addition to this, rats have higher initial levels of mixed function oxidase activity and consequently less adaptation of the rate of flux through the pathway is necessary. In man however, basal levels of mixed function oxidase activity are low as compared to the rat. Consequently, greater induction has to occur and, since the enzymes of the pathway are also present in reduced amounts, the pathway has to have many more intermediates fed into it at the level of ALA than in the rat.

ALA-D activity in rat liver was reduced by CBZ treatment with features similar to the reduction of activity in human erythrocytes. There was a relationship between the dose of CBZ administered and the extent of the depression. The reduction in activity could not be reversed by treatment with activators. Although the effect on activity was not immediate, it occurred more rapidly than in man.

This was probably a consequence of the greater rate of drug turn-over in the rat. As was the case in man, it was not possible to define the mechanism whereby CBZ treatment caused reduction of ALA-D activity. PBG-D and URO-D were unaffected by treatment with CBZ.

Whether or not the effects of CBZ treatment on ALA-S and ALA-D activities have any influence on the urinary excretion of porphyrins and precursors is examined in the following section.

5.3 THE EFFECTS OF CBZ TREATMENT ON THE EXCRETION OF PORPHYRINS AND PRECURSORS IN RAT URINE

This section consists of quantitative and qualitative investigations into the effects of CBZ treatment on the excretion of porphyrins and the porphyrin precursors, ALA and PBG, in rat urine. The purpose of these investigations was to determine if the effects of CBZ treatment on ALA-S and ALA-D activity described in the previous section would influence the urinary excretion of porphyrins and precursors in rats in the same way as in man (see sections 3.3 and 4.3). The section is divided into three sub-sections. The first of these deals with quantitative aspects and the second with qualitative aspects of porphyrin and porphyrin precursor excretion. The third summarises this section.

5.3.1 QUANTITATIVE ANALYSIS OF THE EFFECTS OF CBZ TREATMENT ON THE EXCRETION OF PORPHYRINS AND PRECURSORS IN RAT URINE

As has been described for human urine, 3 quantitative analyses were performed on rat urine:

(i) 24 hour ALA excretion

(ii) 24 hour PBG excretion

(iii) 24 hour total porphyrin excretion

The results of urine analysis from rats treated with 75mg CBZ/kg body weight b.d. for 7 days and then 150mg CBZ/kg body weight for a further 7 days are shown in table (26). These results are from the rats whose enzyme activities following sacrifice were presented as group 3 in table (25). Urine samples were stored in the freezer at -80°C for 1 month without additives. This was not ideal, as neither ALA nor PBG are particularly stable and some deterioration may have occurred. However control samples were stored under identical

TABLE (26)

QUANTITATIVE ANALYSIS OF PORPHYRINS AND PRECURSORS IN THE URINE OF CBZ TREATED RATS

	ALA[a]	PBG[b]	TOTAL PORPHYRIN[c]
CONTROL (n=12)	110 ± 87	21±18	2.19 ± 2.0
CBZ 5 days (n=6)	47 ± 93	63 ± 71*	5.11 ± 2.19**
CBZ 12 days (n=6)	561 ± 226***	13 ± 17	12.25 ± 8.98***

* P<0.05, ** P<0.01, *** P<0.001 as compared to controls.

Each result presented in the table is the mean ± 1 S.D. for the group. CBZ treated animals were given 75mg/Kg CBZ b.d. for 7 days then 150mg/Kg b.d. for a further 7 days after which they were sacrificed. Urine collections were made at the times indicated in the table.

a units are μmoles ALA excreted/24 hours

b units are μmoles PBG excreted/24 hours

c units are mmoles porphyrin excreted/24 hours

conditions and therefore the results, though not absolute, should be valid. Two urine collections were obtained from each of the six control rats. CBZ treated animals were compared against the combined data from these two sets of results. The results presented in table (26) are now discussed.

ALA

There was no increase in ALA excretion after 5 days (in fact there appeared to be a decrease) but there was a highly significant increase after 12 days, CBZ treated rats having an ALA excretion 510% that of controls ($P<0.001$). It is surprising that ALA excretion was increased after 12 days and not after 5 days. The increased excretion observed at day 12 can only be due to reduced ALA-D activity since at this stage of treatment ALA-S activities had returned to baseline (see table 24). Therefore an increased ALA excretion would also be expected after 5 days treatment since in addition to ALA-D activity being reduced (albeit not to the same degree as at day 12) ALA-S activity was increased to 352% of control values. The reasons for this apparent anomaly are not known.

PBG

PBG excretion was significantly increased to 300% of control values after 5 days treatment, ($P<0.05$) but not after 12 days treatment. This would suggest that it may be linked to ALA-S activity as this was shown to be increased after 5 days but to have returned to baseline after 12 days (table 24). This could occur as follows. After 5 days treatment, ALA-D would be only slightly affected, but ALA-S activity would be greatly increased. Since there was no increase in urinary ALA excretion at this time, it must be presumed that ALA-D could cope with the extra ALA, converting it to

PBG. However, next to ALA-S, PBG-D has the lowest endogenous activity of all the enzymes in the pathway and consequently would be unable to cope with the extra substrate, some of which would be excreted. After 12 days, ALA-S activity had returned to baseline but ALA-D activities were depressed to 69% of control activities, leading to decreased PBG synthesis. This could explain why PBG excretion was reduced after 12 days treatment, though not to a significant extent.

Total Porphyrin

Total porphyrin excretion was significantly increased after 5 days of CBZ treatment ($P<0.01$) and even more so after 12 days treatment ($P<0.001$), mean values being 233% and 559% of mean control values respectively. Since an increase in porphyrin excreted could only occur through increased porphyrin synthesis and since ALA-S is the rate limiting enzyme of the pathway, it follows that it should only be possible to increase total porphyrin excretion as observed here by an increase in the levels of ALA-S activity. However whereas this may be the case after 5 days treatment, it is not for 12 days treatment. Indeed table (24) shows that ALA-S activity at this time had returned to control values. How then can total porphyrin excretion be increased? One possible explanation is that CBZ metabolites in urine interfere with the quantitative measurement of porphyrins by fluorescence. If this were so it could explain why total porphyrin excretion seemed to be raised in the second week, during which the CBZ dosage was increased from 75mg/kg b.d. to 150mg/kg b.d. To investigate if this was so, solutions of CBZ and CBZ-E in HCl were prepared and a scan of the fluorescence emmission spectrum was run using an excitation wavelength of 396nm, the

wavelength used for quantifying porphyrins by fluoresence. This revealed that CBZ-E, but not CBZ, was highly fluorescent under the conditions used. Although the maximum emmission wavelength was around 450nm, there was still considerable fluoresence at 594nm which lay in the tail end of this peak. This is the wavelength at which the fluorescence of porphyrin is measured. Therefore the presence of CBZ-E, and presumably other metabolites of CBZ, can produce increased fluorescence readings at the wavelengths used for porphyrin measurements, resulting in over-estimation of the amount of porphyrin present. To investigate if this was in fact the case, an attempt was made to see if the quantities of CBZ and metabolites in urine could be related to the apparent total porphyrin concentration. This was not possible using the HPLC method described in sub-section 2.4.3 because urine from control animals contained peaks which interfered with determination of CBZ and CBZ-E and also because urine from CBZ treated rats contained many peaks, presumably metabolites of CBZ, which could not be quantified due to the lack of suitable standards. No other means of quantifying CBZ and metabolites in urine was available, (the EMIT system used for other analyses in this thesis was suitable only for plasma drug measurement). Therefore it was not possible at this stage to say conclusively that the apparently increased porphyrin content of these urine samples was attributable to the presence of CBZ-E or other fluorescent metabolites of CBZ. However the following experiment shows that this is probably true. Epoxide was added directly to rat urine, (adjusted to pH 6.7 as described in sub-section 2.3.3) to investigate if CBZ-E would be retained on the Dowex ion exchange resin used in the assay. After adding the urine to the

column, washing and eluting, the sample spiked with CBZ-E gave an increased fluorescence reading indicating that CBZ-E is not separated from porphyrin using Dowex resin at this pH.

In a separate study in which rats were treated with 75mgCBZ/kg b.d. for 12 days, urinary total porphyrins were measured without adjusting the pH. The results of these analyses are shown in table (27). Urinary total porphyrin excretion was measured at different intervals after starting treatment and at no time was there a significant difference between treated and control animals. This indicates that in this case CBZ-E and other metabolites responsible for the increase in fluorescence observed in the results shown in table (26) are separated from porphyrin by loading onto the ion-exchanger at a higher pH (rat urine is about pH 9.0).

To summarise, the effects of CBZ treatment on urinary total porphyrin excretion are not clear. In one experiment where there appeared to be an increase, it was shown that this was very probably due to the presence of CBZ-E and/or other metabolites. In the second experiment, CBZ-E at least and presumably some other similar compounds which may interfere with fluorescence were removed and no change in urinary porphyrin excretion was found. However even this is uncertain as the pH varied between samples and this may cause differences in the extraction of porphyrins from different samples.

Conclusions on quantitative analysis of the effects of CBZ treatment on the excretion of porphyrins and precursors in rat urine

Treatment with CBZ appeared to cause an increase in urinary ALA excretion after 12 days treatment which may be due in part to a reduction in ALA-D activity. CBZ treatment also produced an increase

in the urinary excretion of PBG after 5 days and a decrease in the same after 12 days, though the latter was not significant. These changes could be explained in terms of the relative activities of ALA-S and ALA-D. From table (27) (presented in the following sub-section), there appeared to be no effect of CBZ treatment on urinary total porphyrin excretion. This was in direct contrast to the results obtained in table (26). The former is probably the case as it was shown that under the conditions used in the determination of porphyrin content in the latter, the presence of CBZ-E could have resulted in an over-estimation of porphyrin content of the sample.

5.3.2 QUALITATIVE ANALYSIS OF THE EFFECTS OF CBZ TREATMENT ON PORPHYRINS IN RAT URINE

This sub-section made a qualitative analysis of urinary porphyrins in both CBZ-treated and control rats, to investigate if the increase in the % uroporphyrin (URO) found after initiating treatment with CBZ in man (see Section 4.3) occurred in the rat. Rats were given 75mg CBZ/kg body weight b.d. for a period of 12 days during which time urine was collected over 24 hour periods and a qualitative analysis of the porphyrins present was made as described in sub-section 2.3.4. The results are presented in table (27), and are taken from the rats whose enzyme activities in the course of this particular study were presented in table (24). Two urine collections were obtained from each of the 6 control rats. CBZ treated animals were compared against the combined data from these two sets of results.

The results show that treatment with CBZ produced some significant changes with respect to controls, but these were very

TABLE (27)

QUANTITATIVE AND QUALITATIVE ANALYSIS OF PORPHYRINS IN THE URINE OF CBZ TREATED RATS

		TOTAL PORPHYRIN[a]	PERCENTAGE PORPHYRIN COMPOSITION					
			2-COOH	4-COOH	5-COOH	6-COOH	7-COOH	8-COOH
CONTROLS (n=10)		4.78 ± 3.57	26.0 ± 10.1	49.8 ± 10.7	10.0 ± 3.4	4.0 ± 1.0	6.1 ± 2.0	4.1 ± 1.8
CBZ	2 Days (n=5)	4.52 ± 2.78	22.9 ± 6.6	50.4 ± 6.9	10.9 ± 1.4	3.5 ± 0.7	5.9 ± 0.7	6.2 ± 2.2*
	4 Days (n=5)	5.14 ± 3.64	35.6 ± 14.1	40.4 ± 5.8*	10.4 ± 4.0	2.9 ± 1.8	5.6 ± 3.6	5.1 ± 2.7
	7 Days (n=5)	3.53 ± 1.41	39.8 ± 7.4**	43.3 ± 6.7	7.6 ± 1.6	2.7 ± 1.4*	3.2 ± 1.7**	3.3 ± 2.2
	12 Days (n=5)	4.79 ± 2.71	27.5 ± 4.8	66.2 ± 5.3**	2.7 ± 1.5***	1.2 ± 0.7***	1.4 ± 0.8***	0.9 ± 0.6**

* P<0.05, ** P<0.01 ***P<0.001 as compared to controls

Each result in the table is the mean ± 1 S.D. for the group. CBZ treated animals were given 75mg CBZ/kg b.d. for the duration of the experiment. Qualitative and quantitative analyses of urinary porphyrin excretion were performed at the times indicated.

[a] units are nmoles porphyrin excreted/24 hours.

generally small, with the exception of the results after 12 days. The only effect of interest in relation to man was a slight increase in the % URO after 2 days treatment with CBZ ($p<0.05$). The pattern found in the remaining analyses was confusing but the general trend seemed to be an increase in the % protoporphyrin, up to day 7 of treatment, with corresponding decreases in the other fractions. These results cannot be satisfactorily explained with the data at hand, but are of limited interest because they show that the qualitative effects of CBZ treatment on the porphyrin composition of rat urine is not the same as in man.

5.3.3 SUMMARY AND GENERAL DISCUSSION OF SECTION 5.3

This section described quantitative and qualitative investigations of the effects of CBZ treatment on urinary porphyrin and porphyrin precursor excretion in the rat. Quantitatively it was shown that CBZ treatment caused an increase in urinary ALA excretion at day 12. The reasons for this are not clear, as the depression of ALA-D activity was not sufficient to account for the enormous increase in excretion. Also at this time ALA-S activities had returned to baseline. Therefore there should have been an even larger increase earlier when ALA-S activities were increased after 5 days treatment. One possible explanation for this is that the urines were stored without adjustment of the pH. Rat urine is basic and ALA is not stable in basic solutions.

PBG excretion was shown to be increased after 5 days of CBZ treatment, probably due to derepression of ALA-S. After 12 days there was a non-significant drop in PBG excretion which could have been due to the loss of ALA-D activity caused by CBZ treatment.

The situation as regards quantitative excretion of porphyrins was uncertain but it would seem that total porphyrin excreted in urine was not increased by CBZ treatment, despite derepression of ALA-S. The reasons why derepression of ALA-S activity produces an increase in total urinary porphyrin in man but not in the rat is unclear, but may be due to differences in the relative levels of different enzyme activities. It was shown that if CBZ-E and presumably therefore other metabolites of CBZ were present in samples in which a quantitative measurement of porphyrin was to be made, then an over-estimation of the porphyrin content of the sample would result. The significance of this latter observation in determining total porphyrin concentration in 24 hour urine collections from human patients taking CBZ is not as great as in rats because there is less drug present per given volume of urine although porphyrin concentrations are equivalent.

Qualitatively, the changes in the porphyrin composition of rat urine samples were small and did not show the main effect found in the initial stages of treatment in man, i.e. large increases in the % URO. As was stated in sub-section 3.2.4 it was thought that this increase in % URO arose from non-enzymatic polymerization and cyclization of PBG molecules in the urine, present in excessive amounts due to derepression of ALA-S. It was shown in table (26) that increases in PBG excretion did occur, providing scope for such a reaction, with consequent increase in urinary URO. The fact that this did not occur may be because of two things: (i) rat urine is more basic than human urine which may slow the PBG $\rightarrow$ URO reaction and (ii) samples were frozen immediately after collection unlike human samples which may

have been left at room temperature for up to 24 hours before either freezing or analysis.

Overall, the results of this section parallel the findings made in man in some respects (ALA and PBG) but not in others (total porphyrin and qualitative analysis of total porphyrin), and so are of limited value in understanding the nature of the effects of CBZ treatment on the excretion of urinary porphyrins and precursors in man.

5.4 CBZ AND CYTOCHROME P450

Cytochrome P450 (cytP450) is the name given to the family of inducible haemoproteins which are the terminal components of the hepatic microsomal mixed function oxidase system responsible for the oxidative metabolism of endogenous and exogenous substances. The latter category includes a great many drugs of which CBZ is one. The primary product of CBZ metabolism by this system is the epoxide. Many of the drugs which are metabolized by the hepatic mixed function oxidase system induce de novo synthesis of cytP450 which subsequently causes derepression of ALA-S in the liver. The experiments described in this section studied the effects of CBZ treatment on the levels of cytP450 in microsomal preparations from the livers of treated rats as described in methods 2.11.1.

5.4.1 THE EFFECTS OF CBZ TREATMENT ON CYTOCHROME P450 IN RAT LIVER

Initial experiments established that CBZ did induce cytP450. The absorption peak of the induced, carbon-monoxide treated protein was 450nm, indicating that the induced protein belonged to the same family of cytP450s as that induced by phenobarbitone and not to the P448 type family of cytochromes induced by 3-methylcholanthrene.

The results of 3 studies are summarised in table (28). The figures show that in all 3 cases cytP450 levels were significantly increased during CBZ treatment and reached maximal levels of induction 3 to 4 days after initiation of treatment with CBZ. In studies (B) and (C), it can be seen that the increase in cytP450 levels was preceded by a rise in hepatic ALA-S activity. The relationship between ALA-S and cytP450 levels is shown in fig. (26) using the

TABLE (28)

THE EFFECTS OF CBZ TREATMENT ON RAT LIVER CYTCHROME P-450 AND ITS RELATIONSHIP TO ALA-S ACTIVITY OVER FIVE DAYS

		1	2	3	4	5
Study A						
cytP-450[a]	control	0.76 ± 0.22	0.80 ± 0.21*	0.68 ± 0.14	0.79 ± 0.11	0.92 ± 0.06
	CBZ	0.67 ± 0.10	1.08 ± 0.18	0.86 ± 0.17	0.85 ± 0.21	0.91 ± 0.12
Study B						
cytP-450[a]	control	0.75 ± 0.18	0.89 ± 0.23	0.75 ± 0.07*	0.74 ± 0.17*	0.75 ± 0.16
	CBZ	0.90 ± 0.12	1.0 ± 0.15	1.16 ± 0.16	0.95 ± 0.06	0.91 ± 0.07
ALA-S[b]	control	40.7 ± 5.8	43.2 ± 7.1***	41.8 ± 4.3***	39.7 ± 4.9***	43.1 ± 6.2***
	CBZ	43.0 ± 7.9	91.3 ± 11.8	121.0 ± 19.6	112.4 ± 21.2	100.8 ± 19.3
Study C						
cytP-450[a]	control	1.0 ± 0.15	0.98 ± 0.15	1.03 ± 0.08*	1.31 ± 0.13**	1.03 ± 0.08*
	CBZ	0.82 ± 0.15	1.07 ± 0.11	1.36 ± 0.27	1.52 ± 0.20	1.38 ± 0.23
ALA-S[b]	control	71.2 ± 4.9	73.8 ± 8.1**	70.5 ± 9.2***	68.9 ± 12.1***	75.0 ± 7.9***
	CBZ	73.6 ± 6.0	128.3 ± 20.3	338.2 ± 52.9	296.6 ± 63.1	192.9 ± 30.6

* P<0.005 **P<0.005 ***P<0.001 as compared to controls

Each result is the mean ± 1 S.D. for measurements from 4 animals. Doses were as follows:

Study A 50mg/Kg; Study B 50mg/Kg b.d.; Study C 60mg/Kg b.d.

