A POSSIBLE ROLE FOR △-AMINOLAEVULINIC ACID IN ACUTE INTERMITTENT PORPHYRIA

by

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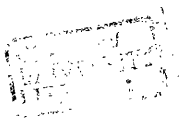

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SUMMARY

From a consideration of past work on acute intermittent porphyria, it appeared that the porphyrin precursor most likely to be involved in the production of the clinical manifestations of the disease was δ-aminolaevulinic acid (ALA).

A series of experimental investigations on ALA was carried out on both human and animal systems. The main findings were as follows:-

a) The blood ALA concentrations of a number of porphyrics in remission, were significantly higher than the concentrations in normal subjects. In one porphyric subject ALA was detected in the cerebro-spinal fluid.

b) Rats which were made experimentally porphyric by increasing ALA synthetase activity by treatment with ally-isopropyl-acetamide; were significantly more susceptible than untreated rats to convulsions induced chemically by isonicotinyl hydrazide. Rats pretreated with ALA however, did not show this increased susceptibility to induced convulsions.

c) ALA was found to be capable of passing the blood-brain barrier at blood concentrations known to occur in acute porphyria. Acute ethanol intoxication inhibited this process. ALA was capable of remaining unchanged in the brain tissue against a brain-blood concentration gradient, for up to three days.

d) ALA produced behavioural changes when administered to experimental animals. In mice a single dose of ALA given intraperitoneally, caused an initial significant depression in spontaneous activity, which was soon followed by a significant increase in spontaneous activity, which lasted for at least ninety minutes after ALA administration. Mice chronically pretreated with ethanol demonstrated a similar pattern of response to ALA.

Rats chronically pretreated with ALA, showed signs of severe behavioural disturbance. Spontaneous activity and all parameters measuring inquisitiveness were severely depressed. Withdrawal and righting reflexes were, however, normal.

e) The substance 2,4 dimethyl-3-ethylpyrrole, which is commonly found in schizophrenics and which may be formed by a reaction involving ALA, was found to be present in the urine of subjects with porphyria, but not in the urine of normal subjects. None of these porphyric subjects showed signs of mental abnormality.

f) At blood concentrations known to occur in acute porphyria, ALA was found to be capable of penetration into the following tissues:- heart, brain, liver, kidney, spleen, ileum and mesenteric fat.

g) In the anaesthetised and pithed rat preparations, ALA was found to cause a fall in blood pressure. It was considered unlikely, on the basis of supportive evidence from experiments, that ALA did this by acting centrally, neurally on cardiac muscle on adrenergic or cholinergic receptors, or by histamine release. The action thought most likely to account for this fall was a histamine like action on the capillaries.

h) On the isolated perfused rabbit ear artery, ALA produced no statistically significant changes in the response of the preparation to injected noradrenaline or to periarterial sympathetic nerve stimulation.

i) Using the isolated guinea pig ileum, it was found that ALA had no statistically significant effect on the response of the muscle to injected acetylcholine or histamine, or to electrical field stimulation.

j) ALA was found to be capable of inhibition of the total ATPase fraction of the guinea pig ileum. It did not, however, as might be expected on this basis, significantly increase the neural output of acetylcholine from muscle strips of ileum.

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The significance of these findings is discussed with particular emphasis on the possibility that ALA may play a role in the production of the clinical manifestations of acute intermittent porphyria.

SECTION I

GENERAL INTRODUCTION

Background

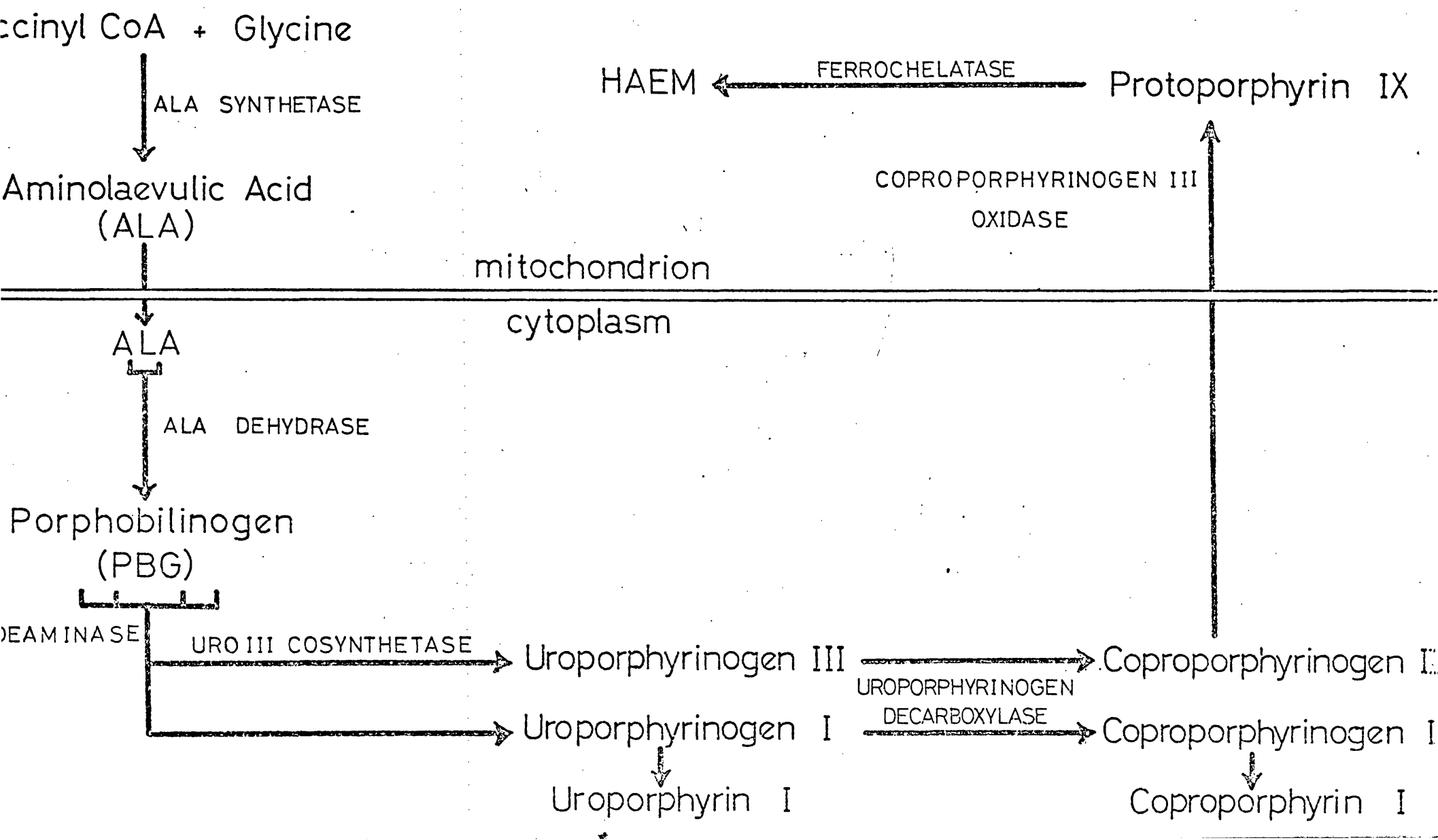
The word 'porphyria' literally means 'purple urine'. The porphyrias are a group of hereditary metabolic disorders of haem and porphyrin biosynthesis which often result in the excessive passage of the porphyrins and their precursors in the urine, hence the derivation of the term. They are classical examples of those diseases generally termed 'inborn errors of metabolism'.