(a) μmoles cytP-450/g microsomal protein (b) nmoles ALA produced/h/g protein

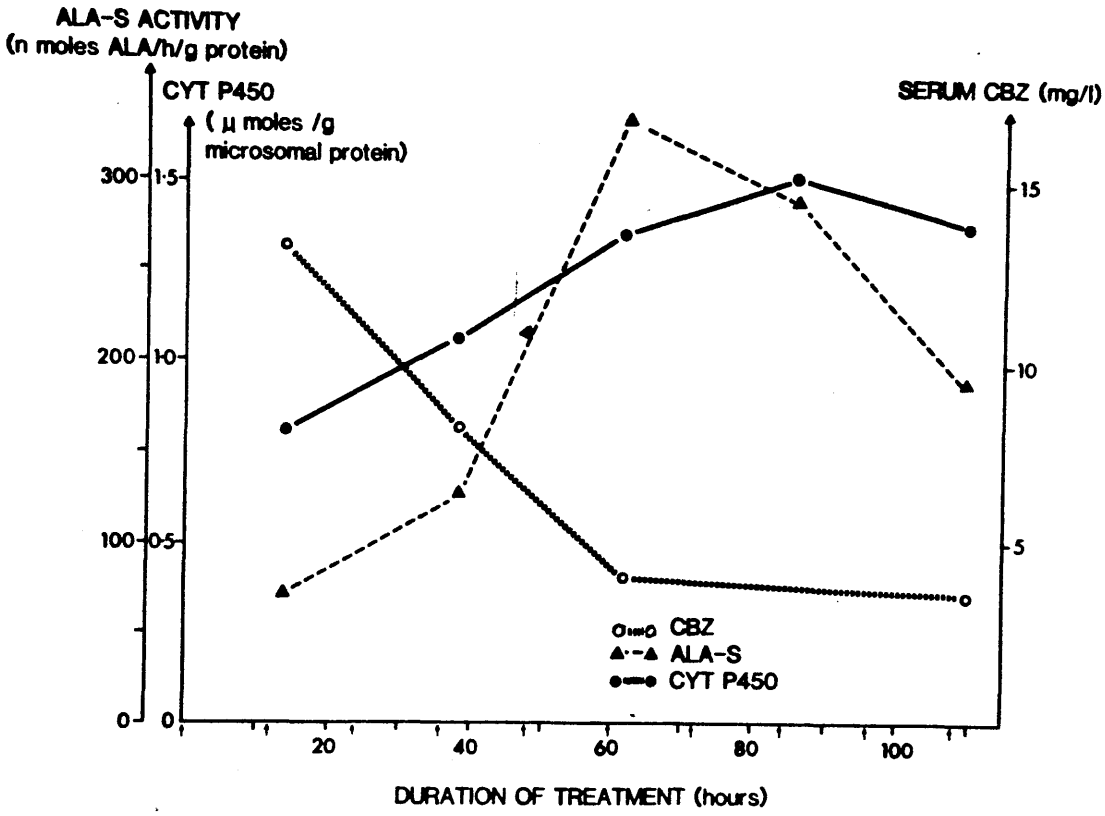


FIG. (26)

THE RELATIONSHIPS BETWEEN DEREPRESSION OF ALA-S, INDUCTION OF OF CYTOCHROME P450 AND SERUM CBZ CONCENTRATION IN CBZ-TREATED RATS

The figure shows the effects of CBZ treatment (60 mg/kg b.d.) on serum CBZ concentration, hepatic ALA-S activity and microsomal cytP450 concentration. As treatment progresses, ALA-S is derepressed in order to produce supplementary haem to satisfy the increased requirement. This haem is used in the synthesis of microsomal cytP450 , leading to increased concentrations of this protein. As cytP450 is induced in this way, serum CBZ concentrations fall.

Each point on the figure is the mean result of measurements from 4 animals. The arrows on the baseline indicate the times at which CBZ was administered.

results from study C. Mean serum CBZ concentrations are also plotted. Peak levels of cytP450 were preceded by the peak levels of ALA-S activity. This pattern occurs because metabolism of drug may initiate de novo synthesis of cytP450 apoprotein (as discussed in 1.3.9. The newly synthesized apoprotein binds haem from the proposed "free haem pool" in the cell. This is limited, and the fall in the intracellular free haem levels causes derepression of ALA-S, which in turn leads to increased synthesis of haem. This newly synthesized haem binds cytP450 apoprotein to form functional P450 leading to the peak levels of the complex as detected at day 4. How does treatment with CBZ induce cytP450? One possible mechanism of induction is destruction of cytP450 in the course of metabolism. Many olefinic substances are metabolized by cytP450 to form an epoxide group from a carbon-carbon double bond, such as occurs with CBZ. The majority of olefins metabolized in this way cause alkylation of the prostethic haem group of the cytochrome, rendering the enzyme inactive. CBZ however does not act in this way (Ortiz de Montellano et al, 1981; Ortiz de Montellano and Correia, 1983). CBZ-E also does not have any effect on cytP450 concentrations (Jung, Bentley and Oesch, 1980). It is interesting to note that in both studies (A) and (C) there was a slight decrease in the mean levels of cytP450 2 hours after the second dose of CBZ as compared to mean control values at this time, suggesting that CBZ may have some sort of destructive effect on the cytochrome. This is argued against by the papers quoted above and by the fact that cytP450 was still significantly elevated after 12 days of treatment, even though ALA-S activities had returned to normal, table (29). If CBZ was destroying cytP450 at this time, when ALA-S activity was not derepressed, the levels could

TABLE (29)

THE EFFECTS OF CBZ ON RAT LIVER CYTOCHROME P450 AFTER 5 DAYS AND AFTER 12 DAYS TREATMENT

	cyt P450[a]	ALA-S[b]	LIVER WEIGHT AS % BODY WEIGHT
CONTROL	0.92 ± 0.18	88.4 ± 3.0	3.1 ± 0.2
5 DAYS CBZ	1.53 ± 0.29***	311.1 ± 177.2***	3.7 ± 0.4**
12 DAYS CBZ	1.37 ± 0.18***	74.4 ± 18.2*	3.6 ± 0.2***

* P<0.05, ** P<0.005 *** P<0.001 as compared to controls.

Each result is the mean ± 1.S.D. for measurements from 6 animals.

a units are μmoles cytP450/g microsomal protein

b units are nmoles ALA produced/h/g protein

not be maintained and would fall. As was just stated, this does not occur, leading to the conclusion that CBZ does not in fact destroy cytP450. The actual extent of the reductions in the levels of cytP450 in these studies was small (12% and 18% for studies A and C respectively) and could be simply a consequence of the small numbers used.

To return to the effects of cytP450 induction on CBZ metabolism, it was shown in sub-section 5.1.1 that the half-life of CBZ was reduced during treatment with the drug, and it was suggested that this may be due to induction of cytP450, leading to a faster rate of metabolism and consequently a faster rate of clearance. Fig. (26) shows this to be the case. When mean serum CBZ concentrations are plotted on the same graph as cytP450 levels, it can be seen that as the levels of cytP450 in these animals increases, the mean serum concentrations (two hours after the last dose in each case) fall and then level out once cytP450 is maximally induced. The fact that this rate of clearance is maintained was proven by a separate experiment in which mean serum CBZ concentrations were shown to be essentially the same after 12 days treatment as they were after 5 days treatment, table (24).

5.4.2 SUMMARY AND GENERAL DISCUSSION OF SECTION 5.4

Results from this section revealed that CBZ treatment induces cytP450 synthesis in rat liver and that the cytochrome produced belongs to the same family as that induced by phenobarbitone. The induction of cytP450 synthesis would appear to be the underlying mechanism whereby ALA-S is derepressed (5.2.1). Furthermore, the increased levels of cytP450 provide the explanation for the increased

rate of clearance of CBZ noted in sub-section 5.1.1. Considering these facts and bearing in mind that Rapeport et al, (1983) showed that antipyrine clearance (an index of hepatic mixed function oxidase activity), was increased during CBZ treatment in man, it would be reasonable to assume that the derepression of ALA-S which is found in man during chronic CBZ treatment (see Section 3.1) was due to induction of cytP450. A further point in favour of this was the increased 6B-hydroxycortisol excretion found in these patients. The mechanism of induction is unknown, but is probably not due to CBZ-mediated destruction of existing cytP450.

5.5 CBZ AND GLUTATHIONE

Glutathione (see 4.1.5) is a cysteine containing tripeptide found in cells which acts as a sulphydryl buffer, maintaining the thiol groups of intracellular proteins in the reduced state. The effects of CBZ treatment on glutathione concentrations in rat liver were studied for two reasons, both of which were related to the fact that ALA-D is susceptible to loss of activity through oxidation of sensitive thiol groups. These reasons are (a) if CBZ or a metabolite caused a depletion of GSH levels this would lead to reduced ALA-D activity and (b) if CBZ caused a loss of ALA-D activity by indiscriminate reaction with thiols, then the concentration of glutathione would also be reduced. It could be argued that if the former possibility was true, activity would be restored by treatment with activators as described elsewhere. Although no changes were found in glutathione concentrations of human erythrocytes, rat liver glutathiones were measured for completeness.

5.5.1 THE EFFECTS OF CBZ TREATMENT ON RAT LIVER GLUTATHIONE

The effects of CBZ treatment on rat liver glutathione concentrations were investigated. The method used measured total glutathione (i.e. both oxidized and reduced forms) and is described in sub-section 2.10.2. For the reasons described in sub-section 4.1.5, proposed complexes of glutathione and CBZ should not interfere with the assay. The results of two studies are presented in table (30). The first part of the table, study (A), reveals that treatment with 100mg CBZ/kg body weight b.d. for 5 days reduced mean liver glutathione concentrations by 49% with respect to mean control values. ALA-D activity was also measured and, as expected, was reduced. No

TABLE (30)

THE EFFECTS OF CBZ TREATMENT ON RAT

LIVER GLUTATHIONE CONCENTRATIONS

	LIVER GLUTATHIONE[a]	ALA-D[b]	SERUM [CBZ][c]
Study A			
Control	6.3 ± 2.8	13.0 ± 3.9	-
CBZ	3.2 ± 0.9**	9.0 ± 2.1*	7.3 ± 0.8
Study B			
Control	7.3 ± 1.3	7.8 ± 0.9	-
Group 1	6.7 ± 1.0	7.1 ± 0.3	3.7 ± 0.5
Group 2	5.6 ± 1.2*	6.4 ± 0.5**	5.3 ± 0.6
Group 3	4.5 ± 1.3**	5.4 ± 0.4***	8.1 ± 1.9

* $P<0.05$, ** $P<0.005$, *** $P<0.001$ as compared to controls

Each result is the mean ± 1.S.D. of measurements in 6 animals.

Study A CBZ treated rats were given 100mg/kg b.d. for 5 days.

Study B rats were treated with 75 mg/kg b.d. for 7 days. The following doses were then administered for a further 7 days: Group 1, 50mg/kg b.d.; Group 2, 75mg/kg b.d.; Group 3, 150mg/kg b.d.

a units are μmoles glutathione/g protein

b units are μmoles PBG produced/h/g protein

c units are mg/l

correlation was found between hepatic glutathione levels and serum CBZ concentrations, but as was the case with rat liver ALA-D in sub-section 5.2.1, this was probably due to the fact that CBZ concentrations were all very similar. The second part of table (30), which presents the results of study (B), shows that the extent of the reduction in rat liver glutathione was in fact dependent on the dose administered and the resultant serum CBZ concentration. These results would suggest that treatment with CBZ produces an indiscriminate reduction of intracellular thiol groups. This, however, contradicts the results obtained in sub-section 4.1.5. Why should CBZ cause depletion of glutathione in rat liver and not in human erythrocytes? One possible explanation is that the higher levels of epoxide or some other CBZ metabolite in the rat cause the loss of glutathione, but because these are present in lower concentrations or absent in man, glutathione concentrations were unaffected. This is improbable because ALA-D activity would then be more severely affected in the rat and this is not the case. The most probable explanation for the differences lies in the nature of the tissues being examined. In the liver, in addition to maintaining intracellular thiols in the reduced state, glutathione has an important role to play in the detoxification of the metabolites of xenobiotics. This role involves conjugation of glutathione to such compounds by the action of glutathione transferases of which several are known (Ketterer et al, 1984). This renders them more easily eliminated. No glutathione conjugates of CBZ or its metabolites have been reported. This however does not exclude the possibility of their formation as many drug-glutathione conjugates are very unstable.

This proposed mechanism of glutathione depletion would not be applicable to erythrocytes as they lack the necessary enzymes: Consequently, erythrocyte glutathione concentrations were unaffected by treatment with CBZ.

There was no correlation between glutathione concentrations and the levels of ALA-D activity (or any other of the haem biosynthetic enzymes) in rat liver.

5.5.2 SUMMARY OF SECTION 5.5

In contrast with the findings on the effects of CBZ treatment on erythrocyte glutathione in man, rat liver glutathione was reduced in a dose dependent manner during CBZ treatment. However, it is believed that this was a consequence of the action of glutathione transferases conjugating glutathione to CBZ and its metabolites to facilitate their elimination, rather than an indiscriminate attack of CBZ or a metabolite thereof on glutathione. Bearing in mind the results from 4.1.5, the conclusion is that ALA-D activity is not reduced as a consequence of glutathione depletion or a non-specific destruction of thiol groups by CBZ or its metabolites.

5.6 SUMMARY OF CHAPTER 5

This chapter investigated the potential of the rat as a system for further study of the effects of CBZ on haem biosynthesis in man. The results of this chapter are summarised below.

The effects of CBZ on the enzymes of haem biosynthesis in rat liver were in general very similar to those observed in peripheral blood cells in man: a dose-dependent derepression of ALA-S; a dose-dependent, irreversible reduction in ALA-D activity and no effects on PBG-D or URO-D. However, in contrast to man, there was no sustainment of the derepression of ALA-S in rat liver.

With regard to the urinary excretion of porphyrins and precursors in the rat, treatment with CBZ caused some changes in ALA and PBG excretion but did not at any time produce the large increases in the uroporphyrin fraction found in the early stages of treatment in man. No satisfactory results were obtained on the effects of CBZ treatment on the quantitative excretion of porphyrin.

Treatment with CBZ induced *de novo* synthesis of cyt P450 in rat liver. This induction was linked to derepression of ALA-S and was inversely related to the serum concentration of CBZ. This indicates that, in the rat, the derepression of ALA-S produced by CBZ is mediated through induction of cyt P450 which is required for metabolism of the drug. This is probably also the case in man.

Hepatic glutathione was reduced in the rat as a result of CBZ treatment. This was probably due to the involvement of this compound in detoxification processes.

In conclusion, these studies of the effects of CBZ on haem biosynthesis in the rat have shown that in some aspects at least, the pathway is affected in the same way as in man and have been of use in explaining some of the findings made in earlier chapters.

CHAPTER 6

CHAPTER 6

CARBAMAZEPINE AND ALA-D:SOME IN VITRO STUDIES

Preceding chapters have shown that CBZ treatment results in reductions in the levels of ALA-D activity in both human erythrocytes and rat liver. However the mechanism whereby activity is lost is unknown. This chapter describes attempts to reproduce the depression of activity which occurs *in vivo* during CBZ treatment using several *in vitro* systems. The objective was to find a system which could be used as a model to study the mechanism whereby CBZ causes a loss of ALA-D activity. The problem was approached in several ways using the following 3 basic systems:

(1) human blood

(2) preparations of rat liver

(3) purified bovine liver ALA-D

None of the experiments in this chapter were capable of showing that the observed effects occurred through an indirect action of CBZ or its metabolites, or that they were due to an alteration in the rate of synthesis of ALA-D. Rather, because all of the above systems are metabolically inert, the experiments were designed purely to test for a direct effect of CBZ (or the metabolites studied) on the ALA-D enzyme protein.

The drugs studied were CBZ and its principal metabolite, CBZ-E. In some experiments, 10,11-dihydrocarbamazepine (H_2CBZ) was also included.

The chapter is split into four sections. Three of these are devoted to each of the aforementioned systems. The fourth summarises and discusses the significance of the results obtained in the chapter.

6.1 THE IN VITRO EFFECTS OF CBZ AND METABOLITES ON ERYTHROCYTE ALA-D

The effects of added CBZ, CBZ-E and H_2CBZ on ALA-D activity in human blood were investigated under two sets of conditions:

(1) following addition of drugs

(2) following heating of samples to which drug had been added

In addition to these experiments, ALA-D activity was measured in erythrocytes reconstituted with control plasma or plasma from CBZ treated patients. Each of these are discussed in the following 3 sub-sections. The fourth and final sub-section summarises the results. In all experiments, the blood used was freshly drawn and heparinised. When isolated erythrocytes and plasma were required, they were prepared from fresh heparinised blood. Two methods of adding drugs were tried: drugs were dissolved in either acetone or methanol and added directly to whole blood or were added to potassium phosphate buffer (0.15M,pH7.2) which was added to washed erythrocytes. Both these methods gave identical results.

6.1.1 THE IN VITRO EFFECTS OF ADDED CBZ, CBZ-E AND H_2CBZ ON ERYTHROCYTE OF ALA-D ACTIVITY IN WHOLE BLOOD

The effects of added CBZ, CBZ-E and H_2CBZ on both circulating and restored ALA-D activities in whole and sonicated blood were investigated. The concentration of the drugs used varied from pharmacological concentrations of 10mg/l to levels of 50mg/l, far in excess of any concentration liable to occur during treatment. Enzyme activity in both sonicated and whole blood was measured for several days following additions of the drugs. In all of these experiments there was no effect on ALA-D activity as compared to controls. Some typical results are presented in tables (31) and (32). The fact that

TABLE (31)

THE EFFECTS OF CBZ, CBZ-E AND H_2CBZ ON ALA-D ACTIVITY IN WHOLE BLOOD OVER 4 DAYS

ADDITIONS	EXPOSURE TIME (HOURS)			
	2	26	50	74
Acetone	999	1016	850	814
CBZ[a]	1002	962	887	833
CBZ-E[b]	1056	1029	870	817
H_2CBZ[b]	1010	992	873	806

All additions were made in 300µl acetone to 6ml whole blood.

[a] present at a final plasma concentration of 50mg/l

[b] present at a final plasma concentration of 25 mg/l

ALA-D activities are expressed as nmoles PBG produced/h/ml erythrocytes.

Samples were stored at 4°C between measurements of activity.

TABLE (32)

THE EFFECTS OF CBZ, CBZ-E AND H_2CBZ ON ALA-D ACTIVITY IN WHOLE AND SONICATED BLOOD

ADDITIONS	CIRCULATING ALA-DACTIVITY	ALA-D ACTIVITY IN SONICATED CELLS	RESTORED ALA-D ACTIVITY
Acetone	1184	1080	1905
CBZ	1233	1095	1878
CBZ-E	1197	1077	1911
H_2CBZ	1200	1065	1920

All additions were made in 100µl of acetone to 2ml whole blood.

All drugs were present at final plasma concentrations of 50mg/l.

ALA-D activities were determined after 4 hours incubation at room temperature following the addition of drug.

ALA-D activity was not affected means that either CBZ, CBZ-E or H_2CBZ do not act directly on the enzyme to reduce activity, or if they do, then the reaction is slow. This latter possibility is investigated in the following sub-section.

Conclusions on the effects of added CBZ, CBZ-E, and H_2CBZ on erythrocyte ALA-D activity in whole blood

None of the compounds tested had any effect on circulating or restored ALA-D activity in whole or sonicated blood either immediately or after several days exposure to the drug.

6.1.2 THE EFFECTS OF HEAT TREATMENT ON ERYTHROCYTE ALA-D ACTIVITY IN WHOLE BLOOD FOLLOWING ADDITION OF CBZ OR CBZ-E

Due to the absence of any change in ALA-D activity following the addition of CBZ, CBZ-E or H_2CBZ, whole blood to which CBZ or CBZ-E had been added was heated to investigate if this would accelerate what may have been a slow reaction between either of these compounds and the enzyme. Heat treatment of whole blood usually results in an increase in erythrocyte ALA-D activity. (Chiba, 1976; Ushio *et al*, 1975). Reasons for this are unknown, but the most popular explanation is that heating results in the destruction of a heat labile inhibitory factor (Sakai, Yanagihara and Ushio, 1981; Trevisan *et al*, 1983; Kondo, Kajimoto and Urata, 1983). Preliminary studies established that when blood was incubated at 60°C, the maximal increase in activity occurred after 5 minutes. Thereafter activity declined. Therefore this temperature and incubation period were routinely used. Heat treatment also caused haemolysis therefore it was necessary to determine the packed cell volume beforehand. Following additions of drugs and heat treatment, ALA-D activity was measured in two ways. (a) without further treatment and (b) following the addition of

dithiothreitol (DTT) and $ZnCl_2$. Activities were determined immediately after heating, then again after 24 hours. Between assays the samples were stored at 4°C. The results of CBZ additions are shown in table (33) and the results of CBZ-E additions in table (34).

The results presented in these tables show that there was no effect of CBZ or CBZ-E on either untreated or restored enzyme activites following heat treatment of blood to which either compound had been added. Even after a further 24 hours, no loss of activities with respect to controls had occurred.

These findings suggest that if the loss of ALA-D activity shown to occur during CBZ treatment is caused by CBZ itself or CBZ-E, then it does not arise through a direct action of either compound on the enzyme. This does not exclude the possibility that the observed effect is a direct result of the action of some other minor metabolite. This possibility is explored in the following sub-section.

Conclusions on the effects of heat-treatment on erythrocte ALA-D activity in whole blood following the addition of CBZ or CBZ-E

Heat treatment of ALA-D in blood to which CBZ or CBZ-E had been added failed to produce any effect on untreated or restored enzyme activities.