The first allusion to porphyrins was made by Scherer in 1841 and the first classification of the porphyrias in terms of diseases of porphyrin metabolism was made by Gunther in two papers published in 1911 and 1922. Since this time research into these diseases has been extensive; for although they themselves are comparatively rare (about 1.5 cases per 100,000 for Acute Intermittent Porphyria) they have many aspects in common with other diseases such as lead poisoning, and hence they are of general interest. Our knowledge of all aspects of the porphyrias has progressively increased in concert with the gradual refinements of research techniques, and continues to do so.

The primary lesion of the porphyrias is considered to be one concerning haem and porphyrin metabolism. Thus as an initial approach to a study of these diseases the haem biosynthetic pathway will be described.

Fig. 1

THE HAEM BIOSYNTHETIC PATHWAY



Haem Biosynthesis. (FIGURE 1)

As a result of work both on mammalian and non-mammalian systems, the general mechanisms of haem biosynthesis are now well established. The first recognisable units of haem are glycine, which is readily available in the cell, and succinyl CoA, which comes from the Kreb's cycle. These are condensed to form the pathway's primary precursor δ-aminolaevulinic acid (ALA). This condensation reaction, which occurs in the mitochondria, is catalysed by the enzyme ALA synthetase; the reaction requires as cofactors pyridoxal phosphate (Schulman and Richert, 1956) and ferrous iron (Brown, 1958). The production of ALA is by way of a transient intermediate α-amino-β-keto-adipic acid, which, being very labile, rapidly decarboxylates to give ALA.

ALA synthetase is considered to be the rate limiting enzyme of the pathway (Lascelles, 1964; Levere and Granick, 1965) and is thus critically important in determining the rate of haem synthesis. There is evidence that haem regulates the activity of synthetase both by feed-back inhibition (Burnham and Lascells, 1963; Scholnick et al, 1969) and by repression of its synthesis (Hayashi et al, 1968; Marver, 1969; Sassa and Granick, 1970).

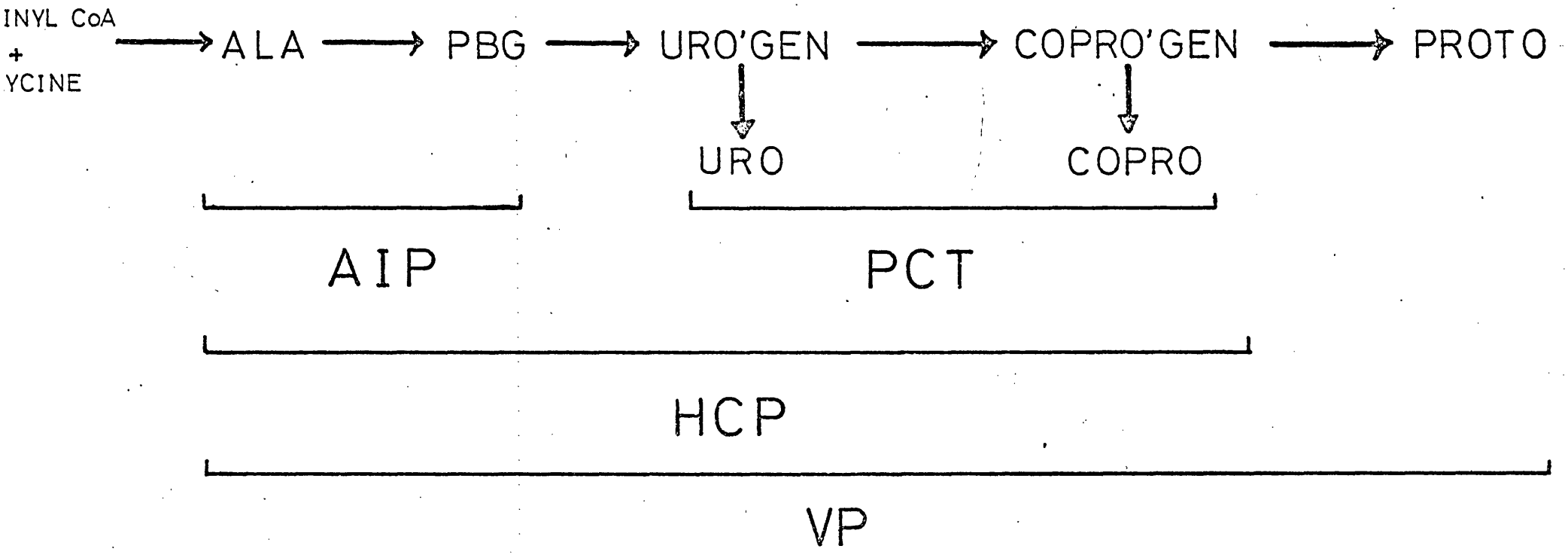
The next step of the sequence takes place in the cytoplasm, and consists of the condensation of 2 molecules of ALA and, with the elimination of water, the formation of the monopyrrole porphobilinogen (PBG). It is from PBG that the tetrapyrrolic porphyrins are subsequently synthesised.

Still in the cytoplasm, 4 molecules of PBG, in the presence of PBG deaminase and uroporphyrinogen I synthetase, undergo transformation to an unidentified intermediate which <u>can</u> result in the production of uroporphyrinogen I, but is normally converted to uroporphyrinogen III. The series III porphyrins are the ones most commonly found in nature.