6.1.3 THE EFFECTS OF RECONSTITUTION WITH CONTROL AND CBZ TREATED PLASMA ON ERYTHROCYTE ALA-D ACTIVITY

This sub-section looked at the effects of reconstituting control erythrocytes with fresh control plasma and fresh plasma from CBZ treated epileptic patients, on ALA-D activity. The purpose of these experiments was to investigate if there was some other constituent in

TABLE (33)

THE EFFECTS OF HEAT TREATMENT ON ALA-D ACTIVITY FOLLOWING THE ADDITION OF CBZ TO WHOLE BLOOD

CBZ[a]	UNTREATED ALA-D ACTIVITY[a]		RESTORED ALA-D ACTIVITY[b]	
	DAY 1	DAY 2	DAY 1	DAY 2
0	1110	801	1323	1383
2	1143	804	1311	1314
4	1107	777	1311	1368
6	1119	831	1299	1281
8	1113	819	1302	1389
10	1107	855	1317	1407
15	1098	789	1305	1374
20	1086	795	1311	1371

Blood was incubated at 60°C for 5 minutes following addition of CBZ.

ALA-D activities were determined immediately after this treatment (Day 1 results) and after a further 24 hours at 4°C (Day 2 results).

[a] units are mgCBZ/l plasma

[b] units are nmoles PBG produced/h/ml erythrocytes

TABLE (34)

THE EFFECTS OF HEAT TREATMENT ON ALA-D ACTIVITY FOLLOWING THE ADDITION OF CBZ-E TO WHOLE BLOOD

CBZ-E[a]	UNTREATED ALA-D ACTIVITY [a]		RESTORED ALA-D ACTIVITY[b]	
	DAY 1	DAY 2	DAY 1	DAY 2
0	1080	768	1581	1440
1	1104	750	1641	1425
2	1089	729	1623	1413
3	1107	747	1638	1470
4	1056	759	1617	1449
5	1077	783	1617	1431

Blood was incubated at 60°C for 5 minutes following addition of CBZ-E.

ALA-D activities were determined immediately after this treatment (Day 1 results) and after a further 24 hours at 4°C (Day 2 results).

a units are mg CBZ-E/l plasma

b units are mmoles PBG produced/h/ml erythrocytes

the blood of CBZ treated patients responsible for the loss of activity which occurs during treatment. It may appear that these experiments were unnecessary because it has been shown in earlier sections that even when there were high plasma concentrations of CBZ and CBZ-E, erythrocyte ALA-D activity was unaffected over the first few days of treatment. Indeed these experiments would be senseless if it was believed that the effect was caused directly by CBZ or CBZ-E as this possibility has already been tested and rejected in the two preceding sub-sections of this chapter. The purpose of the experiments was to investigate if ALA-D activity was directly reduced by some minor metabolite of CBZ, only produced in effective quantities once induction of hepatic mixed function oxidase activity has occurred. If this was true, then plasma from a patient who has been receiving CBZ treatment for some time should contain some of this effector and may cause a reduction of ALA-D activity when added to control erythrocytes.

Experiments were conducted as follows. Plasma was prepared from fresh heparinised blood from control subjects and from CBZ-treated epileptic patients who exhibited reduced erythrocyte ALA-D activities. These plasmas were used to reconstitute erythrocytes from control and CBZ treated subjects. Activities were determined 16 hours and 40 hours after reconstitution. Between assays, samples were stored at $4^{o}C$.

The results shown in table (35) are typical of all the experiments conducted in this sub-section. They show two things. The first of these is that the ALA-D activity in the erythrocytes of CBZ treated patients was not restored by the addition of control

TABLE (35)

THE EFFECTS OF ADDING PLASMA FROM CBZ-TREATED PATIENTS ON ALA-D ACTIVITY IN CONTROL ERYTHROCYTES AND VICE VERSA

PLASMA / ERYTHROCYTES	ALA-D ACTIVITY[a] 16 HOURS AFTER MIXING			ALA-D ACTIVITY[a] 40 HOURS AFTER MIXING		
	CBZ	CONTROL 1	CONTROL 2	CBZ	CONTROL 1	CONTROL 2
CBZ	48	48	51	33	39	36
CONTROL 1	816	792	824	558	576	606
CONTROL 2	1221	1215	1152	1125	1128	1140

[a] Units are nmoles PBG produced/h/ml erythrocytes

Plasma was prepared from fresh heparinised blood from a CBZ-treated epileptic patient and from two control subjects, then each was individually mixed with erythrocytes from the same sample and from the other two.

plasma. This would be expected because other attempts at reversing the loss of activity have failed. The second is that, even after 40 hours, ALA-D activity in control erythrocytes was unaffected by reconstitution with plasma from CBZ-treated patients.

Conclusions on the effects of reconstitution with control and CBZ treated plasma on erythrocyte ALA-D activity

The effects of CBZ treatment on erythrocyte ALA-D activity could not be repeated by reconstitution of control erythrocytes with plasma from a CBZ-treated epileptic patient with reduced enzyme activity.

6.1.4 SUMMARY OF SECTION 6.1

Under no circumstances could a direct effect of CBZ, CBZ-E or H_2CBZ be demonstrated on ALA-D activity in human erythrocytes. These included the use of non-sonicated and sonicated cells, the use of drug concentrations greatly in excess of those encountered *in vivo*, measurement of activity over a number of days following addition of drug, measurements of the effects on both untreated and restored activities, heating of drugs with whole blood and the use of plasma from chronically treated patients as a source of drug.

6.2 THE IN VITRO EFFECTS OF CBZ, CBZ-E AND H_2CBZ ON ALA-D ACTIVITY IN RAT LIVER

This section investigated the effects of added CBZ, CBZ-E and H_2CBZ on ALA-D activity in preparations of rat liver.

The experiments were performed on samples of rat liver prepared as described in sub-section 2.2.2 for routine determination of ALA-D activity. Drugs were dissolved in acetone and 50µl of these solutions was added to 2ml fractions of liver preparation to give final CBZ, CBZ-E and H_2CBZ concentrations of 250, 125 and 125mg/l respectively.

The results of two such experiments are presented in table (36) and reveal that none of these compounds had any effects on ALA-D activity. These results show that the depression of ALA-D activity in rat liver shown to occur during treatment with CBZ was not caused by a direct action of any of the above compounds. This provides confirmation of the results obtained in sub-sections 4.1.2 and 5.2.2 where it was shown that ALA-D activities were unaffected in the initial stages of treatment even in the presence of high concentrations of CBZ and CBZ-E.

Conclusions on the in vitro effects of CBZ, CBZ-E or H_2CBZ on ALA-D activity in rat liver

Neither CBZ, CBZ-E or H_2CBZ had any demonstrable effect on rat liver ALA-D activity *in vitro* even though concentrations far in excess of those occurring during treatment were used.

TABLE (36)

THE IN VITRO EFFECTS OF CBZ, CBZ-E AND H_2CBZ ON ALA-D ACTIVITY IN PREPARATIONS OF RAT LIVER

ADDITION	ALA-D ACTIVITY[a]	
	RAT 1	RAT 2
Buffer	13.1	10.6
Acetone	12.1	10.6
CBZ[b]	11.9	10.6
CBZ-E[c]	12.0	10.5
H_2CBZ[c]	12.1	10.5

a units are umoles PBG produced/h/g protein

b present at a final concentration of 250mg/l

c present at a final concentration of 125mg/l

6.3 THE IN VITRO EFFECTS OF CBZ, CBZ-E AND H_2CBZ ON PURIFIED BOVINE LIVER ALA-D

The effects of added CBZ, CBZ-E or H_2CBZ on the activity of ALA-D purified from bovine liver as described in sub-section 2.9.1 were investigated. Although the *in vivo* effects of CBZ treatment on this enzyme are not known, there is no reason to believe that the response would be any different to that observed in human erythrocytes or rat liver.

The results of two experiments are presented in table (37). In the first of these, drugs were added to a solution of enzyme as described in sub-section 2.7.4 and activity measured after 4 hours incubation at room temperature. The second experiment used higher concentrations of drugs and the samples to which drugs had been added were incubated at 37°C for 30 minutes, then at room temperature for a further 3 hours 30 minutes before determination of activity. The drug concentrations used in both of these experiments are shown in the table. Examinations of the results reveals that none of the treatments described resulted in any changes in activity with respect to controls. This is perhaps not surprising in view of the lack of effect of these compounds on enzyme activity in human erythrocytes and rat liver as described in the previous sections. In those experiments, enzyme activities were determined in the absence of DTT and $ZnCl_2$. However it was necessary to include these compounds in the assay of purified enzyme otherwise no activity was detectable. Experiments identical to those described above but to which DTT and $ZnCl_2$ had been added before addition of the drugs were also performed. These experiments were also unable to show any effect on ALA-D activity.

TABLE (37)

THE EFFECTS OF ADDED CBZ, CBZ-E AND H_2CBZ ON PURIFIED BOVINE LIVER ALA-D

ADDITION	STUDY 1		STUDY 2	
	[DRUG][a]	ALA-D ACTIVITY[b]	[DRUG][a]	ALA-D ACTIVITY[b]
Buffer	0	100.0 ± 6.8	0	100.0 ± 2.4
Acetone	0	100.2 ± 1.9	0	100.3 ± 2.4
CBZ	50	98.3 ± 1.5	500	98.2 ± 2.6
CBZ-E	50	102.5 ± 1.5	250	99.3 ± 3.2
H_2CBZ	50	99.1 ± 1.9	250	99.3 ± 1.8

Study 1 Activity was measured after 4 hours incubation at room temperature following addition of drug.

Study 2 After the addition of drugs, samples were incubated at 37°C for 30 minutes, then at room temperature for a further 3 hours 30 minutes before measuring activity.

[a] units are mg/l

[b] results expressed as percentages of controls

Conclusions on the in vitro effects of CBZ, CBZ-E and H_2CBZ on purified bovine liver ALA-D

ALA-D purified from bovine liver was not affected by the CBZ, CBZ-E or H_2CBZ either in the presence or absence of DTT and $ZnCl_2$.

6.4 SUMMARY OF CHAPTER 6

This chapter described attempts to find an *in vitro* model system to study the mechanism of the reduction in the levels of ALA-D activity shown to occur during treatment with CBZ in both man and rat. This was done by looking for a direct action of CBZ or two of its metabolites on ALA-D activity in a number of different systems. It may be said that a direct effect of CBZ or CBZ-E on the enzyme was unlikely, as it has been shown in earlier sections that in both man and rat, ALA-D activity can be unaffected by concentrations of CBZ and CBZ-E which correlate with loss of activity in other patients or animals. However, there are many metabolic processes taking place in these *in vitro* systems which complicate the overall picture and which may mask a direct effect. Therefore experiments such as these were necessary to prove if in fact the effect is direct.

The conclusion of the chapter is that none of the systems studied provide a model for studying the effects of CBZ treatment on ALA-D activity. The results indicate that in all probability, the loss of ALA-D activity is not caused by a direct effect of CBZ, CBZ-E or H_2CBZ on the enzyme. The possibility that it is direct but is slow was eliminated. The results do not exclude the possibility of the effect occurring through a direct action of some metabolite of CBZ not tested here. However the results obtained would indicate that if this were true then this metabolite must be short lived. As was stated in the introduction to this chapter, these experiments did not test for indirect effects of CBZ or any of its metabolites. The experiments described in the following chapters attempt to investigate these possibilities.

CHAPTER 7

CHAPTER 7

CARBAMAZEPINE AND HAEM BIOSYNTHESIS IN CULTURED HUMAN LYMPHOCYTES

In the preceding chapters, the effects of CBZ on several aspects of haem biosynthesis, in particular on ALA-D, in several different systems have been investigated. These included the study of the effects of the drug on *in vivo* systems, (man and rat), and on *in vitro* systems, such as isolated human erythrocytes, crude rat liver preparations and purified bovine liver ALA-D. Each of these has several drawbacks as far as elucidating the mechanism of the CBZ-mediated reduction in ALA-D activity is concerned. For instance, in complex entities such as man and the rat, there are many other processes taking place which may affect or be affected by ALA-D. There is extensive metabolism of CBZ which generates uncertainty as to which metabolite is responsible. The fact that individual persons and animals differ in their response to treatment with CBZ, shown by the ranges of drug concentration in plasma and the varying extent of the reduction in ALA-D activity, is also a complicating factor. The major problem with all of the *in vitro* systems studied so far is that they are metabolically inert. For example, red cells are not actively respiring, synthesizing proteins or performing any of the other processes commonly associated with living cells. The same is true of the preparations of rat liver and of purified enzyme. The use of cell culture eliminates several of these problems. For instance, it is possible to precisely define variables such as drug concentration, there are no problems due to different rates of uptake, metabolism and excretion of drug, and the cells are metabolically active. Mitogen stimulated human lymphocytes were used because the

cells were readily available in sufficient quantities for the proposed experiments and also because there are many reports in the literature describing methods for measuring the activities of the relevant enzymes in these cells. Human monocytes were evaluated as a potential system of study, but these were not available in sufficient numbers. This problem could have been overcome by the use of a lymphocyte tumour cell line. This was rejected because activities of enzymes were low compared to lymphocytes and without liquid nitrogen storage facilities it would have been both costly and troublesome to maintain the continuous culture which the use of these cells would have dictated. This latter reason also ruled out Epstein-Barr virus transformed lymphocytes.

The chapter is split into four sections, the first three of which are concerned with the effects of both CBZ and CBZ-E on (1) ALA-D activity, (2) PBG-D activity and (3) the capacity of the cells to synthesize porphyrins. As described in methods sub-section 2.2.1, the methods tested for the determination of ALA-S activity in these cells were not satisfactory and therefore no ALA-S results are presented. The final section summarises the chapter.

In all the experiments described, cultures were prepared and maintained as described in section 2.6 and enzyme activities were determined as described in the relative parts of section 2.2.

7.1 THE EFFECTS OF CBZ AND CBZ-E ON ALA-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

The effects of both CBZ and CBZ-E on ALA-D activity in cultured human lymphocytes were investigated. This section is divided into four sub-sections. The first of these investigates the effects of fixed doses of CBZ and CBZ-E on ALA-D activity, the second investigates the effects of varying doses of CBZ-E on enzyme activity and the third investigates the effects of varying the exposure time of cultures to CBZ-E on ALA-D activity. The fourth summarises the section.

7.1.1 THE EFFECTS OF CBZ AND CBZ-E ON ALA-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

This section describes the first investigations into the effects of CBZ and CBZ-E on enzyme activity in mitogen-stimulated lymphocytes. The results of five experiments were collected and are presented in table (38). Activities are shown as percentages of mean control values in order to eliminate the spread of activities found in cells from different individuals. In all of these experiments, CBZ was present at a concentration of 30mg/l and CBZ-E was present at 15mg/l. In both instances, the cells were exposed to the drugs for 96 hours, after which ALA-D activity was measured. The results show that both CBZ and CBZ-E reduced ALA-D activity, but that CBZ-E was more effective in doing so. Activity in CBZ treated cells was reduced by a mean value of 16.9% ($P<0.001$) whereas half as much CBZ-E reduced activity by 77.4% ($P<0.001$) on average. These results would suggest that CBZ-E is the species responsible for the reduction in ALA-D activity in man and in the rat and that CBZ only results in depression

TABLE (38)

THE EFFECTS OF CBZ AND CBZ-E ON ALA-D ACTIVITY

IN CULTURED HUMAN LYMPHOCYTES

	CONTROL	CBZ (30mg/l)	CBZ-E (15mg/l)
ALA-D ACTIVITY[a]	100 ± 5.3 (n=13)	83.1 ± 5.8* (n=11)	22.6 ± 4.1* (n=10)

[a] activities are expressed as percentages of control values

* $P<0.001$ as compared to control

of the levels of ALA-D activity because it is metabolized to the epoxide. However, when samples of medium were analysed by HPLC, no metabolism of CBZ or CBZ-E had occurred. This proves that CBZ has the capacity to reduce ALA-D activity in its own right. The addition of an epoxide group potentiates this effect. These results do not prove whether the effects of CBZ and CBZ-E on the enzyme are direct or indirect.

To check that this inactivation of ALA-D was a specific property of CBZ and CBZ-E, phenobarbitone and sodium valproate were added to cultures to give final concentrations of 10mg/l and 50mg/l respectively. These two drugs do not affect ALA-D *in vivo*, and, as expected, did not affect activity in these cultured lymphocytes.

Conclusions on the effects of CBZ and CBZ-E on ALA-D activity in cultured human lymphocytes

Both drugs caused a reduction of ALA-D activity in treated cells, but CBZ-E was a more potent effector than CBZ. No metabolism of the drugs occurred, indicating that it is the basic structure of CBZ which is responsible for the depression of activity. Phenobarbitone and sodium valproate did not affect activity. Further investigations using CBZ-E alone now follow. In the course of these, it was assumed that CBZ would act in the same way: the epoxide was used because its effects were more potent and therefore more easily measured.

7.1.2 THE EFFECTS OF VARYING CONCENTRATIONS OF CBZ-E ON ALA-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

The effects of a range of concentrations of CBZ-E (up to 15mg/l) on ALA-D activity in mitogen stimulated human lymphocytes were studied to investigate if there was a relationship between drug concentration and enzyme activity. The results of exposing the cells to various

concentrations of epoxide for 96 hours are shown in fig. (27). This reveals that the effect was dose dependent. It would be reasonable to assume, bearing in mind the results of the previous sub-section, that CBZ also affects activity in a dose-dependent manner. The largest concentrations used resulted in activity which was only 26.7% of control values. This compares well with the results from sub-section 7.1.1 (see table (38)). Loss of ALA-D activity was found at epoxide concentrations which would occur during treatment with CBZ: the lowest concentration used was 3.75mg/l, and this produced a 41% reduction in enzyme activity. This concentration of drug is in the upper range of normal plasma values. It may seem reasonable to make this comparison as the lymphocytes were cultured at a density which approximates their concentration in vivo. However it must also be remembered that the epoxide concentration used here may be much higher than that effectively encountered in vivo because in the plasma there is a high degree of binding to plasma proteins which does not occur to the same extent in culture.

Conclusions on the effects of varying concentrations of CBZ-E on ALA-D activity in cultured human lymphocytes

It was shown that there was a dose dependent relationship between the concentration of CBZ-E in the medium and the activity of ALA-D in the cells. Presumably this would also apply to CBZ, but as was shown in sub-section 7.1.1, the effects would be much less marked.

7.1.3 THE TIME COURSE OF THE CBZ-E MEDIATED REDUCTION OF ALA-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

Studies in earlier chapters demonstrated that the reduction in the levels of ALA-D activity produced during CBZ treatment was a slow process. This experiment investigated the rate at which activity was

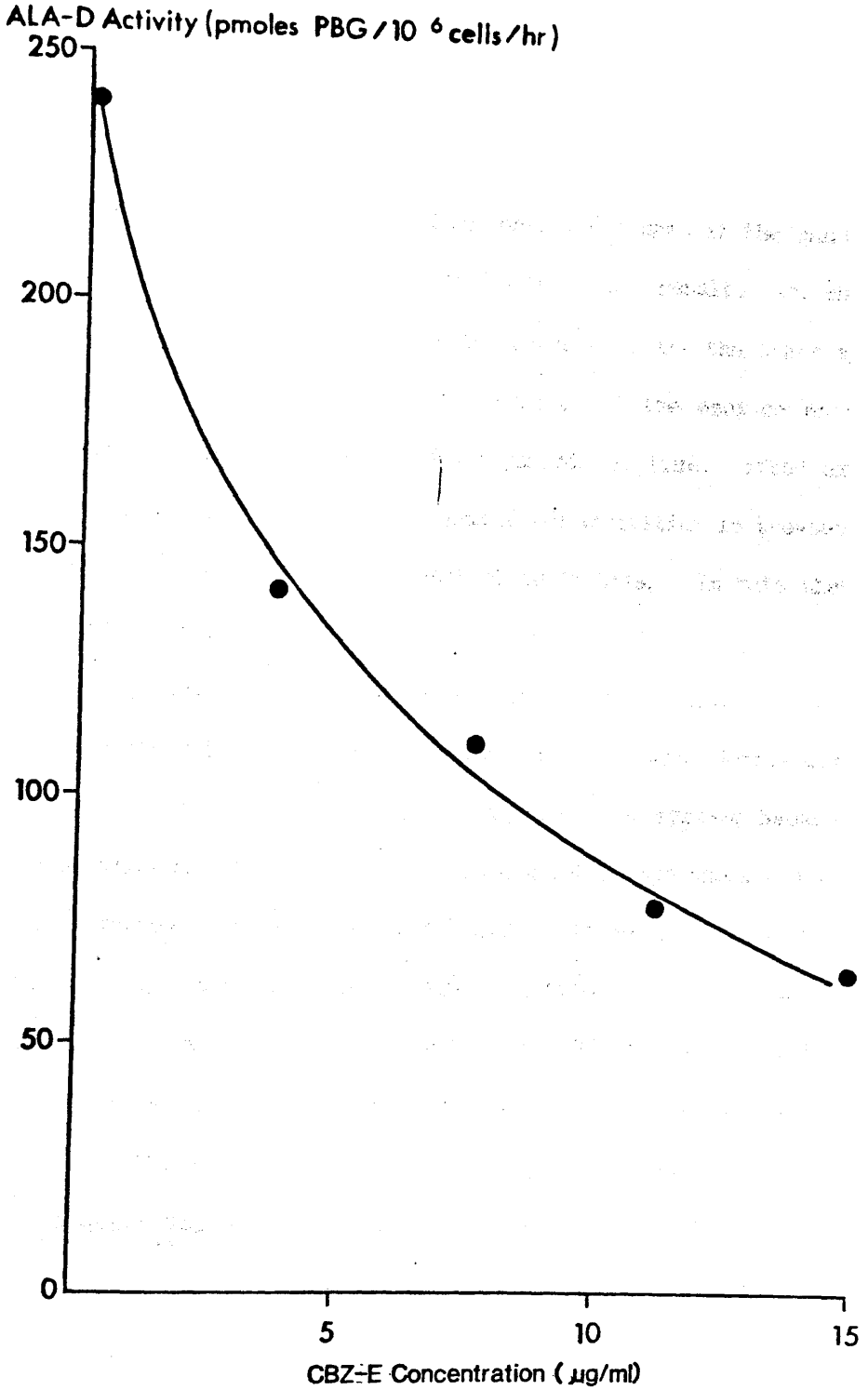


FIG.(27)

THE EFFECT OF CBZ-E ON ALA-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

CBZ-E was added to 10ml cultures of lymphocytes (cell density = 0.75 x 10^6 cells/ml) to give final concentrations of 0,3.75,7.5,11.25 and 15 µg/ml. ALA-D activity was measured after incubation for 96 hours under the conditions described in sub-section 2.6.2. Each point is the mean of 3 observations.

The figure shows that CBZ-E causes a reduction in ALA-D activity in the cells which is related to the concentration of the drug added.

lost in cultured cells by measuring ALA-D activity after varying periods of exposure to CBZ-E (15mg/l). Activity was measured in samples which had been exposed to the epoxide for periods ranging from 0 to 92 hours. DMSO was added to control cultures at the same times as CBZ-E was added to test cultures. The results are shown in fig. (28). The graph shows that in contrast to all the other systems studied in previous chapters, the effects of the epoxide on enzyme activity are detectable after short periods of time: after exposure to CBZ-E for only 5 hours, the mean ALA-D activities in treated cells were 74% of the corresponding control activities. In rats there was no detectable difference in the activity of hepatic ALA-D until 4 days after initiation of treatment and in man there was no change in erythrocyte activity for at least two days following dosage with CBZ. The response may occur more rapidly in this system because the concentrations of CBZ-E used were much greater than those which might occur during treatment and also because there are no complicating factors such as elimination or further metabolism of the drug. After the longest period of exposure to the drug (92 hours), mean activities were reduced to 21.9% of control values. This compares favourably with results gained in sub-section 7.1.1 (see table (38)).

Conclusions on the time course of the CBZ-E mediated reduction of ALA-D activity in cultured human lymphocytes

CBZ-E caused a reduction of ALA-D in activity at a much greater rate in this lymphocyte cell culture system than CBZ treatment did in man or in the rat.

7.1.4 SUMMARY AND GENERAL DISCUSSION OF SECTION 7.1

CBZ and CBZ-E both caused a reduction in the levels of ALA-D activity in cultured, mitogen-stimulated human lymphocytes. CBZ-E

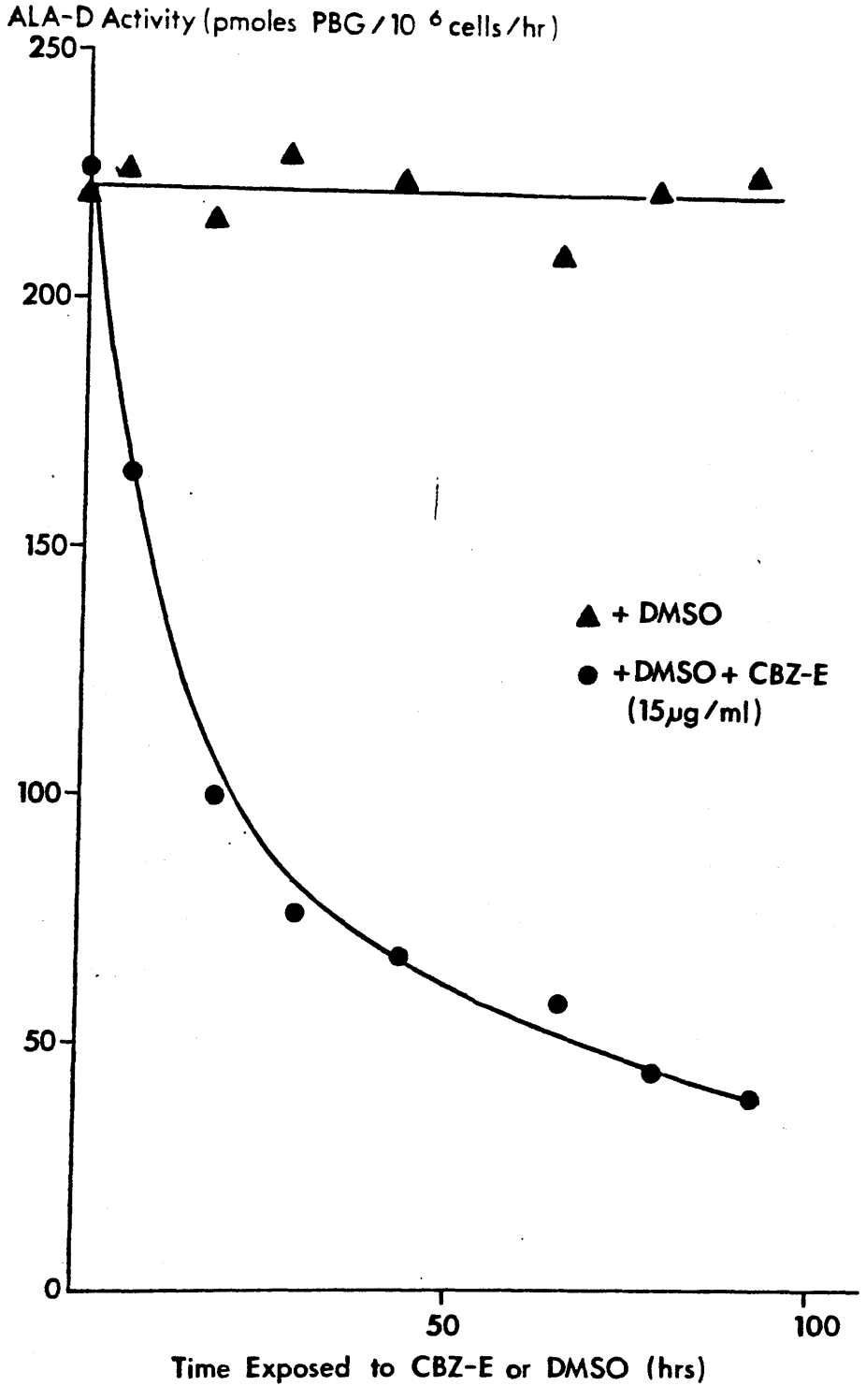


FIG. (28)

TIME COURSE OF THE EFFECT OF CBZ-E ON ALA-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

CBZ-E was added to 10ml cultures of lymphocytes (cell density = 0.75×10^6 cells/ml) to give a final concentration of 15µg/ml. The cultures were exposed to CBZ-E for 0,5,18.5,29,44,65,78.5 and 91 hours prior to assay for ALA-D activity, 96 hours after establishing the culture. Each point is the mean of 3 observations. The figure shows a progressive reduction in ALA-D activity during exposure to the drug.

was much more effective in reducing activity than was CBZ, and because of this it was used in further experiments to demonstrate that the depression of ALA-D activity was both dose and time dependent. Effects were found at pharmacological concentrations and were evident after a relatively short exposure to the drug as compared to other systems studied. In view of this powerful effect of CBZ-E, it is tempting to assume that this is the species responsible for the inhibition of ALA-D in man and in the rat. However it was demonstrated that CBZ could also reduce activity, albeit to a lesser extent, without any detectable metabolism. Thus it would seem that the presence of the epoxide group potentiates the effect. While these results would suggest that activity is reduced directly by CBZ and CBZ-E, the situation cannot be that straightforward, otherwise the simple addition of these compounds to the systems described in Chapter 6 would have produced a loss of activity. The fact that the system requires to be metabolically active suggests that some form of bioactivation occurs, the nature of which can only be speculative. This proposal would not of course be applicable if ALA-D activity was lost as a consequence of a reduced rate of enzyme synthesis. Indeed this would explain why enzyme activity was not affected in the experiments described in Chapter 6. However this section has demonstrated that this cannot be the case for the following reason (assuming that the lymphocyte and erythrocyte enzymes are identical). If ALA-D activity was depressed due to a reduction in the rate of synthesis, then activity could only be lost as quickly as observed in sub-section 7.1.3 if the enzyme had a very short half-life (17.5 hours from fig. (28). However this is not the case, otherwise activity

would rapidly be irreversibly lost in erythrocytes removed from circulation, which it is not.

A further point which must be made is that although it would seem from the results presented in sub-section 7.1.1 that CBZ-E plays a more significant role in the depressson of ALA-D activity in man than CBZ, both probably make a significant contribution <u>in vivo</u> as CBZ-E accounts for only 10-20% of the total drug concentration.

The following section is concerned with the effects of CBZ and CBZ-E on PBG-D in cultured cells.

7.2 THE EFFECTS OF CBZ AND CBZ-E ON PBG-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

This section discusses the effects of CBZ and CBZ-E on PBG-D activity. It consists of 5 sub-sections. The first two examine the nature of the effect, the second two attempt to explain it and the fifth and final sub-section summarises the results of the section.

7.2.1. THE EFFECTS OF CBZ AND CBZ-E ON PBG-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

This sub-section presents the results of preliminary investigations into the effects of CBZ and CBZ-E on PBG-D activity in mitogen stimulated lymphocytes. The results of 5 experiments were collected and are presented in table (39). Activities are expressed as percentages of mean control values in order to eliminate the spread of activities found in cells from different individuals. In each of these experiments CBZ was present at a concentration of 30mg/l and CBZ-E was present at 15mg/l. In both instances, cells were exposed to the drug for 96 hours before determination of activity. Examination of the results shown in table (39) reveals that CBZ-E seemed to cause an increase in the levels of PBG-D activity. The mean CBZ-treated value was not significantly different from the mean value of controls. The reasons why PBG-D activity should be affected by CBZ-E in this system but not apparently in man or the rat will be discussed in the final part of this section.

Conclusions on the effects of CBZ and CBZ-E on PBG-D activity in cultured human lymphocytes

CBZ-E seemed to cause an elevation of PBG-D activities whereas CBZ did not. Further investigations are described in the following sub-sections, the objectives of which are to determine if the increase

TABLE (39)

THE EFFECTS OF CBZ AND CBZ-E ON PBG-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

	CONTROL	CBZ (30mg/l)	CBZ-E (15mg/l)
PBG-D ACTIVITY[a]	100.0 ± 10.8 (n=12)	99.5 ± 29.3 (n=8)	130.4 ± 16.0* (n=11)

[a] activities are expressed as percentages of control values.

* $P<0.001$ as compared to controls.

is (a) related to drug concentration and (b) related to the inactivation of ALA-D described in the previous section.

7.2.2 THE EFFECTS OF VARYING CONCENTRATIONS OF CBZ-E ON PBG-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

The effects of a range of concentrations of CBZ-E (up to 10mg/l) on PBG-D activity in mitogen stimulated human lymphocytes were studied to determine whether or not there was a relationship between drug concentration and enzyme activity. The results of exposure to the various concentrations of drug for 96 hours are shown in fig. (29). The graph shows that the increase in PBG-D activity was related to the concentration of CBZ-E in the incubation medium.

In conjunction with the results of the previous sub-section, these findings would seem to indicate that CBZ-E did indeed produce a rise in PBG-D activity. The only plausible way in which this could have occurred would be through an increase in the amount of enzyme, i.e. an increase in enzyme synthesis. If this were the case, it could be produced either directly by CBZ-E or as a consequence of the inactivation of ALA-D. This latter possibility is investigated in the following sub-section.

7.2.3 INVESTIGATIONS INTO THE POTENTIAL RELATIONSHIP BETEEN INACTIVATION OF ALA-D AND INCREASED PBG-D ACTIVITY

The effects of lead and 4,6-dioxoheptanoic acid (DHA, succinyl acetone) on PBG-D activity in mitogen stimulated lymphocytes were studied to investigate if the apparent increase in the levels of PBG-D activity produced by CBZ-E treatment was a consequence of a reduction in the levels of ALA-D activity. Both of these compounds are potent

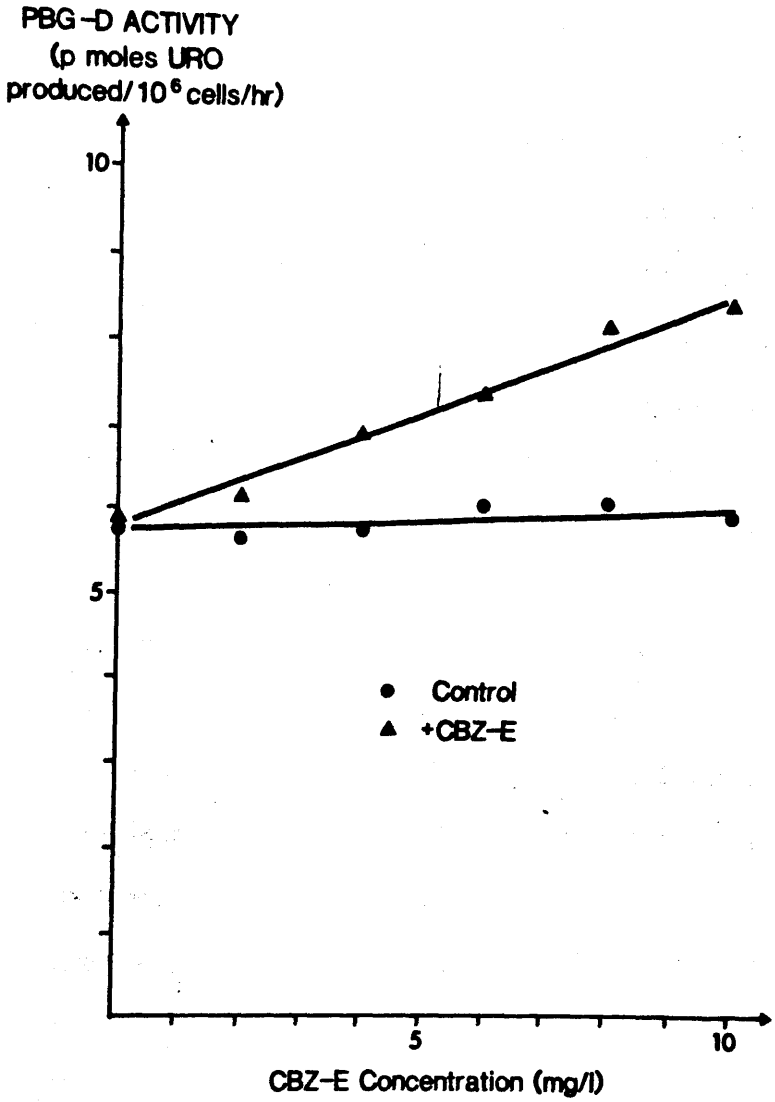


FIG. (29)

THE EFFECT OF CBZ-E ON PBG-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

CBZ-E was added to 10ml cultures of lymphocytes (cell density 0.75 x 10^6 cells/ml) to give final concentrations of 0,2.5,5,7.5 and 10µg/ml. PBG-D activity was measured after incubation for 96 hours under the conditions described in subsection 2.6.2. Each point is the mean of 3 observations. The figure shows an apparent increase in PBG-D activity which is related to the concentration of the drug added.

inhibitors of ALA-D and therefore should result in an increase in PBG-D activity if this occurs as a consequence of inactivation of ALA-D.

The additions were made to cultures prepared in the usual way. Lead was added as lead acetate to a concentration of 25 μM. DHA was added to a concentration of 50μM. Cells treated with DHA exhibited slightly increased mortality, this was corrected for when calculating results. It had previously been established, using purified enzyme, that these concentrations were effective in reducing ALA-D activity.

The results of treatment with these compounds on ALA-D and PBG-D activities are shown in table (40). The results show that only DHA appeared to reduce ALA-D activity in the cultured cells: lead appeared to have no effects. This however may be because inhibition of ALA-D by lead is reversible by treatment with dithiothreitol and Zn^{2+} ions, both of which were included in the assay. In neither case was there any increase in the levels of PBG-D activity. Therefore the apparent increase in PBG-D activity noted during treatment with CBZ-E cannot have been due to a loss of ALA-D activity, but must have occurred through some other effect of the drug. This may have been either direct or indirect. However, it is also possible that CBZ-E itself interferes with the quantification of uroporphyrin produced in the assay resulting in an inaccurate estimation of porphyrin levels as shown in sub-section 5.3.2. This is investigated in the following sub-section.

Conclusions on investigations with the potential relationship between inactivation of ALA-D and increase of PBG-D activity

These investigations revealed that there was no relationship between the loss of ALA-D activity and apparent increase in PBG-D

TABLE (40)

THE EFFECTS OF CBZ-E, LEAD ACETATE AND 4, 6, DIOXOHEPTANOIC ACID (DHA) ON ALA-D AND PBG-D ACTIVITIES IN CULTURED HUMAN LYMPHOCYTES

ADDITION	ALA-D ACTIVITY[a]	PBG-D ACTIVITY[b]
DMSO	231.1 ± 12.6	6.7 ± 0.7
CBZ-E (15mg/1)	43.2 ± 9.7	8.8 ± 1.3*
Lead acetate (25μM)	234.3 ± 10.0	6.8 ± 0.9
DHA (50μM)	20.9 ± 6.8*	6.9 ± 0.7

* $P<0.001$

[a] units are pmoles PBG produced/10^6 cells/hour

[b] units are pmoles URO produced/10^6 cells/hour

Each result represents the mean ± 1 S.D. of 4 measurements.

activity. This means that the inactivation of ALA-D by CBZ-E does not explain the apparent increase in PBG-D activity.

7.2.4 THE EFFECTS OF CBZ-E ON THE ASSAY USED FOR DETERMINATION OF PBG-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

As was shown in sub-section 5.3.2, CBZ and CBZ-E absorb at the wavelengths used for measurement of porphyrins and fluoresce following excitation with light of these wavelengths. Part of the emmission spectrum falls in the range used for detection of porphyrin fluorescence. This sub-section, therefore, looked at the effects of CBZ-E on the assay used for determining PBG-D activity in mitogen-stimulated lymphocytes to investigate if, under the assay conditions employed, CBZ-E interfered with the fluorometric quantification of uroporphyrin which is the basis of calculating enzyme activity.

The experiment was conducted in the following way. 6 tubes were set up: 2 containing 25µl DMSO,2 containing 25µl of 20mg CBZ/ml in DMSO, and 2 containing 25µl of 10mg CBZ-E/ml DMSO. The tubes were then treated as PBG-D assay blanks as described in sub-section 2.2.3, except that no PBG was added. At the end of the experiment, the fluorescence of the samples was measured at the same wavelengths used for calculating the amount of produced in the PBG-D assay. A fluorimeter expansion 10X less sensitive than that used in the assay was used. The results are presented in table (41) and they show that if CBZ-E is present, then the fluorescence of the samples is greatly increased. The same is true to a much lesser extent for CBZ. Thus the apparent increase in PBG-D activity can be explained by uptake of CBZ-E by the cells in the course of the incubation and retention of the drug during washing of the cells prior to assay. The CBZ-E

TABLE (41)

THE EFFECTS OF CBZ-E ON THE ASSAY USED FOR DETERMINATION OF PBG-D ACTIVITY IN CULTURED HUMAN LYMPHOCYTES

ADDITION	MEAN FLUORESCENCE READING*
DMSO	0.5
CBZ (250µg)	0.8
CBZ-E (250µg)	7.7

* mean of 2 measurements.