Uroporphyrinogen III is then decarboxylated, in the presence of uroporphyrinogen decarboxylase, to form coproporphyrinogen III. Once again within the mitochondrian, this is oxidised and decarboxylated, in the presence of coproporphyrinogen oxidase, to form protoporphyrin IX. Finally ferrous iron is introduced into protoporphyrin IX by haem synthetase (ferro-chelatase) to form haem.

Fig. 2

orphyrins and precursors excessively excreted during the acute phases of each of the hepatic porphyrias



In this figure, each of the hepatic porphyrias is classified in terms of the porphyrins and their precursors, which are excreted during the acute phase.

The Porphyrias

The porphyrias have been classified by Schmid et al, (1954) into two types: the erythropoietic and the hepatic porphyrias. In any of these the disturbed porphyrin metabolism may lead to over-production and excretion of the porphyrins and their precursors. In the erythropoietic porphyrias, the site of this disturbed metabolism is the bone marrow and in the hepatic porphyrias it is the liver. A useful classification of the different types of porphyrias is given in Figure (2).

As this thesis is mainly concerned with acute intermittent porphyria (AIP) this will be discussed in some detail. As the other porphyrias have certain similarities, however, they will be briefly described individually before consideration of AIP.

The Erythropoietic Porphyrias

Congenital (Erythropoietic) Porphyria:

Although not proven, it is thought that this disease may be transmitted by a Mendelian recessive character. The most important manifestation is skin photosensitivity, exposure of the skin to sunlight often causing severe mutilations. Because of the large porphyrin content, the bones and teeth are often coloured a reddish-brown, and

in most cases there is splenic enlargement which may be associated with haemolytic anaemia and in some cases thrombocytopenia. Kramer et al (1965) suggested that the anaemia was due to ineffective erythropoiesis and shortened red cell survival.

Large quantities of uroporphyrin I are excreted in the urine and less in the stools. Coproporphyrin I is excreted predominantly in the stools. Uroporphyrin I, coproporphyrin I and increased amounts of protoporphyrin, are found in circulating erythrocytes. Uroporphyrin, coproporphyrin and protoporphyrin can be detected in the plasma. Taddeini and Watson (1968) have indicated that there is induction of ALA synthetase in the young red cells of the bone marrow, in this disease, but the aetiology of the disease has yet to be defined.

Erythropoietic Protoporphyria.

This disease is transmitted genetically and is the most common of the erythropoietic porphyrias. Its main feature is photo-sensitivity which manifests itself as a burning sensation on exposure to sunlight and which may have associated oedema. In children there is sometimes a vesicular crusted eruption on exposure to sunlight, but in adults the oedema, erythaeria and sometimes urticaria,

appear to be the only acute changes.

The urine in this disease contains normal amounts of porphyrin, the main biochemical feature being excessive quantities of protoporphyrin in the red cells, which may be accompanied by excessive faecal excretion of coproporphyrin and protoporphyrin. A significant increase in ALA synthetase activity has been demonstrated in this disease (Miyagi, 1967).

Congenital Erythropoietic Coproporphyria.

This disease would appear to be very rare only two cases, one being the mother of the original patient, having been described. Again there is skin photo-sensitivity. The urine and stool contain normal amounts of porphyrin, but the erythrocytes contain excessive coproporphyrin III. It has been suggested that this disease is transmitted by a Mendelian dominant character (Heilmeyer and Clotten, 1964).

The Hepatic Porphyrias

Porphyria Variegata.

This disease is inherited as Mendelian dominant character. In attack the manifestations are similar to those of acute intermittent porphyria although, unlike acute porphyria, in male patients light sensitivity often occurs. Attacks are much more common

among the female population, who do not often, however, develop photo-sensitivity.

During acute states there is a high urinary excretion of coproporphyrin, ALA and PBG. In most cases of acute attack, and in remission, faecal copro- and protoporphyrin is raised. These findings, in particular, distinguish porphyria variegata from acute porphyria. Dowdle et al (1967) has demonstrated that hepatic ALA synthetase is elevated in this disease.

Cutaneous Hepatic Porphyrias.

This sub-group of the hepatic porphyrias may result from different aetiological factors. Thus they can be of a hereditary origin or arise from hepatoxic processes such as over-indulgence in alcohol or from hepato-toxic agents such as hexachlorobenzene. In all these diseases excess porphyrins, synthesised in the liver, pass via the bile to be excreted in the faeces. When there is hepatic disfunction the porphyrins are diverted into the systemic circulation and are then mostly excreted in the urine. Again this disease exhibits skin manifestation:- photosensitivity, excessive fragility, hyper-pigmentation and hypertrichosis. Hereditary forms in particular can show similarities to acute

porphyria, and again hepatic ALA synthetase activity is elevated.

Hereditary Coproporphyria.

This is a relatively rare disease which may remain symptomless. If an attack occurs however, it resembles acute porphyria although photosensitivity can occur on occasion. Attack can be provoked by certain drugs such as barbiturates. In attack there is excessive urinary excretion of ALA and PBG. The characteristic biochemical finding is the massive urinary and faecal excretion of coproporphyrin III.

Experimental Porphyria

Experimental porphyria produced in animals by means of drugs is the subject of recent reviews by Goldberg (1968) and Tschudy & Bonkowsky (1972). The essential feature of experimental porphyria is the development of a disturbed porphyrin metabolism similar to that occurring in the hepatic porphyrias.

Many drugs and metabolic products of a structurally heterogeneous nature can produce distortion of porphyrin biosynthesis similar to that found in AIP. The principal of these are allylisopropylacetamide (AIA) (Goldberg, 1953), dicarbethoxydihydro-collidine, griseofulvin and hexachlorobenzene. All these 'porphyrinogenic' drugs are known to increase the activity of hepatic ALA synthetase.

Although experimental porphyria emulates closely the disturbed porphyrin metabolism of AIP, the most common manifestations of the disease are not seen in these animals. Thus in an extensive study of experimentally porphyric rabbits, Goldberg and Rimington (1954) indicated that the only visible clinical findings were loss of appetite, weight loss and constipation, with gaseous distention of bowel loops in one animal.

More recently however Kosower and Rock (1968) have found that rats pretreated with AIA show distinct neurological changes and they have suggested that this may serve as a model system for acute porphyria.