CBZ or CBZ-E was added to tubes set up for PBG-D assay blanks (except that no PBG was added) to assess if the presence of either compound would interfere with the assay. The fluorescence expansion was 10 x less sensitive than that used in the assay.

present is sufficient to cause an artificially high estimation of uroporphyrin produced leading to over-estimation of PBG-D activity. Whether or not this fluorescence of CBZ-E is a problem in the measurement of the porphyrin-synthesizing capacity of the cells is discussed in the next section.

Conclusions on the effects of CBZ-E on the assay used for the determination of PBG-D activity in cultured human lymphocytes

The presence of CBZ-E can cause an over-estimation of the quantity of uroporphyrin produced in the assay. This fact is the likely explanation for the apparent increase in the levels of PBG-D activity produced following the addition of CBZ-E to cultures as described in sub-sections 7.2.1 and 7.2.2.

7.2.5 SUMMARY OF SECTION 7.2

This section investigated the effects of CBZ and CBZ-E on PBG-D activity in cultured human lymphocytes. Preliminary results seemed to indicate an increase in PBG-D activity during CBZ-E treatment, but not during CBZ treatment. This result was unexpected because no changes in PBG-D activity had been found during CBZ treatment in either man or rat in the studies described in Chapters 4 and 5. However it is interesting to note that a positive linear correlation between plasma CBZ concentration and erythrocyte PBG-D activity in man was noted by Wright *et al* (1985), although there was no overall significant difference in the activities of control and CBZ-treated individuals. The possibility that the increase in activity was linked to the loss of ALA-D activity was investigated by the use of inhibitors of ALA-D. These failed to produce any increase in PBG-D activity. Therefore the apparent effect of CBZ-E on PBG-D activity was not mediated by ALA-D. This observation, coupled with the facts

that PBG-D activity was unaffected during CBZ treatment in man and rats and that CBZ-E can have some fluorescence at the wavelengths used for quantitative porphyrin analysis, prompted an examination of the effects of CBZ-E on the assay. This revealed that CBZ-E itself was fluorescent at the wavelengths used in the assay. The presence of any CBZ-E in an assay would therefore result in an overestimation of PBG-D activity. This would be especially true when the fluorimeter was set at the sensitive levels required for the measurement of PBG-D activity in mitogen-stimulated human lymphocytes.

In conclusion, neither CBZ nor CBZ-E had any effect on PBG-D. The apparent increase in activity produced by CBZ-E as described in sub-sections 7.2.1 and 7.2.2 was due to retention of epoxide by the cells with subsequent interference of the fluorimetric measurement of porphyrin produced.

7.3 THE EFFECTS OF CBZ AND CBZ-E ON PORPHYRIN SYNTHESIS IN CULTURED HUMAN LYMPHOCYTES

This section looked at the quantitive and qualitative effects of CBZ and CBZ-E on porphyrin synthesis in cultured human lymphocytes following incubation of the cells with ALA as described in sub-section 2.6.4. In all parts of this section, cells were prepared and cultured as described in 2.6.1 and 2.6.2. Drug additions were made in dimethyl sulphoxide (DMSO). For quantitative analyses, porphyrin retained in cells was determined by fluorimetry following solution in acid. Porphyrin which had been released into the medium was first isolated by ion-exchange chromatography on Dowex columns and then measured by fluorimetry. Both these methods are described in sub-section 2.6.4. For qualitative analyses, porphyrins in these samples were esterified and then separated by HPLC. The relative proportions of each were then calculated. The method used was as described in sub-section 2.3.4.

This section is composed of four sub-sections. The first of these presents a quantitative and qualitative comparison of the porphyrins retained by the cells and porphyrins released into the medium. The second sub-section investigates the quantitative effects of CBZ and CBZ-E on porphyrin synthesis and the third examines the qualitative effects of the same. The fourth and final sub-section summarises the results of this section.

7.3.1 QUANTITATIVE AND QUALITATIVE ANALYSES OF PORPHYRINS SYNTHESIZED BY CULTURED HUMAN LYMPHOCYTES

Before investigating the effects of drugs on porphyrin synthesis, quantitative and qualitative studies of the porphyrins produced by cultured human lymphocytes from ALA were made. As some of the

porphyrin produced by the cells escapes into the medium, it was necessary to perform the analyses on both cells and medium. This sub-section makes a quantitative and a qualitative comparison of the porphyrins found in these different samples and is thus divided into two parts.

(i) Quantitative analysis of porphyrins in cells and in cell culture medium

The results of 3 studies are presented in table (42). The table shows that although most porphyrin remains in the cells, a considerable amount of the porphyrin produced is lost into the medium. This represented 36, 30 and 25% of the total porphyrin produced in studies 1,2 and 3 respectively, demonstrating that there is some degree of variability in the quantity of synthesized porphyrin lost to the medium. It would however be correct to say that cells contained 2 to 3 times as much porphyrin as was found in the medium. The reasons for this variability will be discussed shortly.

(ii) Qualitative analysis of porphyrins in cells and in culture medium

The relative proportions of each of the porphyrins in the mixture of porphyrins from cells and from samples of medium were determined. Figure (30) shows typical chromatograms of the porphyrin composition of cells and of medium. Table (43) contains data from three separate experiments. With regards fig. (30), the most striking difference in porphyrin composition of cells and of medium is that a considerable proportion of the total porphyrin in the latter was uroporphyrin (URO), whereas the cells contained little or no URO. This would be expected because URO in cells would be converted to coproporphyrin (COPRO)

TABLE (42)

QUANTITATIVE ANALYSIS OF PORPHYRIN SYNTHESISED IN CULTURED HUMAN LYMPHOCYTES

STUDY No.	TOTAL PORPHYRIN IN CELLS	TOTAL PORPHYRIN IN MEDIUM	TOTAL PORPHYRIN PRODUCED
1	3.2 ± 0.4	1.8 ± 0.1	5.0 ± 0.3
2	3.7 ± 0.5	1.6 ± 0.1	5.3 ± 0.4
3	3.8 ± 0.5	1.3 ± 0.2	5.1 ± 0.6

Each value is the mean ± 1 S.D. for 4 measurements.

Results are presented as ng total porphyrin produced/10^6 cells/24 hours.

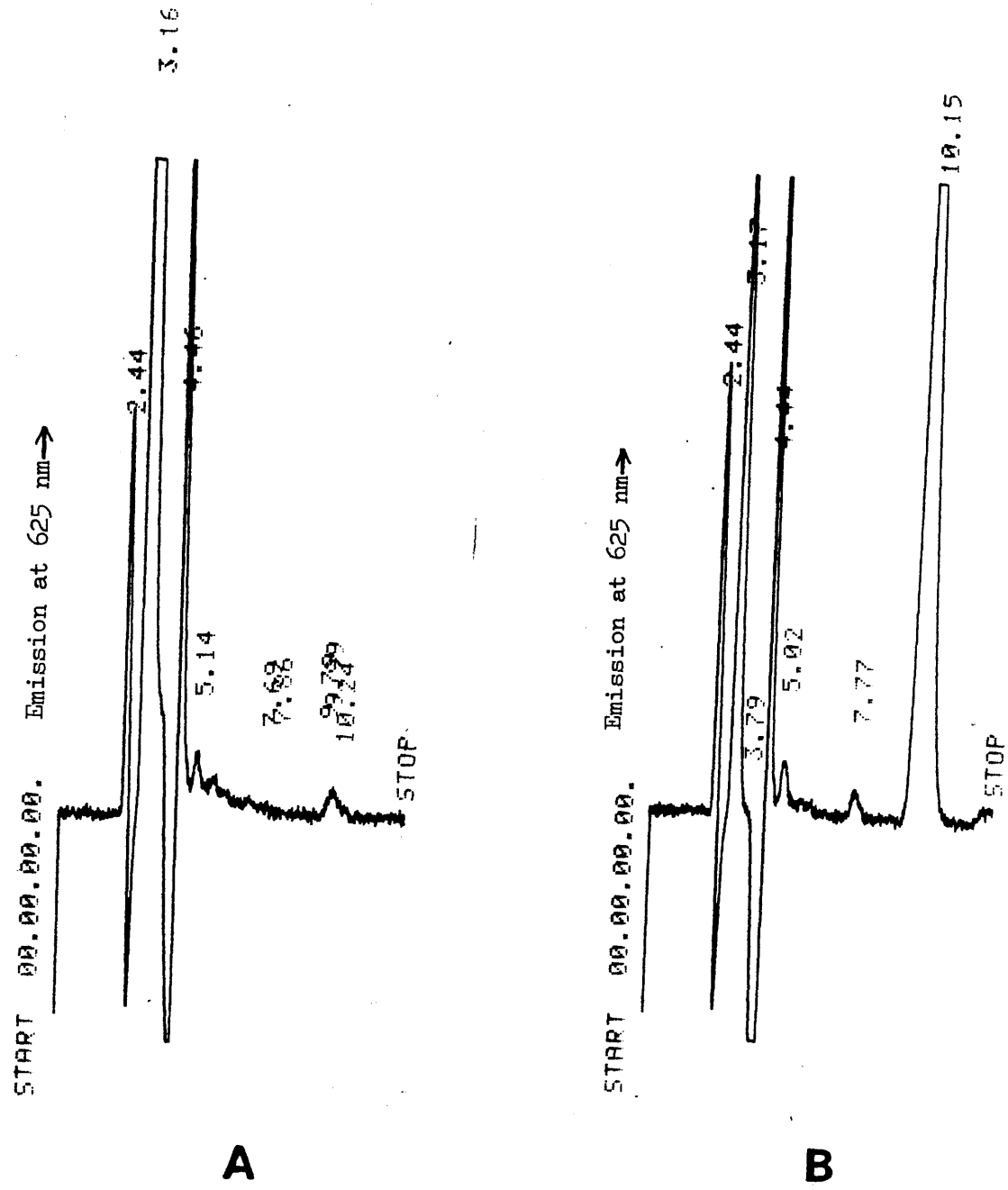


FIG. (30)

COMPARISON OF THE PORPHYRINS FOUND IN CELLS AND IN MEDIA FOLLOWING INCUBATION OF CULTURED HUMAN LYMPHOCYTES WITH ALA

Chromatogram A is a porphyrin extract from cells incubated with ALA. B is an extract from the medium these cells were growing in. Any porphyrin present in B must have been released from the cells. There is a clear difference in the porphyrin composition of the two extracts: in A the major peak is PROTO, but in B the major peak is URO.

A

TIME	PEAK	% OF TOTAL
3.16	PROTO	88.0
4.46	COPRO	11.6
5.14	PENTA	TRACE
10.24	URO	0.4

B

TIME	PEAK	% OF TOTAL
3.17	PROTO	38.4
4.44	COPRO	21.2
5.14	PENTA	TRACE
7.77	HEPTA	TRACE
10.15	URO	40.0

TABLE (43)

QUALITATIVE ANALYSIS OF PORPHYRIN SYNTHESIZED

IN CULTURED HUMAN LYMPHOCYTES

STUDY		% PORPHYRIN COMPOSITION					
		2-COOH	4-COOH	5-COOH	6-COOH	7-COOH	8-COOH
1	CELLS	88	12	-	-	-	Trace
	MEDIUM	42	20	-	-	-	37
2	CELLS	67	32	-	-	-	1
	MEDIUM	5	29	-	-	3	63
3	CELLS	80	17	-	-	-	3
	MEDIUM	19	30	-	-	-	51

Results of HPLC analysis of porphyrins synthesized from ALA added to cultured human lymphocytes.

and protoporphyrin (PROTO). A further difference between the two is that there was much more PROTO in cells than there was in the medium. Again this was anticipated because COPRO in cells would be converted to PROTO, but COPRO released into the medium would not. Thus the relative amounts of COPRO and PROTO differ between cells and medium. This is shown more clearly in table (43) which also shows that although the relative proportions of these compounds may vary, the overall pattern is always the same: considerable amounts of URO in the medium but little or none in cells, and a higher PROTO/COPRO ratio in cells than in medium.

Discussion of quantitative and qualitative aspects of porphyrin synthesis in cultured human lymphocytes

These two sets of experiments have shown that, following incubation of mitogen-stimulated lymphocytes with ALA, there are quantitative and qualitative differences in the porphyrins found in cells and in the medium. Furthermore the results prove that these cells have all the enzymes necessary to synthesize PROTO from ALA. In both sets of experiments there were differences in, for example, the fraction of total porphyrin which was in the medium and the proportions of the individual porphyrins. These differences can only be attributed to slightly different relative activities of the enzymes in each sample of lymphocytes. Having established these basic results, the following sub-sections investigate the effects of CBZ and CBZ-E on porphyrin synthesis.

7.3.2 QUANTITATIVE ANALYSIS OF THE EFFECTS OF CBZ AND CBZ-E ON PORPHYRIN SYNTHESIS IN CULTURED HUMAN LYMPHOCYTES

This sub-section investigated the effects of CBZ and CBZ-E on the quantities of porphyrin produced from added ALA in mitogen-stimulated lymphocytes.

In these experiments, cells were exposed to CBZ or CBZ-E at concentrations of 30mg/l and 15mg/l respectively for 96 hours prior to setting up porphyrin synthesis as described (2.6.4). The results of quantitative porphyrin determinations and ALA-D activities in these experiments are presented in table (44). There are several interesting results. The first of these is that both CBZ and CBZ-E significantly reduced the total amounts of porphyrin formed in both cells and in medium. This effect is probably due to the effects of these drugs on ALA-D activity. Since the starting point for porphyrin synthesis in these experiments was ALA, then it follows that the amounts of subsequent intermediate for porphyrin synthesis will be dependent solely on the levels of ALA-D activity. Examination of total porphyrin produced reveals that in the case of CBZ where ALA-D activity was only 83% of control values, total porphyrin production was 79% of control values, which supports the foregoing explanation. However with CBZ-E, although porphyrin synthesis was reduced, it was not depressed to the same extent as was ALA-D activity, treated values being 54% and 23% of control values respectively. This is almost certainly due to the presence of CBZ-E in the cells and in the medium, because as described in the previous section, CBZ-E absorbs light of the wavelengths used to examine porphyrins and part of the emission spectrum falls in the range at which porphyrin fluoresence is measured. However the effect is not as marked in this instance

TABLE (44)

QUANTITATIVE ANALYSIS OF THE EFFECTS OF CBZ AND CBZ-E ON PORPHYRIN SYNTHESIS IN CULTURED HUMAN LYMPHOCYTES

ADDITION	PORPHYRIN IN CELLS[a]	PORPHYRIN IN MEDIUM[a]	TOTAL PORPHYRIN PRODUCED[a]	ALA-D ACTIVITY[b]
DMSO	3.7 ± 0.5	1.6 ± 0.1	5.3 ± 0.4	100 ± 5.3
CBZ	3.1 ± 0.1	1.1 ± 0.1	4.2 ± 0.1	83 ± 5.8
CBZ-E	2.0 ± 0.1	0.9 ± 0.1	2.9 ± 0.1	22.6 ± 4.1

Values are mean ± 1 S.D. for 4 determinations

CBZ and CBZ-E were present at concentrations of 30mg/l and 15mg/l respectively.

[a] units are ng porphyrin produced/10^6 cells/24 hours

[b] ALA-D activities were expressed as percentages of control activities.

because the fluoresence readings of the porphyrin produced in this assay were much higher than those found in the PBG-D assay. Consequently the drug does not make such a large contribution to the overall fluorescence.

To further investigate this, the effects of a range of CBZ-E concentrations on both ALA-D activity and the quantities of porphyrin synthesised were examined. The results are shown in table (45). As before, ALA-D activities are presented as % of control activities and the quantities of porphryin produced are also shown as percentages of controls. The results show that the total quantity of porphyrin produced was reduced following exposure to CBZ-E. This was achieved by reduction in the amount synthesized in both cells and medium. Furthermore, the higher the concentration of CBZ-E, the less porphyrin was produced and less ALA-D activity remained. Both did not seem to be affected to the same extent: the more CBZ-E added, the greater was the discrepancy between the two sets of data (see table 45). This would suggest that CBZ-E was indeed interfering with the determination of the amount of porphyrin produced.

Conclusions on a quantitative analysis of the effects of CBZ and CBZ-E on porphyrin synthesis in cultured human lymphocytes

Both CBZ and CBZ-E reduced the amount of porphyrin synthesized in mitogen-stimulated lymphocytes. This effect was a likely consequence of the loss of ALA-D activity: there was a good comparison between the effects of CBZ on ALA-D and the effects on porphyrin synthesis. No such comparison was found with CBZ-E due to the interference of this compound with the fluorimetric quantitation of porphyrins. This resulted in an overestimation of the porphyrin content in any sample in which CBZ-E was present.

TABLE (45)

THE EFFECTS OF VARYING DOSES OF CBZ-E ON ALA-D ACTIVITY AND PORPHYRIN SYNTHESIS IN CULTURED HUMAN LYMPHOCYTES

[CBZ-E][a]	(A) ALA-D[b] ACTIVITY	TOTAL PORPHYRIN[c] IN CELLS	TOTAL PORPHYRIN[c] IN MEDIUM	TOTAL PORPHYRIN[c] PRODUCED	(B) TOTAL PORPHYRIN AS % OF CONTROL	(B)/(A)
0	100 ± 9	4.0 ± 0.5	1.4 ± 0.2	5.4 ± 0.6	100 ± 11	1
3.3	68 ± 13	3.6 ± 0.1	1.2 ± 0.1	4.8 ± 0.1	89 ± 2	1.3
6.6	55 ± 13	3.3 ± 0.3	1.1 ± 0.1	4.3 ± 0.4	80 ± 7	1.5
10	42 ± 6	2.9 ± 0.2	0.9 ± 0.1	3.8 ± 0.2	70 ± 4	1.7

a units are mg/l

b activities are expressed as percentages of control values

c units are ng porphyrin produced/10^6 cells/24 hours

The ratio of B, (total porphyrin produced as a percentage of control) to A, (ALA-D activity as a percentage of control) reveals that these two parameters are not affected to the same extent by CBZ-E, and that the greater the concentration of CBZ-E, the greater the discrepancy.

7.3.3 QUALITATIVE ANALYSIS OF THE EFFECTS OF CBZ AND CBZ-E ON PORPHYRIN SYNTHESIS IN CULTURED HUMAN LYMPHOCYTES

The effects of CBZ and CBZ-E on the relative proportions of the individual porphyrins produced from ALA were investigated. The porphyrin composition of both medium and cells were studied. The results of one such experiment are shown in table (46). CBZ or CBZ-E were present at concentrations of 30mg/l and 15mg/l respectively for 96 hours prior to commencement of the assay of porphyrin synthesis.

The assays were performed in duplicate and therefore the results shown in the table are mean values.

There was very little difference in the porphyrin composition of cells treated with CBZ or CBZ-E when compared to that of control cells. However analysis of the porphyrins present in the medium revealed some changes with respect to controls. The changes were slight following CBZ treatment, but were great following CBZ-E treatment. Media to which CBZ-E had been added exhibited a reduced percentage of URO, but increased percentages of COPRO and PROTO when compared to controls. This was expected because, due to the reduced levels of ALA-D activity found following exposure of cells to CBZ-E, there would be fewer pathway intermediates. Consequently the cells would have been able to convert a greater proportion of the URO produced to COPRO and PROTO. This would have meant that less URO was available to diffuse into the medium, leading to an increase in the percentages of COPRO and PROTO. The experiment was repeated and similar results were obtained.

The unchanged porphyrin composition in cells treated with the drug in the face of reduced porphyrin synthesis in the same cells

TABLE (46)

QUALITATIVE ANALYSIS OF THE EFFECTS OF CBZ AND CBZ-E ON PORPHYRIN SYNTHESIZED IN CULTURED HUMAN LYMPHOCYTES

FRACTION	ADDITION	PERCENTAGE PORPHYRIN COMPOSITION						TOTAL PORPHYRIN IN SAMPLE	ALA-D ACTIVITY
		2-COOH	4-COOH	5-COOH	6-COOH	7-COOH	8-COOH		
CELLS	DMSO	88.2	11.8	-	-	-	TRACE	3.7 + 0.5	100 + 5.3
	CBZ	85.1	14.8	-	-	-	TRACE	3.1 + 0.1	83.0 + 5.8
	CBZ-E	88.1	11.9	-	-	-	TRACE	2.0 + 0.1	22.6 + 4.1
MEDIUM	DMSO	40.2	20.8	-	-	0.5	38.6	1.6 + 0.1	-
	CBZ	36.5	28.7	-	-	-	34.9	1.1 + 0.1	-
	CBZ-E	49.1	30.2	-	-	-	20.8	0.9 + 0.1	-

The samples analysed were the same ones used for analysis of the quantitative effects of CBZ and CBZ-E treatment shown in table (44). 4 Determinations were made of total porphyrin in the sample. Two of each were combined and duplicate chromatograms run of the various additions. The figures in the table represent the mean of these results.

a units are ng porphyrin produced/10^6 cells/24 hours

b activities are expressed as percentage of control values.

indicates two things. Firstly, the reduction in porphyrin synthesis is indeed due to loss of ALA-D activity, since a reduction in the activities of any of the other enzymes in the pathway would result in changes in the porphyrin composition. Secondly, no change in porphyrin composition was found in this system following treatment with CBZ and CBZ-E, but changes were found in the urinary excretion pattern of these porphyrins during the initial stages of treatment in man, (see sub-section 4.3.2 and Rapeport et al, 1984). This difference could only be due to the one part of the pathway not required for the synthesis of these porphyrins in culture but required in vivo, namely, ALA-S. This finding supports the theory put forward in sub-sections 3.2.2 and 4.3.1, that the increased % URO in human urine during the initial stages of CBZ treatment was due to the very high levels of ALA-S activity produced at that time.

Conclusions on a qualitative analysis of the effects of CBZ and CBZ-E on porphyrin synthesis in cultured human lymphocytes

There were no qualitative differences in porphyrin found in control and CBZ or CBZ-E treated cells although the quantities of porphyrins produced were reduced by the drugs, suggesting that porphyrin synthesis was indeed reduced as a consequence of ALA-D inactivation as was proposed in subsection 7.3.2. This further suggests that the altered porphyrin excretion profile found in man in the initial stages of CBZ treatment was due to derepression of ALA-S. Examination of the porphyrins released into the medium revealed a reduction in the URO fraction following treatment of cells with CBZ-E. This could be explained by the action of CBZ-E on ALA-D.

7.3.4 SUMMARY OF SECTION 7.3

This section has shown that there are quantitative and qualitative differences between the porphyrin found in cells and porphyrin released into the medium following incubation of mitogen-stimulated human lymphocytes with ALA. Furthermore, both CBZ and CBZ-E reduced the quantities of porphyrin synthesized and this was linked to the inactivation of ALA-D caused by these two compounds. As was found in previous sub-sections, the presence of CBZ-E can interfere with quantitative porphyrin determinations. Qualitative investigations of the effects of CBZ and CBZ-E on porphyrins produced from ALA by mitogen-stimulated lymphocytes revealed no changes in the porphyrins in cells but changes in the porphyrin released into the medium, most notably a reduction in the URO fraction. This was particularly so in the case of CBZ-E, and was a likely consequence of reduced ALA-D activity. The fact that the porphyrin composition in cells was unchanged, although the quantities produced were taken as evidence that the quantity of porphyrin produced in this system was indeed dependent on the levels of ALA-D activity. The same observation was also used to suggest that the altered porphyrin excretion profile found in man in the initial stages of CBZ treatment could only be due to the effects of the drug on the one enzyme not studied here, namely, ALA-S.

7.4 SUMMARY OF CHAPTER 7

This chapter examined the effects of CBZ and CBZ-E on several aspects of haem biosynthesis in mitogen stimulated cultured human lymphocytes. Both compounds were shown to cause an irreversible reduction in the levels of ALA-D activity which was due to inactivation of existing enzyme and not to a reduced rate of synthesis. Although it is likely that the mechanism by which enzyme activity was lost here is applicable to the *in vivo* situation, activity was lost at a much greater rate. This is discussed further in sub-section 9.1.4. Phenobarbitone and sodium valproate did not affect ALA-D activity, suggesting that inactivation of the enzyme was a specific property of CBZ and CBZ-E. Inactivation of ALA-D was achieved without detectable metabolism of CBZ or CBZ-E. PBG-D activity was unaffected. Treatment with either CBZ or CBZ-E failed to produce a qualitative change in the porphyrins synthesized in the cells from ALA, though there were quantitative changes. This observation was taken as evidence that the alterations in urinary porphyrin excretion found in 4.3.1 and reported by Rapeport *et al* (1984) in the initial stages of CBZ treatment in man were due to derepression of ALA-S.

CHAPTER 8

CHAPTER 8

IN VITRO METABOLISM OF CBZ: EFFECTS ON ALA-D ACTIVITY

In preceding chapters it was shown that treatment with CBZ resulted in an irreversible reduction in ALA-D activity in both man and the rat. Further experiments revealed that the drop in activity was due to inactivation of the enzyme rather than a modification of activity or a reduction in the rate of synthesis. This inactivation was not the result of a direct action of CBZ itself or the metabolites CBZ-E and H_2CBZ. Results from cell culture studies showed that both CBZ and CBZ-E caused inactivation of ALA-D in mitogen-stimulated human lymphocytes with no detectable metabolism of either compound. There are two possible explanations for this last observation: (i) CBZ and CBZ-E underwent some form of bio-activation in living cells which produced a species capable of inactivating ALA-D; or (ii) CBZ-E inactivated ALA-D through some indirect mechanism, the facilities for which are only found in living cells. Two possible levels at which CBZ treatment could have indirectly affected ALA-D activity which can be excluded at this stage are (a) effects at the level of synthesis and (b) effects on glutathione concentration. This chapter was concerned with the effects of metabolizing CBZ, in the presence of purified bovine liver ALA-D, on the activity of the enzyme. These experiments were designed to test the hypothesis that a bio-activated form of CBZ inactivates ALA-D. This is of interest because other workers have reported that trichloroethylene and bromobenzene, two compounds which irreversibly inhibit ALA-D *in vivo* but not when added to purified enzyme, can inhibit the enzyme *in vitro* when they are

metabolized by purified microsomes containing mixed function oxidase (m.f.o) activity (Fujita et al,1984; Koizumi et al, 1984). The results of these studies suggested that some transient metabolites or activated states of the chemicals were responsible for the inactivation of ALA-D. This chapter looks at the possibility that this is the case with CBZ.

All of the necessary procedures and experimental details for the studies described in this chapter are found in section 2.8 but the basic details were as follows. CBZ was incubated with purified microsomes containing m.f.o. activity and purified bovine liver ALA-D. To drive the m.f.o. system it was necessary to add NADPH as a source of reducing power. Without this the m.f.o. system is inactive. Samples were taken from the incubation vessel at indicated times and ALA-D activity measured. Estimations of enzyme activity were performed in quadruplicate. In the sections which follow a control incubation means one in which there is no metabolism of CBZ taking place. This was usually achieved by omitting NADPH.

The chapter is composed by 4 sections. The first of these investigates if in fact CBZ is metabolized by the system and what effects this may have on ALA-D activity. The second looks at the nature of the effect of CBZ metabolism on ALA-D. The third examines the effect of using CBZ-E in the incubations in place of CBZ and the fourth and final section summarises the chapter.

8.1 THE EFFECTS OF IN VITRO CBZ METABOLISM ON PURIFIED ALA-D - SOME INITIAL STUDIES

This section presents the results of initial investigations into the *in vitro* metabolism of CBZ and the effects of this on purified ALA-D and consists of 5 sub-sections. Four of these are concerned with various aspects of CBZ metabolism and in the effects thereof on ALA-D. The fifth summarises the section.

8.1.1 IN VITRO METABOLISM OF CBZ

Before investigating the effects of CBZ metabolism on purified ALA-D activity, it was necessary to establish if metabolism of the drug was taking place. To this end, rat liver microsomes, CBZ and NADPH were added to a flask and incubated in a shaking water bath set at 37°C as described in sub-section 2.8.3. Aliquots were removed from the incubation mixture at 0, 0.5, 1 and 2 hours after initiation of the reaction and CBZ and its metabolites extracted and analysed by HPLC using the method described in sub-section 2.4.2. Fig.(31) shows the results of two such analyses: one at the start of the incubation and one after 1 hour. It is clear from examination of these results that the above system generates several distinct metabolites of CBZ, the only identifiable one being CBZ-E. The nature of the other products is unknown, but the relative amounts of each appeared to vary in a random fashion as the incubation progressed, suggesting that they were unstable. It was therefore not possible to assign even arbitrary values to these species. However it is unequivocal that some form of metabolism or activation of CBZ occurs. The effects of this on purified ALA-D activity are investigated in the following sub-section.

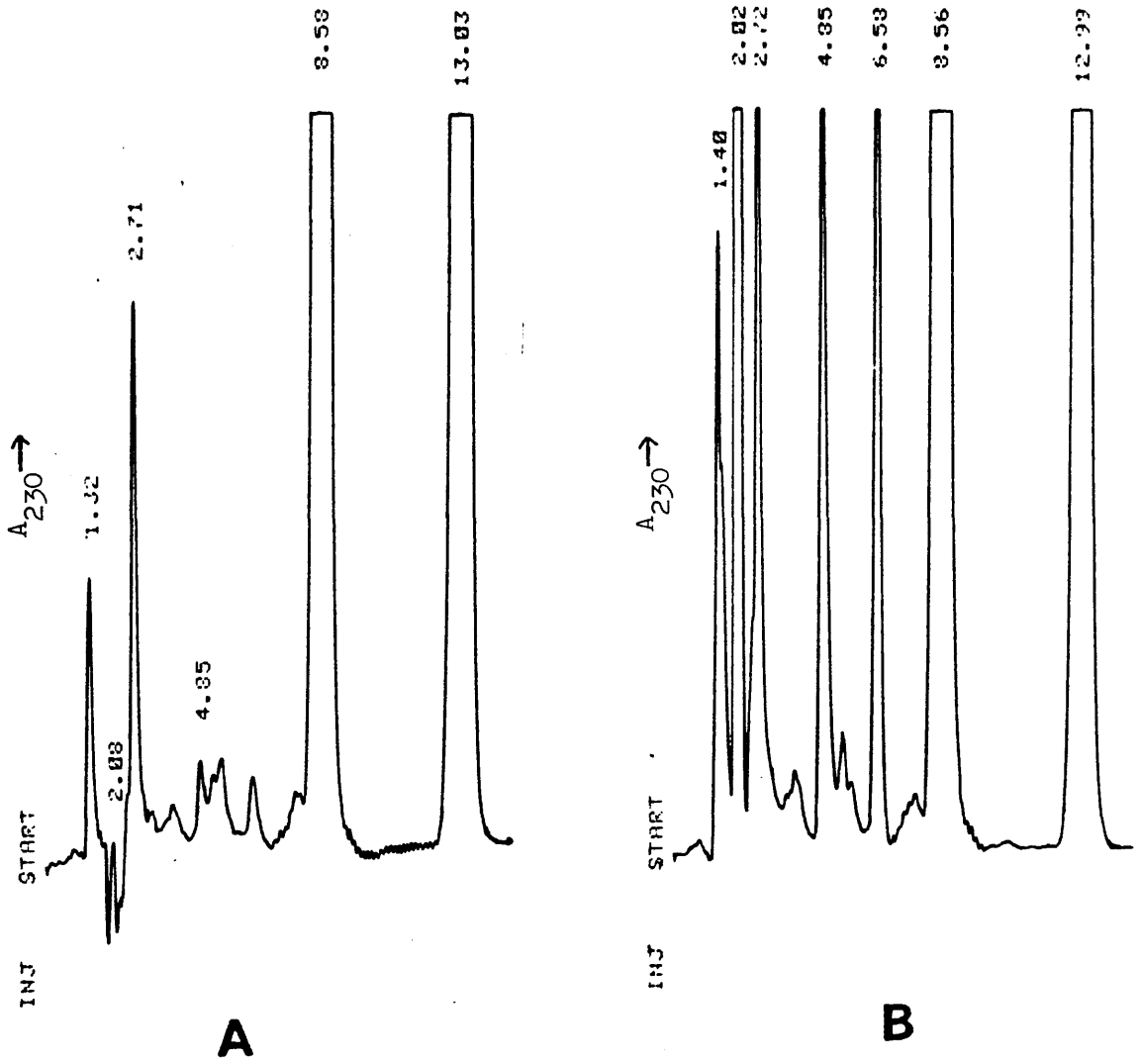


FIG. (31)

IN VITRO METABOLISM OF CBZ

The figure shows the results of HPLC analysis of extracts from incubations of CBZ with an active drug metabolizing system. A is an HPLC chromatogram of an aliquot taken shortly after initiation of the assay and B is a chromatogram of an aliquot from the same incubation 1 hour later. Metabolism of the drug has occurred as evidenced by the extra peaks, the only identifiable one being the epoxide at 4.85 minutes.

Peak	Retention time
CBZ-E	4.85
CBZ	8.58
I.S.	13.03

Conclusions on attempts to metabolize CBZ in vitro

CBZ was metabolized by the system described, resulting in the production of several unidentified, unstable compounds in addition to small quantities of CBZ-E.

8.1.2 IN VITRO METABOLISM OF CBZ: EFFECTS ON ALA-D ACTIVITY

In the previous sub-section it was established that CBZ was metabolized by the *in vitro* system described. This sub-section examined the effects of this metabolism on purified bovine liver ALA-D. For the experiment two flasks were prepared. Each contained CBZ, NADPH and DTT, but only one (the test flask) contained microsomes. The other (the control flask) contained an equivalent amount of buffer. Details of the actual quantities used are listed in sub-section 2.8.3. DTT was included in the incubation to stabilize ALA-D. Aliquots were removed from the flasks 0, 0.5, 1, 1.5, 2 and 3 hours after the initiation of reaction and assayed for ALA-D activity. The results, expressed as percentages of activity at zero time, are shown in fig. (32).

In the control flask (the one lacking microsomes) there was a progressive loss of activity after the incubation had been going for 1 hour. This loss of activity was found in controls in all subsequent studies whether microsomes, CBZ or NADPH were added alone or in a combination of any two of these. However when all 3 were present, as was the case in the test flask, activity was lost at a faster rate than in controls. This indicates that metabolism of CBZ caused inactivation of ALA-D. Another feature of interest is that in the later stages of the incubation, the rate of loss of ALA-D activity in the test flask seemed to approach that of the control. This suggests

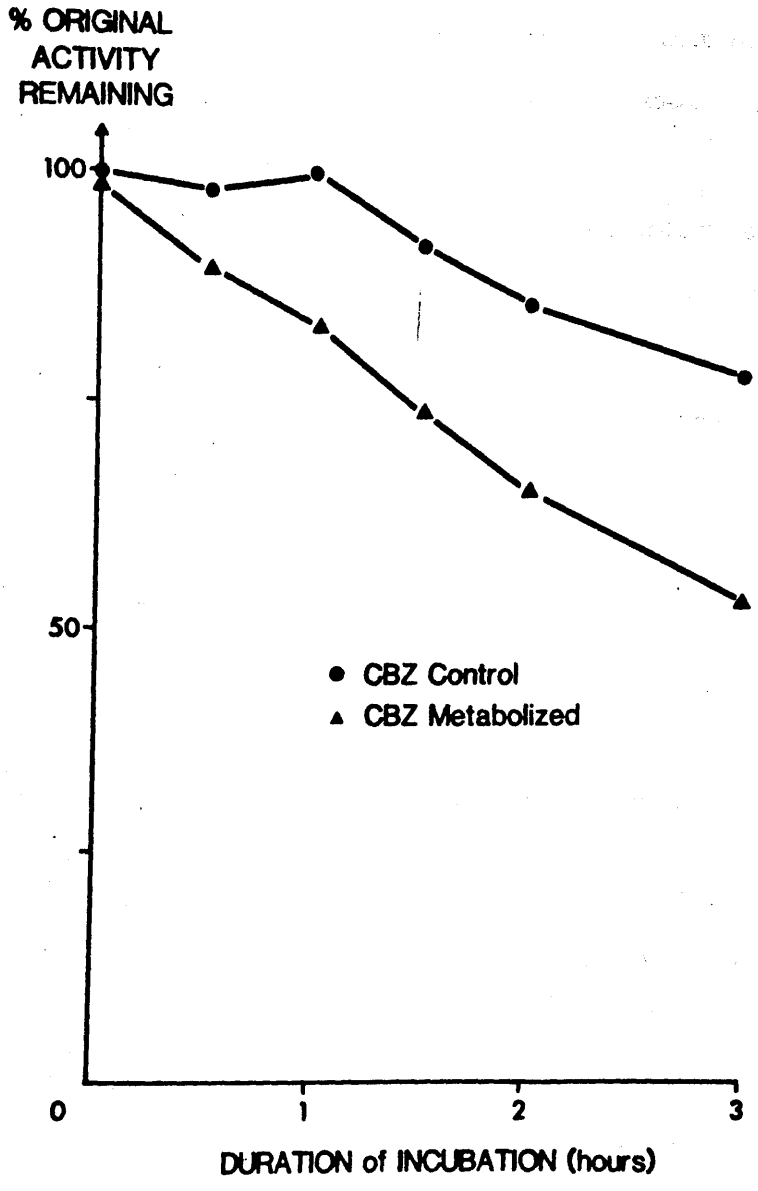


FIG. (32)

THE EFFECTS OF

IN VITRO METABOLISM OF CBZ ON ALA-D ACTIVITY

The figure shows the % of the initial ALA-D activity remaining 0,0.5,1,1.5,2 and 3 hours after starting the experiment. The control lacked NADPH, therefore could not metabolize CBZ. The test flask contained all the requirements for CBZ metabolism. Although activity was lost from the control flask, it was lost at a greater rate from the test flask, indicating that metabolism of CBZ caused inactivation of ALA-D.

that the metabolism of CBZ falls off with time. Unfortunately, due to the inability to quantify the products of the system, it was not possible to verify this. However analysis of the cytP450 content of the microsomes after 3 hours incubation revealed that less than 10% of the original haemoprotein concentrations remained. The mechanism whereby cytP450 was lost is not known.

Although these results show that metabolism of CBZ caused inactivation of ALA-D, they provide no evidence for the effect being due to CBZ or a product of the action of the m.f.o. system on the drug. For instance, it is possible that ALA-D was inactivated by reduced molecular oxygen, a highly reactive species produced by the hepatic m.f.o. system in the course of oxidation of substrates of the system. Also, the results do not show that the effect is specific for ALA-D. These two possibilities are examined in the following two sub-sections.

Conclusions on the effects of in vitro CBZ metabolism on ALA-D activity

Although ALA-D activity was lost from the control, activity was lost at a faster rate from the test flask in which CBZ was being metabolized, but only over the first 1.5 - 2 hours. Thereafter activity was lost at the same rate as in controls. This may be explained by a reduction in the cytP450 content of the microsomes. The results do not confirm that the observed inactivation of ALA-D was due to CBZ metabolism in itself or that the species responsible for inactivation was specific for ALA-D.

8.1.3 IN VITRO METABOLISM OF PRIMIDONE: EFFECTS ON ALA-D ACTIVITY

The aim of this sub-section was to determine if the loss of ALA-D activity found in the course of CBZ metabolism was a feature unique to

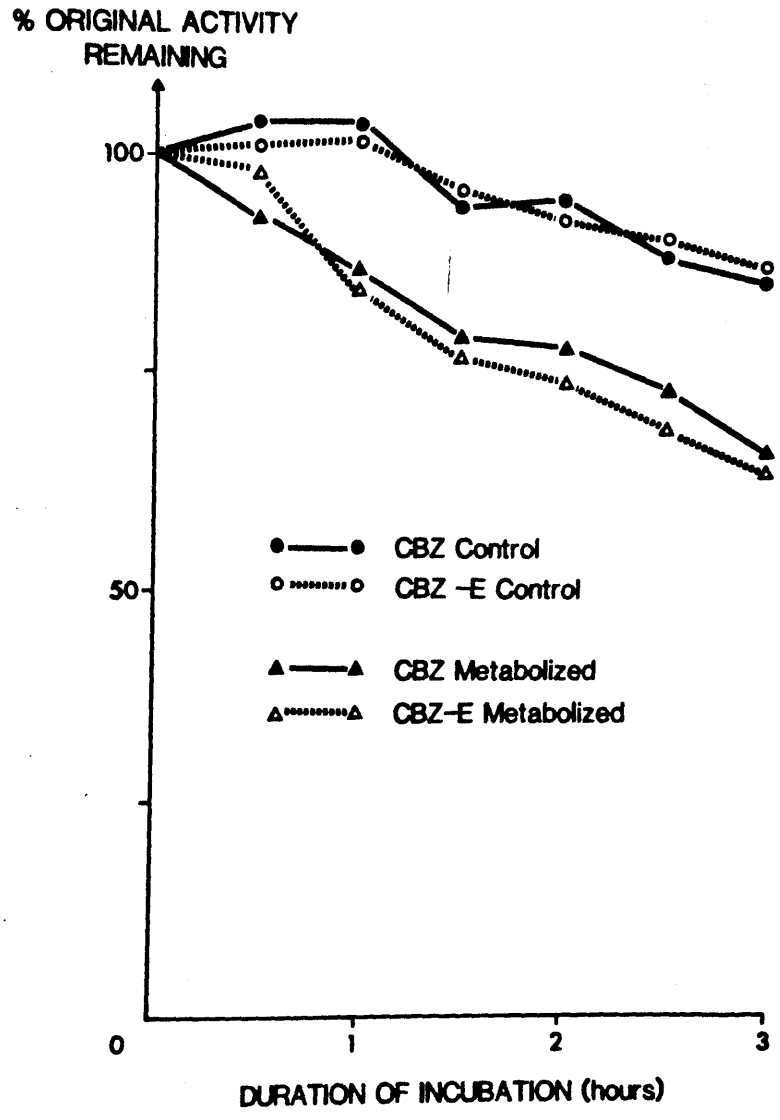


FIG. (33)

COMPARISON OF THE EFFECTS OF IN VITRO METABOLISM OF CBZ AND CBZ-E ON ALA-D ACTIVITY

The figure compares the effects of metabolism of CBZ and CBZ-E on the % of the initial ALA-D activity remaining 0.5, 1, 1.5, 2, 2.5 and 3 hours after starting the experiment. For each compound there was a control flask with no capacity for drug metabolism and a test flask with an active drug metabolizing system. CBZ and CBZ-E behaved identically: the activity in metabolizing system. CBZ and CBZ-E behaved identically: the activity in both test flasks was lost at a greater rate than from the control flasks. The rates of loss of activity in both control and test flasks were the same for both compounds.