Acute Intermittent Porphyria

Acute intermittent porphyria is the most important member of the porphyria diseases. It is hereditary in nature, this fact being established by Waldenstrom in 1937. It is inherited as a Mendelian dominant character and is distinguishable from the other porphyrias by the relative dominance of gastro-intestinal and neuropsychiatric features in the absences of skin photo-sensitivity. In the acute and latent stages of the disease, and often in remission, patients excrete excessive quantities of urinary ALA and PBG (Haeger, 1958).

Although for an holistic appreciation of AIP

the diverse clinical and biochemical characteristics it portrays must be considered together; for the sake of clarity it will be discussed below under four headings:- a) Clinical Features; b) Biochemical Features; c) Intermittency and Precipitation of Attack and d) Possible Origin of Clinical Features.

a) Clinical Features. (Unless otherwise referred to this account is based on the review of 50 patients with AIP given by Goldberg and Rimington (1962).

The main clinical signs and symptoms of AIP are represented on Fig. 3. The most apparent symptoms are of a gastro-intestinal nature. Abdominal pain is very common; it is usually of a short-lasting nature but can continue for several hours or even days. It can be localised to one part of the abdomen or may, occasionally, involve the whole of the abdomen. The pain is very severe, and is accompanied by abdominal tenderness; muscular rigidity, however, is rare. Intestinal spasms, alternating with atoma and dilation, predominantly in the upper tract have been observed at lapatotamy. In association with this abdominal pain there is a high incidence of vomiting, which may be accompanied by constipation. Usually the vomiting occurs after the onset of the abdominal pain. Diarrhoea has been known to be present as well as vomiting.

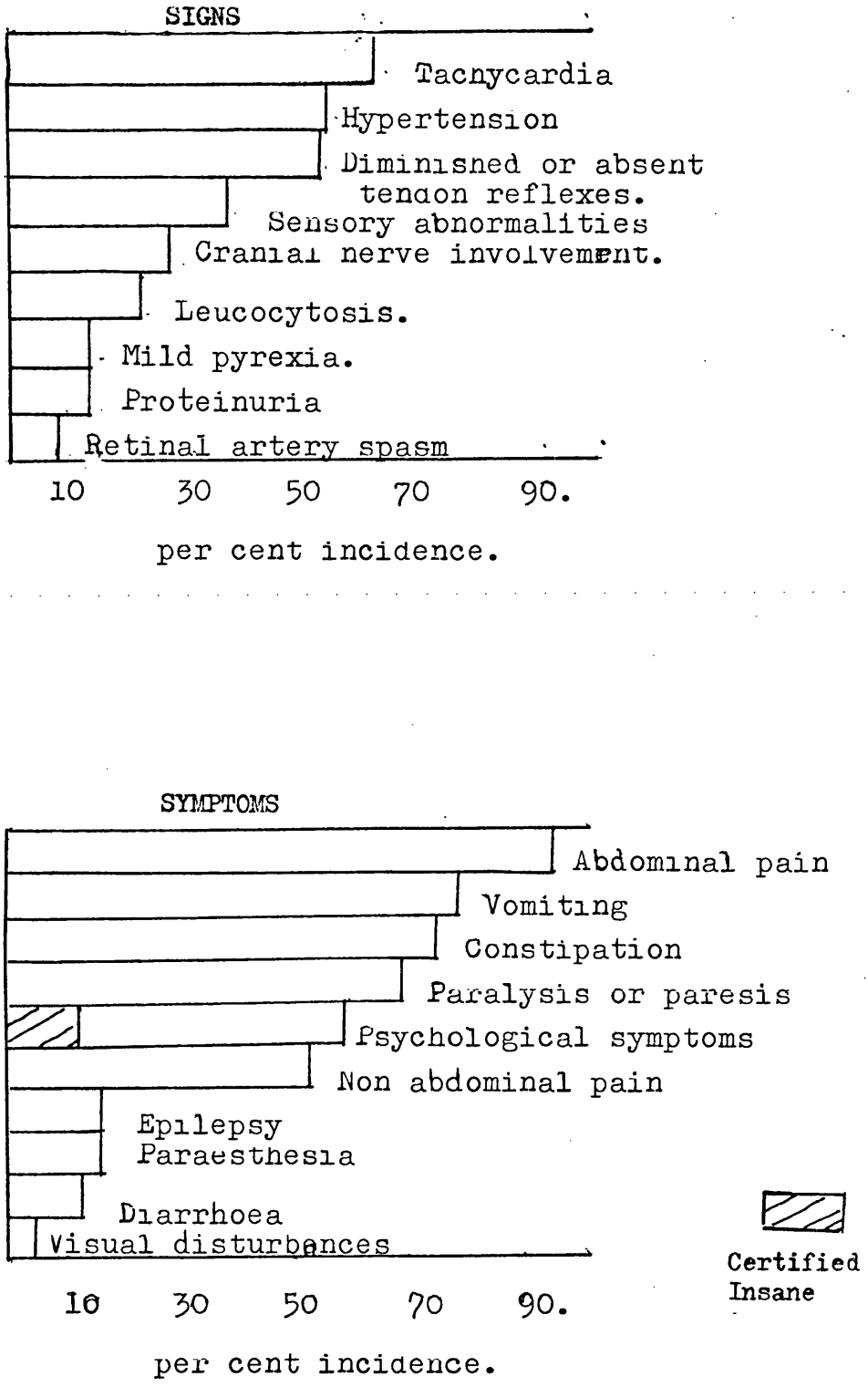


Figure 3. Clinical Signs and Symptoms of AIP.

(After Goldberg & Rimington 1962.)

The most common of the cardio-vascular features is tachycardia which is present in the acute stage of the disease. In most cases the pulse rate is 110-120 per minute, although it has been known to reach transiently as high as 160 per minute. Hypertension is also common with a systolic pressure of around 150mm Hg or more, and a diastolic of 95mm Hg or more. Even in remission some 40% of patients showed some degree of hypertension. (Beattie et al, 1973). On occasion the hypertension can be very severe, and pressures as high as 180/126 can occur. In the active phase of the disease the absence of the carotid-sinus reflex has been observed. Rarely hypotension has been recorded.