CBZ. As stated at the end of the preceding section, it is possible that inactivation could have been caused by reduced molecular oxygen, a highly reactive species released as a by-product of MFO activity. If this was the case, then the metabolism of any drug by this in vitro system should result in the inactivation of ALA-D. Primidone was chosen to test this hypothesis because earlier results indicated that it did not cause inactivation of hepatic ALA-D in rat (results not shown) and that phenobarbitone, one of its metabolites, did not cause a loss of ALA-D in cultured human lymphocytes or loss of erythrocyte ALA-D in man. A further reason was that it was simple to check if this drug was metabolized by the m.f.o. system since both primidone and phenobarbitone can be identified using the same extraction and HPLC system as was used for CBZ. (See sub-section 2.4.2).

Two flasks were prepared. The control contained microsomes, ALA-D and primidone, the test contained all of these plus NADPH. These were incubated at 37°C in a shaking water bath. The actual concentrations used are listed in methods sub-section 2.8.3. ALA-D activities in each flask were measured at the start of the incubation and after 2 hours. Aliquots were also analysed by HPLC. In the control flask, after 2 hours, mean ALA-D activities were 85% of the activities at zero time and there was no detectable metabolism of primidone. In the test sample, there was complete metabolism of primidone, but after 2 hours mean activities were 86% of activities at zero time. This shows that ALA-D activity is not lost as a consequence of the metabolism of any drug but is a specific property of CBZ. After two hours the cytochrome P450 content of the microsomes was only 23% of the starting concentrations. This

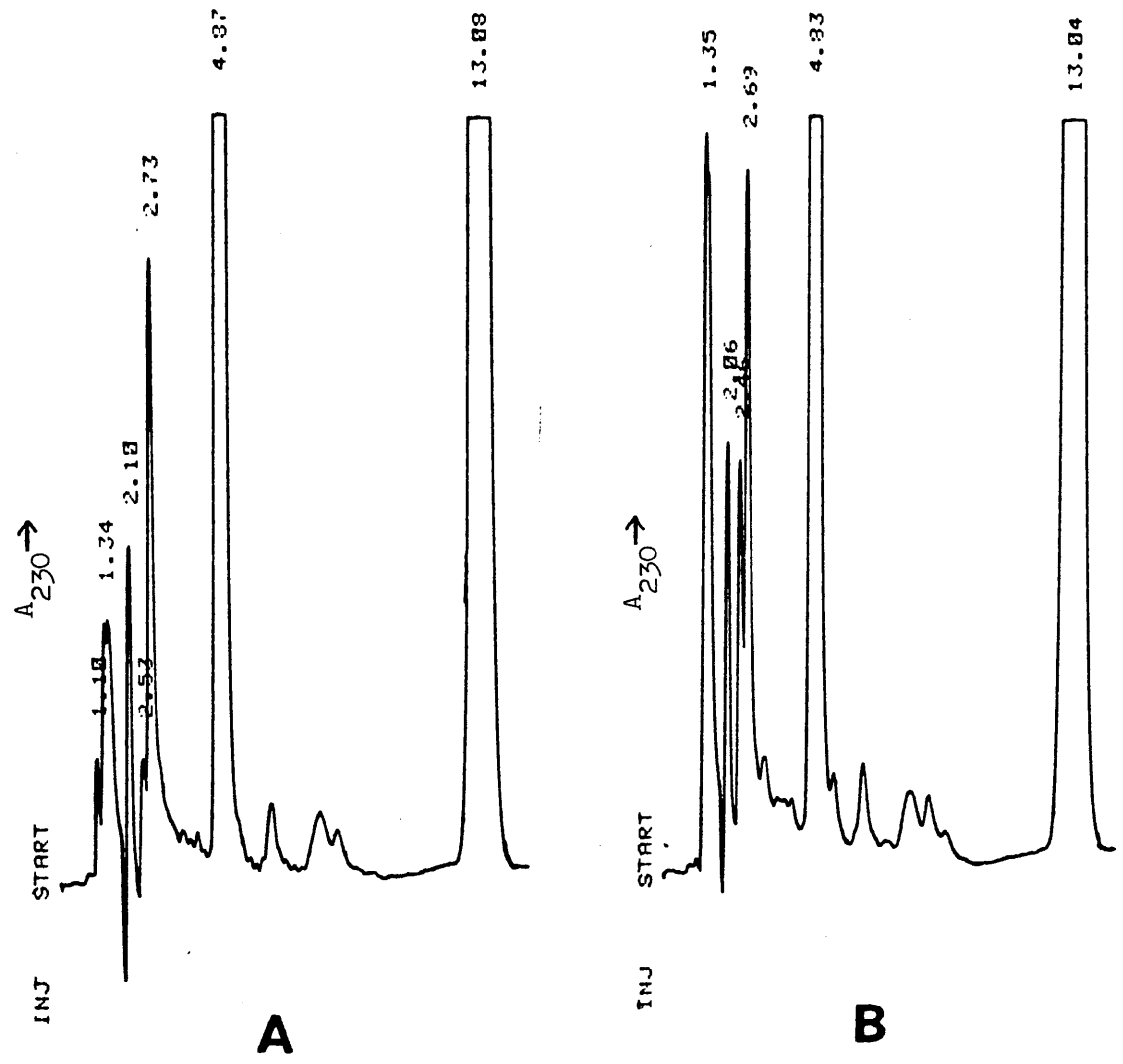


FIG. (34)

IN VITRO METABOLISM OF CBZ-E

The figure shows the results of HPLC analysis of extracts from incubations of CBZ-E with an active drug metabolizing system. A is an HPLC chromatogram of an aliquot taken shortly after initiation of the assay and B is a chromatogram of an aliquot from the same incubation 1 hour later. Metabolism of CBZ-E has occurred as evidenced by the peaks appearing near the start of the trace in B. The nature of these compounds is unknown.

Peak	Retention time	
	A	B
CBZ-E	4.87	4.83
I.S.	13.08	13.04

indicates that the loss of cyt P450 is due to the incubation conditions since this was also shown to be the case when CBZ was added to the system (8.1.2).

Conclusions on effects of the in vitro metabolism of primidone on ALA-D activity

Metabolism of primidone by the in vitro drug metabolizing system described did not affect ALA-D activity whereas metabolism of CBZ did. Therefore the effect is not due to the reduction of molecular oxygen or some other product of drug metabolism but is a specific property of CBZ.

8.1.4 INVESTIGATIONS INTO THE SPECIFICITY OF THE INACTIVATION OF ALA-D PRODUCED DURING IN VITRO METABOLISM OF CBZ

This sub-section investigated if the species responsible for the inactivation of ALA-D during the in vitro metabolism of CBZ was specific for the enzyme. If it was not, then the inclusion of supplementary protein in the incubation should reduce the extent of the inactivation. On the other hand, if it was specific, then the inclusion of other proteins would make no difference. The experiments were performed in the usual way except that bovine serum albumin (BSA,0.5mg/ml) was added to some of the incubations. The results are presented in table (47). The results from flasks 1 and 2 confirm the results of sub-section 8.1.2: an accelerated loss of ALA-D activity was observed when metabolism of CBZ occurred. The lower half of the table shows the results from flasks 3 and 4 which contained BSA. These are no different from the values obtained from flasks 1 and 2. This shows that the presence of BSA offered no protection against inactivation of ALA-D, confirming that the species

TABLE (47)

THE EFFECTS OF ADDED BSA ON THE EXTENT OF ALA-D INACTIVATION

DURING THE IN VITRO METABOLISM OF CBZ

Flask No.	DURATION OF INCUBATION (HRS)				NADPH (1mM)	BSA (0.5mg/ml)
	0	0.5	1.0	1.5		
1	100	93	85	77		
2	100	92	81	70	x	
3	100	95	89	79		x
4	100	92	81	69	x	x

Results were expressed as percentages of initial activity remaining after a fixed time.

Flasks 1 and 3 were controls. Since NADPH was omitted, no metabolism of CBZ could occur.

Flasks 3 and 4 contained bovine serum albumin to investigate if this afforded ALA-D any protection against inactivation.

X = present in the incubation

generated during in vitro metabolism of CBZ which is responsible for the loss of ALA-D activity has some specificity. However, it cannot be said to be absolutely specific on the basis of these results.

Conclusions on the specificity of the inactivation of ALA-D produced during in vitro metabolism of CBZ

This sub-section has shown that the species responsible for the inactivation of ALA-D during in vitro metabolism of CBZ, has some degree of specificity for the enzyme as the inclusion of another protein (BSA) in the incubation did not reduce the extent of the inactivation.

8.1.5 SUMMARY OF SECTION 8.1

This section presented the results of initial studies on the effects of *in vitro* CBZ metabolism on ALA-D activity. Purified microsomes were shown to be capable of metabolizing CBZ to a number of products, including CBZ-E. The nature of these other compounds was not known and they appeared to be transient. When ALA-D was incubated with rat liver microsomes, NADPH and CBZ such that metabolism of CBZ took place, some inactivation of the enzyme occurred. Although in the course of these incubations some ALA-D activity was lost from controls, the amount of activity lost from flasks in which CBZ was being metabolized was always greater. Further experiments showed that inactivation of ALA-D linked to drug metabolism did not occur with primidone, implying that this has a particular property of CBZ. It was also shown that the species responsible for the inactivation of ALA-D was specific for that enzyme, although it was not possible to say whether or not this specificity was absolute.

In conclusion, <u>in vitro</u> metabolism of CBZ results in the generation of a species, the nature of which is unknown, which irreversibly inactivates ALA-D. In the following section, further investigations on the nature of this inactivation are presented.

8.2 FURTHER INVESTIGATIONS ON THE INACTIVATION OF ALA-D PRODUCED DURING IN VITRO METABOLISM OF CBZ

This section investigated several aspects of the inactivation of ALA-D which occurs during the in vitro metabolism of CBZ. It is divided into 3 sub-sections. The first of these examines the effect of having different concentrations of microsomes present in the incubation and also investigates if the presence of DTT in the incubation protects the enzyme against inactivation. The second sub-section attempts to evaluate if Zn^{2+} afforded any protection against inactivation and the third summarises the results of this section.

8.2.1 IN VITRO METABOLISM OF CBZ: EFFECTS OF DTT AND DIFFERENT CONCENTRATIONS OF MICROSOMES ON THE EXTENT OF ALA-D INACTIVATION

Studies in the previous section revealed that ALA-D activity was reduced following incubation of the enzyme with a CBZ metabolizing system. In those studies DTT was present to stabilize the enzyme.

This sub-section investigates the effects of omitting DTT from the incubation. If the omission of DTT resulted in an increased loss of ALA-D activity, it would infer that inactivation was occurring at a sulphydryl group. As part of the same experiment, two different amounts of microsomes were used to determine if the extent of inactivation was related to the quantity of m.f.o. activity present. The quantity of microsomes added is expressed as nmoles cytP450 added. Except for the change in the amount of microsomes added and the omission of DTT, the incubations were set up exactly as described in sub-section 2.8.3. ALA-D activities were measured at the start of the incubation period (zero time) and after two hours in a shaking water bath at 37°C. Results were expressed on the activity remaining

after 2 hours as a percentage of the activity at zero time. The results are presented in table (48). Flask No.1 was the control as it did not contain NADPH and therefore could not metabolise CBZ. The results from this flask show that in the absence of CBZ metabolism, ALA-D activities after 2 hours were 82% of the activities at zero time. The results from flasks numbers 2 and 3 show that in the absence of DTT, even if all the components necessary for metabolism of CBZ were present, there was very little further inactivation of ALA-D compared to the control. However, in flasks numbers 4 and 5, identical to numbers 2 and 3 respectively except that DTT was present, activity was reduced by a further 10 to 20%. This shows that, contrary to expectation, the presence of DTT in the microsomal incubation actually promoted inactivation of ALA-D. This can only be because DTT was required for the maintenace of some component of the microsomal MFO system. For this reason it is not possible to assess whether or not DTT protects the enzyme against the inactivation produced during <u>in vitro</u> metabolism of CBZ.

As stated earlier, this experiment also investigated whether the extent of inactivation was related to the amount of microsomal MFO activity present. Examination of the results from flasks numbers 4 and 5 in table (48) reveals that the addition of microsomes containing 2.5 and 5 nmoles of cytP450 caused ALA-D activities to be reduced to 73% and 63% of the activities or zero times respectively. When these reductions in activity are compared to the control in which 82% of the original activity remained, it can be seen that CBZ metabolism produced additional reductions in activity of 9% and 19% for incubation containing 2.5 and 5 nmoles of cytP450 respectively. This

TABLE (48)

IN VITRO METABOLISM OF CBZ: EFFECTS OF DTT AND DIFFERENT CONCENTRATIONS OF MICROSOMES ON THE EXTENT OF ALA-D INACTIVATION

Flask No.	% INITIAL ALA-D ACTIVITY REMAINING AFTER TWO HOURS	nmoles cyt P450 added	DTT (2mM)	NADPH (1mM)
1	82	5	x	
2	80	2.5		x
3	83	5		x
4	73	2.5	x	x
5	63	5	x	x

Flask 1 was the control since no metabolism of CBZ could occur.

Flasks 4 and 5 were compared to flasks 2 and 3 respectively to investigate if the presence of DTT afforded ALA-D any protection against inactivation.

X = present in the incubation.

shows that twice as much MFO activity results in twice as much inactivation.

Conclusions of sub-section 8.2.1

DTT was shown to be essential for inactivation to occur to any measurable degree, possibly because some component of the microsomal m.f.o. system is sensitive to loss of activity through oxidation of thiol groups. The possible protective role of DTT on the enzyme, therefore, could not be assessed. A correlation was found between the quantity of microsomes added and the extent of ALA-D inactivation.

8.2.2 IN VITRO METABOLISM OF CBZ: THE EFFECT OF Zn^{2+} IONS ON THE EXTENT OF ALA-D INACTIVATION

This sub-section investigated what effect the presence of Zn^{2+} ions has the extent of the inactivation of ALA produced during the *in vitro* metabolism of CBZ. If, in the presence of Zn^{2+}, less ALA-D activity was lost following incubation with a CBZ metabolizing system than was lost in the absence of these ions, it would suggest that the species responsible for inactivation was attacking the enzyme at a zinc binding site. For each experiment two control flasks and 2 test flasks were prepared as described in sub-section 2.8.3. To one of each of these, Zn^{2+} ions were added as $ZnCl_2$ to give a final concentration of 0.5mM. ALA-D activity was measured at the start of the experiment and after incubating the flasks for 2 hours in a shaking water bath at 37°C. Despite repeating this experiment several times, no conclusive results could be obtained. In some experiments the presence of Zn^{2+} ions seemed to prevent any loss of activity from both control and test incubations even although the

results from the other two flasks which did not contain Zn^{2+} were the same as was found in sub-section 8.1.2. i.e. loss of activity in control, but greater loss of activity in the test sample in which CBZ was being metabolized. In other experiments, activity was lost from Zn^{2+} containing control and test incubations at the same rate, and in yet others activity was lost from Zn^{2+} containing test incubations at a faster rate than it was lost from Zn^{2+} containing control incubations. In both of these circumstances however, activity was lost at a slower rate than it was in the corresponding incubations which lacked Zn^{2+}. The reasons for this extensive variation in the effects of Zn^{2+} on ALA-D activity in the presence of a CBZ-metabolising system are unknown.

Conclusions on the effect of Zn^{2+} ions on the extent of ALA-D inactivation produced during in vitro metabolism of CBZ

No conclusions can be drawn about whether or not Zn^{2+} protects against inactivation of ALA-D by a species generated during the in vitro metabolism of CBZ.

8.2.3 SUMMARY OF SECTION 8.2

In this section it was shown that the extent of ALA-D inactivation was dependent on the quantity of m.f.o. activity (as judged by the amount of cytP450 present) added to the incubation. Experiments aimed at determining the nature of the site on the enzyme at which the event leading to inactivation occurs were also performed. However, no conclusions can be made as to whether or not the presence of DTT or Zn^{2+} ions offered any protection against inactivation by the species generated in the course of the incubations.

8.3 IN VITRO METABOLISM OF CBZ-E: EFFECTS ON ALA-D ACTIVITY

In section 7.1, it was shown that the addition of CBZ or CBZ-E to cultures of mitogen stimulated human lymphocytes produce inactivation of ALA-D in the cells, and furthermore, that CBZ-E was much more effective than CBZ at inactivating the enzyme. The effect of incubating CBZ-E with the drug metabolizing system on ALA-D activity was investigated. The aim of the experiment was to determine if under these conditions CBZ-E would cause inactivation of ALA-D and then to compare the results with those obtained with CBZ. Four flasks were set up: two control flasks containing microsomes and ALA-D and two test flasks containing microsomes, ALA-D and NADPH. One control flask and one test flask contained CBZ (50mg/l) and the others contained CBZ-E (50mg/l). The flasks were incubated at 37^{o}C in a shaking water bath. Aliquots were removed at 0, 0.5, 1, 1.5, 2, 2.5 and 3 hours and the percentage of ALA-D activity remaining determined. The results are presented in fig.(33) and show that in both controls (containing CBZ or CBZ-E) activity was lost at the same rate and that in both tests activity was lost at the same rate, though the rate of loss of activity was faster in these than it was in controls. Aliquots from the reaction mixture were also analysed by the HPLC method described in sub-section 2.4.3 to investigate if any metabolism of the epoxide took place. The resultant chromatograms showed the appearance of several new peaks following incubation of the epoxide with microsomes and NADPH but not with microsomes alone. Two of these chromatograms are shown in fig. (34). The nature of these products is unknown.

One puzzling feature of these results is that CBZ-E caused the same amount of inactivation of the enzyme at the same rate as CBZ but in cultured lymphocytes, CBZ-E caused much greater inactivation more quickly than CBZ. The reasons for this difference are unknown. It may be that CBZ and CBZ-E were activated in different ways in the two different systems, or it may be that CBZ-E was activated to the same extent in the drug metabolising system as it was in cell culture, but that greater activation of CBZ occurs in the drug metabolizing system. Whatever the mechanism, it is clear that CBZ-E is activated or metabolised by the in vitro drug metabolising system used for these studies and this results in inactivation of ALA-D.

Conclusions on the effects in vitro metabolism of CBZ-E on ALA-D activity

CBZ-E was metabolized to unknown products by the purified microsomes used in these studies, causing inactivation of ALA-D present in the incubation at the same rate as was found with CBZ.

8.4 SUMMARY OF CHAPTER 8

This chapter investigated if metabolism or bioactivation of CBZ by purified microsomes containing m.f.o. activity would result in the inactivation of ALA-D. This was indeed the case, although the species actually responsible for the inactivation of the enzyme remains unknown. The effect occurred only with CBZ and was not caused by some by-product of the m.f.o system. The inactivating species reacted selectively with ALA-D. The extent of the inactivation of ALA-D was related to the levels of microsomal m.f.o. activity present in the incubation. Further experiments which attempted to elucidate the nature of the site upon which the inactivator acts produced no conclusive results. Surprisingly, CBZ-E was also transformed to some extent by this in vitro drug metabolizing system and this also resulted in inactivation of ALA-D. However, in contrast to the results of Chapter 7, CBZ and CBZ-E were equally effective at inactivating ALA-D. The reasons for this can only be speculative, but it is probable that the inactivating species produced here were different from those produced in the lymphocyte culture system.

The most significant conclusion of the chapter is that the microsomal m.f.o. system acted on CBZ and CBZ-E to generate a metabolite or activated intermediate which inactivated ALA-D. It is likely that this is the mechanism by which ALA-D was inactivated in rat liver, but whether the loss of activity in erythrocytes in man was due to activated species from the m.f.o. system or from other metabolically active cells (which presumably would respond in the same way as cultured lymphocytes) is unknown.

CHAPTER 9

CHAPTER 9

SUMMARY AND GENERAL DISCUSSION

This thesis was primarily concerned with determining and explaining the effects of the anticonvulsant drug carbamazepine on haem biosynthesis in man, although the effects of sodium valproate, phenytoin and phenobarbitone were also studied. Initial studies were concerned with the long-term effects of these drugs on the pathway. These were followed by more detailed investigations into the effects of CBZ itself. This final chapter summarises and discusses the significance of all of these investigations before assessing if the objectives of this thesis, described in section 1.4, were attained.

9.1 SUMMARY AND DISCUSSION OF RESULTS

In the discussion of these results, two issues must be taken into consideration. Firstly, although the effects of drugs were studied in different systems, the primary aim of all of the experiments was a fuller understanding of the effects of the drugs on haem biosynthesis in man. Secondly, the effects of the drugs on haem biosynthesis in peripheral blood cells in man were assumed to be representative of the effects on haem biosynthesis in the liver, the major site of haem biosynthesis in the body.

The effects of each of the drugs studied on haem biosynthesis are now discussed.

9.1.1 PHENOBARBITONE

Only a small number of patients taking phenobarbitone (PB) were studied and consequently the results are limited, but no effect was found on ALA-D, PBG-D or URO-D activities. Whether or not chronic derepression of ALA-S occurs, as was found with VPA and CBZ, was not assessed. However, it is well known that PB is a very potent inducer of the m.f.o. system and would therefore be expected to cause chronic derepression of the enzyme.

9.1.2 PHENYTOIN

The results of all enzyme analyses in patients taking phenytoin were no different from controls, although there was a slight change in the urinary excretion of PBG. However no definitive statements on the effects of long term phenytoin therapy on haem biosynthesis can be made because of the low plasma levels of the drug in the patients studied. The fact that these plasma concentrations were much lower

than expected from the supposed dose suggests a lack of compliance in these patients.

9.1.3 SODIUM VALPROATE

The effects of sodium valproate (VPA) are discussed under individual headings.

ALA-S

ALA-S activity was increased during long-term treatment with VPA. It was also found that short-term treatment with VPA produced increases in ALA-S activity. The mechanism by which most drugs increase ALA-S activity is through an increased haem requirement for the de novo synthesis of cytP450 which is usually required for metabolism of the drug. This may not seem likely in the case of VPA because this drug is not primarily metabolized by the MFO system of which cytP450 is a part. However, the fact that chronic VPA therapy produces increased 6B-OHC excretion (an *in vivo* index of hepatic m.f.o. activity) suggests that an increased cyt P450 requirement is indeed the mechanism by which ALA-S is derepressed in these patients. VPA is known to have inhibitory effects on the m.f.o. system. Therefore the increase in the activity of this system observed here is probably the result of overcompensating for the inhibitory effects of VPA.

ALA-D, PBG-D and URO-D

These enzymes were unaffected by treatment with VPA.

Urinary excretion of porphyrins and precursors

There were increases of the urinary excretion of ALA, PBG and total porphyrin during treatment with VPA, although the increase was only significant as regards PBG and total porphyrin. These increases were believed to be due to increased ALA-S activity. There was no correlation between drug levels and the excretion of any of these.

Qualitative analysis of urinary porphyrins

Long term treatment with VPA resulted in a slight change in the urinary porphyrin excretion profile characterized by an increase in the % coproporphyrin. This was explained by the increased availability of pathway intermediates due to derepression of ALA-S and a difference in the relative activities of URO-D and COPRO-O.

9.1.4 CARBAMAZEPINE

The effects of carbamazepine (CBZ) on haem biosynthesis are discussed under individual headings.

ALA-S

Rapeport *et al* (1983) have shown that short term treatment with CBZ in man resulted in an elevation of ALA-S activity. Results presented in this thesis revealed that the increase in ALA-S activity was sustained during long term treatment with CBZ, although activities were not as high as during the initial stages of treatment. CBZ is metabolized prior to excretion by cytP450 dependent m.f.o. system. It is therefore highly probable that ALA-S was derepressed to produce enough haem to satisfy the increased haem requirements generated by increased synthesis of cytP450 apoprotein according to the mechanism described in sub-section 1.3.9. In support of this, 6B-OHC excretion, (an *in vivo* index of hepatic m.f.o. activity) was increased

in patients receiving CBZ. Further evidence that cytP450 dependent m.f.o. activity was induced in man was noted by Rapeport et al (1983). In subsequent studies in the rat, induction of cyt P450 was shown to be linked to the derepression of ALA-S. Rat liver did not prove to be an ideal system for modelling the effects of CBZ treatment in man since activity soon returned to control levels. This may be due to differences in the relative activites of the other enzymes in the pathway and m.f.o. system or to the fact that activity was measured in a different tissue. The mechanism by which CBZ stimulated the production of cytP450 in the rat and presumably in man is not known, but it was probably not due to CBZ-mediated destruction of cypP450. To return to man, it is possible that in some of the patients studied, the derepression of ALA-S was partly due to an impairment of the flow of metabolites through the pathway as a consequence of reduced ALA-D activity. This was not true for the majority of the patients studied. However, even in those where it may have been applicable, it would not have been as significant as the effect of increased cytP450 requirement. In support of this there was no correlation between ALA-S and ALA-D activities. There was no correlation between ALA-S activity and plasma CBZ, CBZ-E or free CBZ concentrations.

ALA-D.

Long term treatment with CBZ caused a substantial reduction in the activity of erythrocyte ALA-D in man. The reduced activity in these patients was not due to a coincidental high blood lead level. The reduction in activity was irreversible. The Km values for ALA and the optimum assay pH of ALA-D in CBZ-treated patients were identical to controls. These facts suggested that the low activities were probably due to a reduced amount of normal enzyme rather than the

alternative explanation: a normal amount of modified enzyme. In these patients there were correlations between plasma "total" CBZ and CBZ-E concentrations and ALA-D activity, but not between free CBZ levels and ALA-D. Activities were not affected for some time, even after several days in the presence of plasma concentrations of CBZ and CBZ-E found in patients with reduced ALA-D. This suggested that enzyme activity was not lost as a consequence of a direct effect of CBZ or CBZ-E on the enzyme. Loss of activity was not due to a loss of glutathione, a tripeptide responsible for the maintenance of thiol groups sensitive to oxidation in the reduced state. ALA-D has several such groups.

Studies in rats confirmed that CBZ treatment produced an irreversible loss of ALA-D activity and that the extent of the loss was dependent on the dose of CBZ administered. As was the case in man, activity was not immediately affected, even in the presence of high CBZ and CBZ-E concentrations, providing further evidence that activity was not reduced through a direct action of CBZ or CBZ-E on the enzyme. This was confirmed by *in vitro* studies which also showed that the loss of ALA-D activity was not caused by a direct action of any metabolite of CBZ present in plasma or erythrocytes unless the metabolite was very short lived.

In further studies using mitogen-stimulated human lymphocytes, it was shown that both CBZ and CBZ-E caused a specific, irreversible loss of ALA-D activity without detectable metabolism of drug. CBZ-E was much more effective than CBZ. The loss of ALA-D activity in this system could only be explained by inactivation of existing enzyme and not by a reduction in enzyme synthesis. The fact that this occurs

here but does not occur when these compounds are added to metabolically inactive preparations of the enzyme suggests that the inactivating species is produced through some bioactivation of the drugs which occurs in living cells. The nature of this activated species is not known.

Data from *in vitro* drug metabolism studies showed that the action of the cytP450 dependent MFO system on CBZ and CBZ-E generated species which caused inactivation of ALA-D. Whether the species responsible were metabolites or transient intermediates is not known, but it is unlikely that they were the same as those responsible for inactivation of ALA-D in cultured cells. The inactivating species were only generated from CBZ or CBZ-E and showed specificity for ALA-D. The relevance of these results to the *in vivo* situation is doubtful.

In the absence of metabolism, CBZ and CBZ-E only caused inactivation of ALA-D in metabolically active cells. This provides a possible explanation for the length of time taken for erythrocyte ALA-D activities to be affected *in vivo*. Since any bioactivated form of CBZ or CBZ-E must be unstable, it is unlikely that they would be found outwith cells and could therefore only inactivate ALA-D in active cells. ALA-D activity in mature erythrocytes therefore, would be unaffected. However, ALA-D in developing erythroblasts would be inactivated but it would take some time for affected cells to mature and make up a significant proportion of the erythrocyte population. This hypothesis would suggest that ALA-D should have been rapidly inactivated in rat liver. This was not so. Possible explanations for this are the faster rate of enzyme turnover in the rat and the fact that rat liver is very efficient at removing toxic xenobiotics through further metabolism and conjugation. Whereas the relevance of

inactivation of ALA-D in the course of metabolism of CBZ is uncertain (except perhaps in the liver) the activation of these compounds by metabolically active cells with the result inactivation of ALA-D almost certainly occurs *in vivo*.

PBG-D

Previously Rapeport *et al* (1984) and Yeung *et al* (1983(B)) had reported a loss of PBG-D activity during treatment with CBZ. These claims were refuted by Doss and Shafar (1983); Rideout *et al* (1983) and Shanley, (1983).

Initial results showed that PBG-D activities seemed to be reduced by CBZ and that the loss of activity was related to plasma concentrations of the drug. Subsequent studies however revealed that PBG-D activity was not affected by CBZ and that these earlier results were artifacts produced by the fact that the assay used relied on normal levels of ALA-D activity (not present in CBZ treated patients) to generate sufficient substrate for PBG-D. In support of this, PBG-D activity was unaffected in rats during CBZ treatment. CBZ itself did not affect PBG-D in cultured lymphocytes, but the effects of CBZ-E could not be assessed because it interfered with the assay.

This result is important because it demonstrates how the use of a coupled enzyme system can produce false results if the activity of the coupling enzyme is inadequate.

URO-D

URO-D activity was not affected by CBZ treatment.

Urinary excretion of porphyrins and precursors

The urinary excretion of ALA, PBG and total porphyrin was increased by long term CBZ treatment, but not to a significant level.

This was probably due to the derepression of ALA-S already described. The fact that PBG and porphyrin excretion were slightly increased shows that ALA-D activity in general was not reduced to an extent where flux through the pathway was disturbed. There was however a subtle change in the relationship between ALA and PBG concentrations.

The results of studies on the effects of CBZ on ALA, PBG and total porphyrin excretion in rat urine showed changes in ALA and PBG excretion which could be partially explained by the alterations in ALA-S and ALA-D activities. However no conclusions of the effects of CBZ on total porphyrin excretion were made for reasons described in the text.

In tissue culture, both CBZ and CBZ-E caused a reduction in the amount of total porphyrin synthesized from ALA, which correlated with the loss of ALA-D activity. However porphyrin synthesis would not be affected in this way *in vivo*, even if ALA-D activity was reduced to the same extent. This was confirmed by the finding that all the urines studied from CBZ treated patients had normal or slightly raised total porphyrin excretion. The reasons for this difference in the *in vivo* and *in vitro* models is that *in vitro* the rate of porphyrin synthesis depends on the amount of ALA-D activity (since ALA is present in saturating amounts), whereas *in vivo* the rate limiting factor is the amount of ALA produced for ALA-D to act upon. Normally this is well below the km of ALA-D and therefore the rate of porphyrin synthesis is not dependent on the amount of ALA-D present but on the level of ALA-S activity.

Qualitative analysis of urinary porphyrins

No differences were found in the relative proportions of porphyrins in urine from long term CBZ-treated patients and controls,

indicating no changes in COPRO-O, PROTO-O or FERRO-C activity. This did not fit in with the findings of Rapeport et al, (1984) who reported large increases in the URO fraction following short term treatment with the drug. This result was confirmed. Since these changes were transient and occurred at the start of treatment, it may be that they were a consequence of the very high levels of ALA-S present at this time. This would have caused more PBG to be produced than PBG-D could cope with, with the result that the excess would be excreted in the urine where it could undergo non-enzymatic condensation and cyclization to produce URO-I. No increase in the % URO was found in the rat urine. Possible reasons for this are discussed in the text.

In cell culture, the in vivo effects of CBZ and CBZ-E on ALA-D were shown to occur. This did not result in any change in the relative proportions of porphyrins produced from ALA in these cells. This would therefore suggest that the change in urine porphyrin composition in vivo must be due to the one other enzyme shown to be affected by CBZ treatment which would have no effect on this assay, namely ALA-S.

These findings would imply that derepression of ALA-S by other drugs to the very high levels produced by CBZ would also cause an increase in the % URO in the urinary porphyrin excretion profile. It would therefore be prudent when screening for porphyria by analysis of urinary porphyrins to check that the subject is not taking any drugs known to induce m.f.o.activity and consequently raise the levels of ALA-S activity.

APPLICATION OF THESE RESULTS TO THE FINDINGS OF RAPEPORT AND YEUNG

The results presented in this thesis regarding the effects of CBZ on haem biosynthesis can explain the findings made by Rapeport et al (1984) in their study of the effects of CBZ on the pathway, i.e. elevated ALA-S activity, an apparent reduction in PBG-D activity, slightly increased urinary excretion of ALA, PBG and porphyrin and an altered urinary porphyrin excretion profile. However they do not explain some of the results published by Yeung et al (1983). In that paper a patient taking carbamazepine was shown to have a moderately raised ALA-S activity (395 nmolALA/h/g protein) an apparently reduced PBG-D activity (9 nmolURO/h/ml erythrocytes) and elevated urinary excretion of ALA, PBG and URO (171 μmol/l, 194 μmol/l and 1022 nmol/l respectively). Unfortunately values for 24 hour excretions of these compounds are not given and consequently it is difficult to gauge the extent of the increased excretion. The patient also experienced severe abdominal pain for which no cause could be found and passed dark urine.

The ALA-S activity in this patient was not greatly derepressed: many of the patients taking CBZ who were studied as part of this thesis had activities in excess of this. Therefore this effect of CBZ is not unusual. Nor is the apparent reduction in PBG-D activity, which was presumably due to the effects of CBZ on ALA-D as discussed previously. The finding that it took 3-4 weeks for PBG-D activity to return to normal after cessation of CBZ therapy fits in nicely with the observation that it would take this time to renew the erythrocyte population with cells containing unaffected ALA-D. What is difficult to explain is the increased urinary excretion of ALA, PBG and URO. There are two possible explanations. The first is that the excretion

of these compounds was not greatly increased at all, but that the urine was very concentrated. This would also explain the dark colour of the urine and cannot be ruled out as a possibility since 24 hour urine volumes were not given. The second is that the excretion of these compounds was increased because of an impairment of the flow of metabolites further on in the pathway or because the low levels of PBG-D detected were not entirely due to the effects of CBZ on ALA-D. Whichever of these is the case, it would appear that the clinical response of this patient to CBZ treatment was atypical of all of the other patients studied. His many clinical symptoms may have simply been due to extreme sensitivity to the side-effects of CBZ.

9.2 GENERAL CONCLUSIONS

This thesis investigated the effects of CBZ, VPA, DPH and PB on haem biosynthesis in man. As stated in the introduction, the objectives were:

(i) to establish if CBZ, VPA or DPH could be safely used for seizure prophylaxis in individuals with porphyria

(ii) to investigate and elucidate the mechanism(s) whereby CBZ treatment produced an apparent acute intermittent porphyria (AIP) syndrome

and

(iii) to assess the suitability of CBZ treatment as a model for studying AIP

These aims have been satisfied and allow the following general conclusions to be drawn.

(1) CBZ and VPA are contra-indicated in the porphyrias due to the sustained derepression of ALA-S produced by chronic treatment with these drugs. However, they may be tolerated in some patients without adverse effect (Lai, 1981; Peters, 1981; Sergay, 1979 and Brodie et al, 1977,B). Such results reflect the fact that even in the presence of ALA and PBG levels which could be associated with an acute porphyric attack, some individuals remain asymptomatic. Though it was not proven here, other work has shown that DPH is probably unsafe to use for treatment of seizures in porphyria (McColl et al, 1980).

(2) In light of the effects of CBZ on ALA-D, it would be unwise to prescribe this drug to individuals whose levels of ALA-D activity are already chronically reduced by pathological conditions such as lead-poisoning, severe alcoholism or hereditary tyrosinemia.

(3) The altered porphyrin excretion profile in the initial stages of CBZ treatment was a consequence of extreme derepression of ALA-S. Presumably, therefore, other drugs which cause derepression of the enzyme to the same degree would also cause changes in the urinary porphyrin excretion profile. In view of the increasing interestinscreening for porphyria by qualitative analysis of urinary porphyrins, it is important to ensure that the subjects are not taking any such drugs, otherwise a mis-diagnosis of porphyria may result.

(4) CBZ does not cause a loss of PBG-D activity as reported by Yeung et al (1983) and Rapeport et al (1984). These findings were erroneous due to the use of a coupled assay system in which ALA-D was the coupling enzyme, (ALA-D activity is reduced by CBZ).

(5) The effects of CBZ on rat liver and on cultured human lymphocytes were in general the same as in man. The use of these systems allowed further probing of the nature of the effects of CBZ on haem biosynthesis.

(6) CBZ treatment is not a suitable model for studying AIP.

APPENDIX I

PATIENT DETAILS

Control males		CBZ-treated males		
No.	Age	No.	Age	Type of epilepsy*
1	14	1	15	I
2	23	2	18	I
3	23	3	18	I
4	24	4	20	I
5	24	5	21	PC
6	24	6	25	PC
7	24	7	26	I
8	25	8	27	I
9	25	9	27	PC
10	26	10	28	PC
11	26	11	29	PC
12	26	12	29	I
13	27	13	30	PC
14	28	14	30	PC
15	31	15	30	PC
16	32	16	30	PC
17	32	17	32	I
18	35	18	32	PC
19	39	19	33	I
20	39	20	33	PC
21	39	21	35	I
22	39	22	36	I
23	49	23	37	PC
24	51	24	37	PC
25	71	25	43	PC
		26	43	I
		27	46	PC
		28	55	I
		29	59	PC
		30	62	I

* PC = Partial complex

I = Idiopathic

APPENDIX I (cont)

Control females		CBZ-treated females		
No.	Age	No.	Age	Type of epilepsy *
1	14	1	13	PC
2	16	2	14	I
3	17	3	17	PC
4	20	4	18	I
5	20	5	18	PC
6	24	6	19	PC
7	25	7	20	I
8	25	8	21	PC
9	26	9	23	PC
10	29	10	23	PC
11	28	11	24	PC
12	32	12	24	I
13	42	13	26	PC
14	44	14	26	PC
15	49	15	30	PC
16	50	16	37	PC
17	57	17	45	I
		18	47	PC
		19	57	I
		20	57	PC
		21	58	PC
		22	64	PC
		23	75	I

* PC = Partial complex
I = Idiopathic

Phenytoin-treated subjects

No.	Sex	Age	Type of epilepsy *
1	M	20	PC
2	M	27	PC
3	M	27	PC
4	M	42	I
5	M	43	I
6	M	45	I
7	M	53	I
8	F	54	PC
9	F	59	PC
10	M	61	I
11	F	69	PC

Sodium valproate-treated subjects

No.	Sex	Age	Type of epilepsy *
1	F	13	I
2	F	14	I
3	M	14	I
4	F	16	PC
5	M	16	I
6	F	18	I
7	M	24	I
8	M	25	I
9	F	25	I
10	M	28	I
11	F	50	I
12	F	57	I

* PC = Partial complex
I = Idiopathic

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Each of the above transactions was presented in poster form at Biochemical Society Meetings in Leeds (July 1984), Stirling (Sept. 1984) and Oxford (July 1985) respectively.

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IUPHAR 9th International Congress of Pharmacology, London, July-August 1984. Oral Presentation: The effects of carbamazepine on 5-aminolaevulinate dehydratase in rat and man.