Neurological features include paralysis or paresis of limb muscles. This is fairly common and ranges in severity from weakness in a pair of limbs to complete quadraplegia. In most patients paralysis is of the lower motor neurone type; however, the upper motor neurone type has also been described. There is often lack of tendon reflexes with muscular wasting present. Although all cases are bilaterally involved there is usually unequal severity. Cranial nerve involvement can also occur. Urinary incontinence and respiratory paralysis have also been reported, the latter being due to involvement of the diaphragm and/or the intercostal muscles. In the limbs, particularly

the lower limbs, there is often an impairment of sensation, loss of joint and vibrational sense or complete sensory loss. In most cases sensory loss is preceded by muscular paralysis or paresis. Pain can also occur outside the abdomen, e.g. in the limbs,head, neck, large joints, lumbar region or anterior chest region. This is usually associated with abdominal pain.

A few patients exhibit epilepsy, and abnormal encephalograms have been recorded both in patients with, and those without, epilepsy. More common, however, is the presence of psychological aberrations. These take various forms and include simple neurosis, endogenous depression, schizophrenia and various psychoses. These states can be severe enough to have the patients mentally certified.

The course of acute porphyria is variable. In some cases there are no symptoms, but in the most dramatic form there is a sudden explosive attack which may last ten days to ten weeks and ultimately be fatal. Most cases, however, lie between these two extremes. Thus there can be intermittent attacks of similar gravity separated by periods of months or years, or attacks can take a 'step-ladder' pattern, gradually increasing in severity until finally a fatal attack ensues. The most common cause of death is respiratory failure.

b) Biochemical Features

The principal biochemical abnormality in AIP is the excessive urinary excretion of PBG and ALA. Associated with this increased excretion there is an increase in the circulating levels of these substances. In particular ALA which is usually considered to have a value of 0µg/ml in the plasma of normal subjects, is detectable in the plasma of patients in acute attack of porphyria. A concentration of ALA as high as 24µg/ml has been reported under these circumstances (Sweeney et al, 1970).

To account for the increase in circulating and excreted ALA and PBG, one must postulate under utilisation of ALA and PBG as a result of a metabolic block, and/or overproduction of ALA and PBG due to elevated ALA synthetase activity. There is evidence in favour of each of these possibilities and this will now be briefly considered:-

Metabolic Block

In considering the possibility of a metabolic block of haem synthesis in AIP, it is evident that to account for the increased excretion of ALA and PBG this block must occur at some stage in the pathway after PBG synthesis. Unless there is some as yet unidentified pathway by which PBG is converted to an unknown pyrrole (block of which would lead to decreased utilisation of PBG) the first enzymes, block of which could account for increased

ALA and PBG levels, are PBG deaminase and/or uroporphyrinogen I synthetase.

In liver biopsy studies in a series of patients with AIP Strand et al (1970) found that the hepatic conversion of PBG to porphyrins was less than 50% of control levels. Further Meyer et al (1972) have demonstrated a decreased activity of erythrocyte uroporphyrinogen I synthetase in a family of five subjects with AIP. In two siblings however, they found that although the enzyme was decreased in activity, they were apparently unaffected and showed a normal pattern of porphyrin precursor excretion. A single dose of ALA given to these siblings was, however, metabolised in a manner similar to that of patients with AIP. In contrast two siblings with a normal activity of uroporphyrinogen I synthetase, had a normal rate of conversion of ALA to porphyrins. They conclude from these studies that a decreased uroporphyrinogen I synthetase activity reflects the primary genetic defect of AIP.

Although this evidence of a metabolic block in AIP is convincing it is of interest that even with the loss of 100-200mg PBG per day, the disease is not accompanied by anaemia, nor is there any decrease in cytochrome C or liver catalase. These facts would suggest that the decreased activity of uroporphyrinogen I synthetase, in most

cases at least, is not great enough to seriously disturb haem formation. Further, blood from porphyric subjects 'in vitro' can convert PBG to porphyrins in the same way as normal blood 'in vitro' (Goldberg and Rimington, 1962).

Thus it would appear that although metabolic block of uroporphyrinogen I synthetase is most likely a contributing factor in the excessive excretion of ALA and PBG in AIP, it is unlikely to be a sufficient one.

Overproduction

Neuberger et al (1954) found that acute porphyrics could convert a greater amount of a given dose of ALA into PBG than normal subjects, indicating that the activity of ALA dehydrase is possibly elevated in AIP. The most obvious biochemical lesion leading to heme precursor overproduction however, would be the overactivity of hepatic ALA synthetase, the primary rate determining enzyme of the pathway. Such an overactivity of hepatic ALA synthetase has indeed been found in acte porphyria (Tschudy 1965). (Nakao et al, 1966) suggesting that this is also a contributory factor to the excessive production and excretion of ALA and PBG.

There are many theories as to the possible cause of the overactivity of hepatic ALA synthetase in AIP, one of the most likely being the

diversion of haem from its participation in the feedback control of ALA synthetase (Kaufman and Marver, 1970).

In summary then the evidence available at present would suggest that the excessive production and excretion of ALA and PBG in acute porphyria is, to some extent, a result of elevated hepatic ALA synthetase activity and block of some enzyme in the haem pathway, after ALA dehydrase, probably uroporphyrinogen I synthetase.

Although the disturbed porphyrin metabolism is the main biochemical abnormality of AIP, other abnormalities commonly occur. A transient excess in urinary vasopressin has been described (Nielson and Thorn 1965). Hollander et al (1967) have found an increase in the maximal binding capacity of thyroxin binding globulin in some cases of acute porphyria, although the free thyroxin level was normal. In one patient, Waxman et al (1969) have demonstrated defective adrenocorticotrophic hormone secretion during attack. Similarly during attack Schley et al (1970) have demonstrated a large increase in circulating levels of catecholamines. A disturbance of steroid metabolism is also a common characteristic, thus Goldberg et al (1969) have shown significant elevations of the urinary 17-oxosteroids, aetiocholanolone glucoronide, dehydroepiandrosterone glucoronide and sulphate and epiandrone sulphate.

TABLE 1

DRUGS CLINICALLY SHOWN TO PRECIPITATE PORPHYRIA

Barbiturates	Sulphonamides
Sulphonal	Sedormid
Sex Hormones	Oral Contraceptives
Phenytoin and other Hydantoins	Methsuximide and other Sucinimides.
Tolbutamide	Griseofulvin
Aminopyrine	Chlordiazepoxide
Dichloralphenazone	Meprobamate
Hexachlorobenzene	Chloroquine
Methyl Dopa (I)	Ethanol (II)
Imipramine (III)	Chlorpropamide (IV)

All after deMatteis (1967) unless numbered.

(I) Goldberg (1968)

(II) Goldberg (1959)

(III) Goldberg et al (1967)

(IV) Zarowitz and Newhouse (1965)

c) Intermittency and Precipitation

A particularly intriguing aspect of AIP is the intermittency of attack. The cause of this has partly been ascribed to the administration of certain drugs particularly barbiturates. Acute alcohol consumption has also been implicated in precipitation of attack. (Ferguson et al 1970). Oral contraceptives also appear to provoke attack (Rimington and deMatteis, 1965). DeMatteis (1967) has drawn up a table of drugs known to aggravate porphyria; this table is represented opposite (Table I). An interesting feature of these drugs is the fact that some of them are known to increase the activity of hepatic ALA synthetase. Thus this property is shown by ethanol (Shanley et al 1968); barbiturates (Moore et al 1969) and certain steroids (Moore et al 1973).

Factors other than drug administration can provoke attack, thus Waldenstrom (1937) has observed an apparent relation between onset of menstruation and onset of clinical symptoms. Similarly Vine et al (1957) have reported exacerbations of the disease during pregnancies. Diet is also an important factor and this fact has been stressed by Welland et al (1964). An attack can be precipitated by a diminution in carbohydrate and protein consumption, and Knudsen et al (1967) reported the precipitation of attack in a patient placed on a water diet for obesity. Infection

also appears to be a precipitating factor. Goldberg and Rimington (1962), describe a patient who suffered two attacks each associated with acute tonsillitis; this patient underwent tonsillectomy and eight years later showed no further signs of attack.

There is a distinct possibility that certain endogenous factors are capable of precipitating attack in AIP. Recently steroids have attracted much attention as possible endogenous precipitating agents. Granick and Kappas (1967) have described several 5B-H- steroids which strongly stimulate porphyrin synthesis in chick embryo liver cell culture. Goldberg et al (1969) found significant elevations of certain 17-oxosteroids in six patients with AIP. More recently Paxton et al (1973) found the conjugates of one of these steroids, dehydroepiandrosterone, to be significantly elevated in the urine and plasma of subjects with AIP. Further dehydroepiandrosterone has been found to elevate rat hepatic ALA synthetase activity (Goldberg et al 1969). Thus certain of the steroids may well be endogenous precipitating factors of this disease.

d) Possible Origin of Clinical Features

It is now well established that lesions of the nervous system are characteristic features of AIP. The earlier work describing these nervous changes has been summarised by Hierons (1957). Although

the presence of neuropathy is generally agreed upon, the actual form it takes is an issue of some controversy. Thus some workers favour primary axonal damage (Mason et al, 1933; Simpson, 1962) while others favour primary axonal demyelination (Denny-Brown and Sciarra, 1945; Gibson and Goldberg 1956).

The cause of the neuropathy has been ascribed by some workers to neural tissue depletion of pyridoxal phosphate. (Cavanagh and Ridley, 1967). As has been discussed before pyridoxal phosphate is a cofactor in the synthesis of ALA, from glycine and succinyl CoA, by ALA synthetase. These workers suggest that as this enzyme is excessively overactive in AIP, it will deplete other tissue of pyridoxal phosphate resulting in neurological damage, as pyridoxal phosphate is an important factor in nerve metabolism.

Such a theory however, would not appear to be a sufficient explanation for the origin of the neuropathy of AIP. Thus Hamfelt and Wetterberg (1968) in a series of 21 patients with AIP, found that indeed the plasma concentrations of pyridoxal phosphate were significantly lower than a normal control group (in some cases less than 1µg/ml compared with 5-15µg/ml in normals) and they suggest that there may be a disease induced vitamin B deficiency in AIP. They found however, that other non-porphyric patients, with vitamin B_6 deficiency

of the same degree, showed no signs of neuropathy. Nor did they find any correlation between plasma pyridoxal phosphate concentration and neuropathy in patients with AIP, nor improvement in the condition of these patients after administration of this substance. They concluded therefore, that other factors beside pyridoxal phosphate deficiency are probably responsible for the neuropathy of AIP.

Irrespective of the nature or cause of the neuropathy, there is little doubt that all the main clinical features of AIP can be explained on a neuropathological basis. Demyelination of peripheral nerves can explain peripheral paralysis and paresis, and central demyelination can account for the central characteristics, both mental and physical, of the disease. The gastrointestinal features could be due to lesions of the visceral pre-ganglionic motor fibres, and sensory fibre damage in peripheral nerves could explain the origin of non-abdominal pain. Hypertension and tachycardia can be accounted for by the lesions which occur in the vagus and associated central bodies which constitute the cardio-vascular reflex systems.

Goldberg (1959) put forward a hypothesis based on these considerations in which he postulated the existence of a substance, X, of which PBG is a precursor, which is necessary for the

nutrition of myelin. He suggested that in AIP there is a metabolic block of this substance which results in excessive production of ALA and PBG, and in neural demyelination with its consequent pathological correlates. This hypothesis succintly accounts for both clinical and biochemical phenomena associated with AIP. As it does, however, postulate the existence of an unidentified substance 'X', this substance, if the hypothesis is valid, should be capable of detection chemically and/or pharmacologically.

This hypothesis is representative of the general notion that in AIP there is some peccant material which directly or indirectly contributes to the pathology and course of the disease.

The most apparent possibility as to the cause of the manifestations of AIP is that the porphyrins and/or their precursors are pharmacologically active at the elevated levels occurring in the disease. In this case these substances could produce their effect in either an acute or a chronic manner. If an acute action was involved a sudden increase in the biosynthesis and circulating level of the active substance or substances might be expected to precipitate attack. If a chronic action was involved, it might be expected that some pharmacological action of the active substance or substances would, over a long period

of time, lead to pathological changes which would cause attack, or as is more likely, lead to predisposition to attacks precipitated acutely by some other agent. The possibility that the porphyrins or one of their precursors are pharmacologically active has been, and is still being, studied intensively.

Early workers gave what seemed convincing evidence that the porphyrins were pharmacologically active. Waldenstrom (1937, 1939) however, cast doubt on this and suggested instead that certain pyridine derivatives formed from the excessive pyrroles in AIP could affect the nervous system. PBG was also suspected (Lowry et al, 1950) while Denny-Brown and Sciarra (1945) thought there was a circulating vasoconstrictor substance which caused intermittent ischaemia.

These speculative and conflicting reports were resolved to a great extent when Goldberg et al (1954) demonstrated in a series of studies on the anaesthetised cat and isolated organs, that the purified porphyrins and PBG were pharmacologically inactive. Further they showed no effect on the various parameters measured in the anaesthetised cat of an infusion of urine from a patient in a moderately severe attack of porphyria. Although two positive results were obtained in this study, a contraction of the guinea pig ileum with an ultimate refractoriness to acetycholine and histamine, produced by haemato-porphyrin and

a mepyramine-sensitive contraction of the gut produced by porphobilin, it was thought that these were due to contamination, in the latter case at least, by histamine.

At the time of these studies ALA was unobtainable in a purified form. Jarret et al (1956) tested ALA on the guinea pig ileum and the anaesthetised cat and again the results were negative. To reinforce these results and indicate that the porphyrins and their precursors were pharmacologically inactive, were the results in experimentally porphyric animals where although, there was a large excretion of ALA and PBG, there was little sign of clinical change (Goldberg and Rimington, 1954). Similarly there was the fact that many porphyric patients excrete vast quantities of ALA and PBG without any obvious adverse signs. Thus it appeared that these substances were innocuous and had no part in the aetiology of AIP.

In the past few years however, attention has again shifted on to the porphyrin precursors as possible aetiological factors in AIP. Thus Kosower and Rock (1968) have shown animals made experimentally porphyric with AIA are more susceptible than normals to induced convulsions. Feldman et al (1968) have shown that ALA can alter neuro-muscular transmission and cause hyperpolarisation of the motor end-plates and 'in vitro'; ALA has been shown to be capable of red cell and brain ATPase inhibition (Becker et al 1971) and

and inhibition of membrane sodium transport (Eales et al 1971).

Conclusions

The fundamental problem presented by AIP is the relationship between its biochemical and clinical manifestations. As the clinical manifestations can be adequately described in neuropathological terms, this problem resolves itself into one pertaining to the nature of the origin of the neuropathology.

Although, as we have seen, the biochemical disturbances in AIP are varied, the one which is predominant is that of porphyrin metabolism. Now it can be considered that the porphyrin disturbances are entirely incidental to some other obscure lesion, and that they play no role in the disease process. For example a basic lesion of steroid metabolism might be suggested, an indirect result of which would be induction of ALA synthetase with the resultant biochemical picture of porphyrin over-production.

Alternatively, it can be assumed that the manifest disturbances of porphyrin metabolism are a primary feature of, and directly involved in, the pathogenesis of AIP. If this assumption is correct, then the only reasonable choice of a porphyrin precursor which would be sufficiently worth studying as the 'materia peccans' of the

disease appears to be ALA. Thus the greater part of this thesis is concerned with the pharmacology and potential pharmacology of this substance.

SECTION II

GENERAL METHODS, EXPERIMENTS AND CONSIDERATIONS

(1) SOME PHYSICAL AND CHEMICAL ASPECTS OF ALA

Δ-amino laevulinic acid (ALA) (5 amino-4-keto-pentanoic acid) has the chemical structure represented in (fig.4). It has a molecular weight of 131.0, but is normally obtained in the form of the hydrochloride salt which has a molecular weight of 167.5. The salt is a white crystalline solid, which is relatively hygroscopic and therefore is usually kept stored under dessication. The percent yield of true ALA from the salt is therefore $\frac{131.0}{167.5}$ x 100 = 78.2%. The melting point of the salt is 156°-158°C.

ALA when dissolved, gives an acidic solution. It was necessary therefore, in using ALA for biological experimentation, to determine the equivalents of alkali required to neutralise the pH. A pH titration of ALA was therefore carried out.

Method

0.01 molar solutions of ALA and sodium hydroxide (NaOH) were prepared gravimetrically. The NaOH was then titrated against hydrochloric acid (HCl) using phenol red as indicator. The HCl was then diluted with distilled water to the required volume to give a 0.01M solution.

Using a pH electrode, the change in the pH of the 0.01M ALA solution, was determined after addition of 1ml aliquots of either equimolar NaOH or HCl.

Figure 4.

The Chemical Structure of ALA.

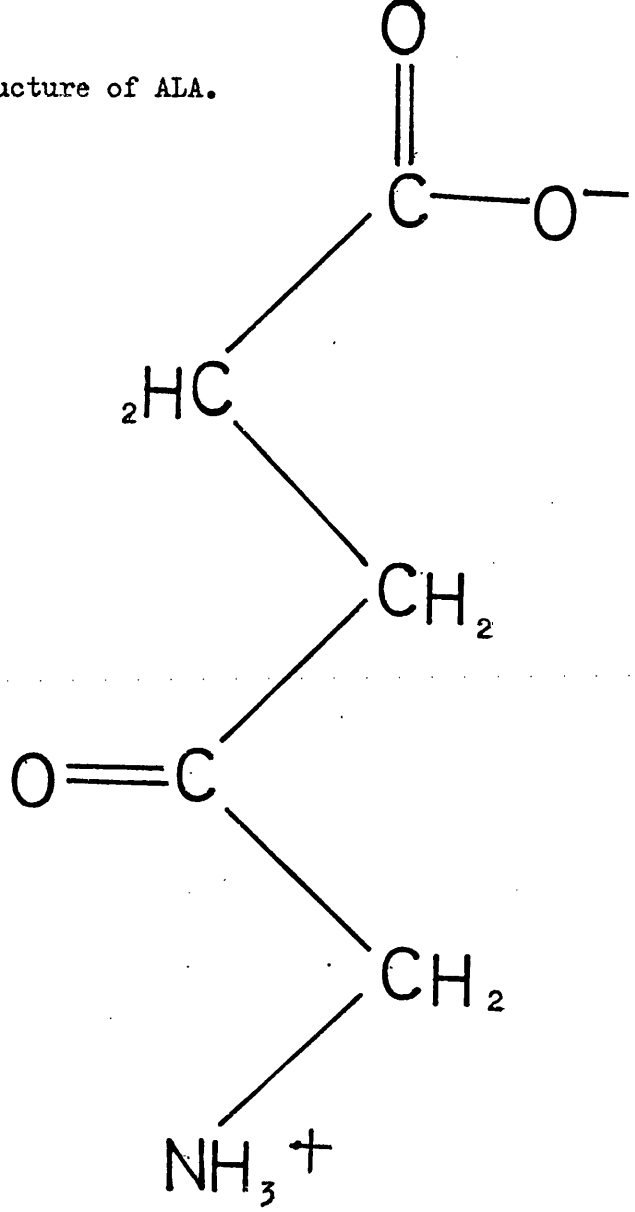
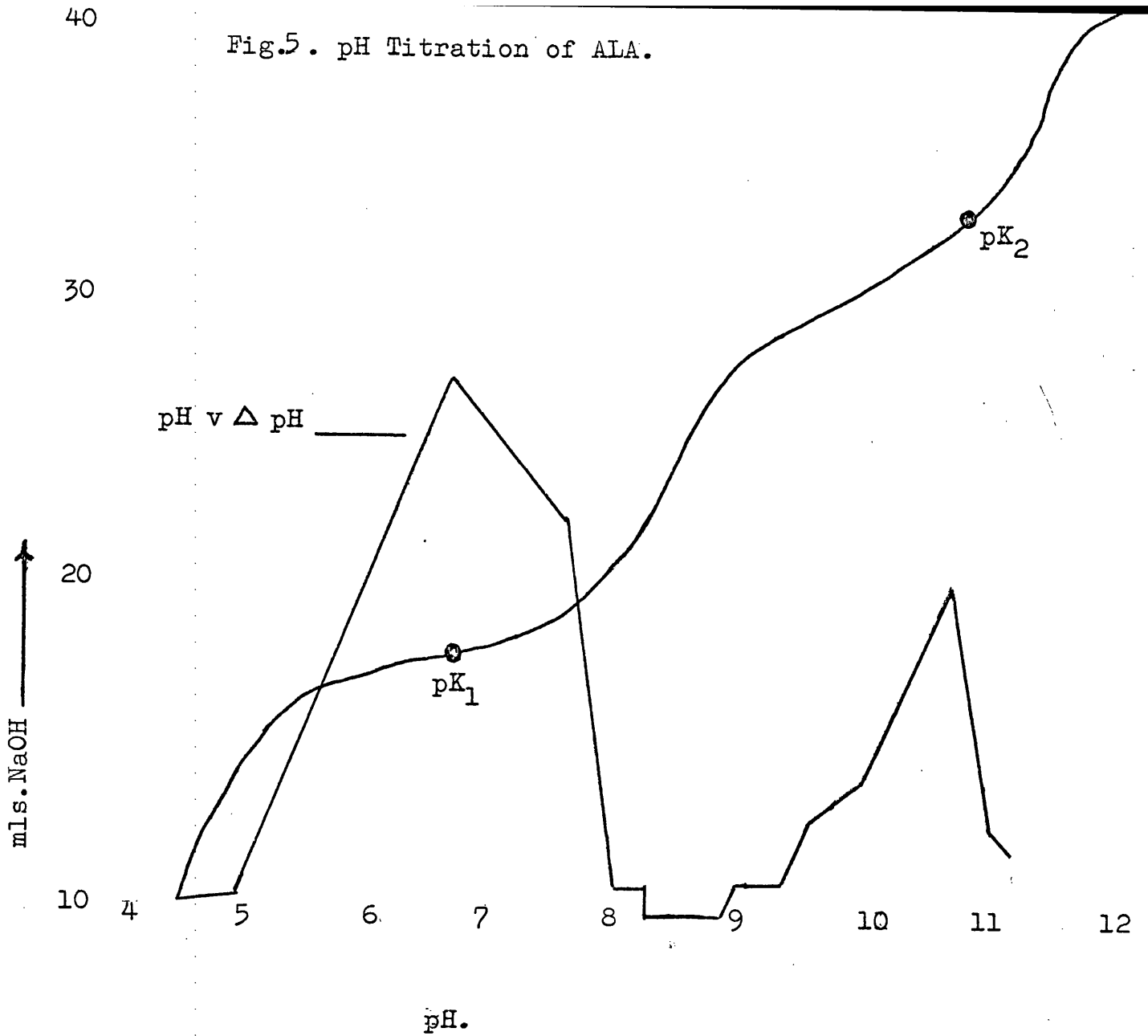


Fig.5. pH Titration of ALA.



Results

The results obtained from the pH titration are shown on fig. 5. The original pH of the 0.01M solution of ALA was 2.55: and the addition of 18-19 mls of equimolar NaOH brought the pH to within physiological range. The greatest changes in pH for an equivalent addition of hydroxide, occurred after the addition of 18 and 33mls NaOH. This indicates that there is a pK value for ALA in each of these regions.

It was observed that at alkaline pH, the ALA/NaOH mixture took on a definite green-yellow colouration, which became increasingly more pronounced as the pH was increased by further addition of NaOH. This process was reversible, in that subsequent reduction in pH by addition of HCl made the solution once more colourless when acid pH values were reached.

Discussion

From the results obtained it can be seen that in order to bring a given solution of ALA to a neutral, physiological pH, some 18-19mls of equimolar hydroxide, or equivalent alkali, must be added. The titration curve can also furnish us with some information concerning the nature of the molecular species of ALA at the various pH values. The points at which there are relatively larger changes in pH for the addition of equivalent volumes of NaOH represent the pK values of ALA. From the differential curve in fig. 5, this can be seen to occur at pH 6.5. and

at pH 10.6. The first pK value is the pH at which half the ALA in solution exists as the molecular form A in fig.6 (1). The second pK value is the pH at which half the ALA exists as form B in fig.6 (1). Thus at pH 6.5 (pK_1) all the ALA molecules have a positively charged amino group, but only half of them have a negatively charged carboxyl group. At pH 10.6 (pK_2), all the ALA molecules have a negatively charged carboxyl group, but only half have a positively charged amino group.

The mean of the two pK values gives the isoelectric point: i.e. the pH at which the ALA shows no net charge. Thus:- $pI = \frac{pK_1 + pK_2}{2} = \frac{6.5 + 10.6}{2} = 8.5$: i.e. at pH 8.5 ALA has no net charge.

Because ALA has a C-4 keto grouping, it is capable of demonstrating a form of molecular resonance:- keto-enol tautomerism (fig.6(2)). The formation of these resonating molecular species is indeed the probable explanation of the green colouration observed in the ALA solution at alkaline pH. The development of this colouration is characteristic of molecules which show keto-enol resonance. The existence of the enol form of ALA might mean that it would have another pK value (pK_3) at which half the OH molecules of the ALA would exist as O-. (fig.6(11)). This process however, is chemically unlikely as the keto-enol forms are stable and would be unlikely to

Fig. 6.1. Change in Molecular Form of ALA With Change in pH.

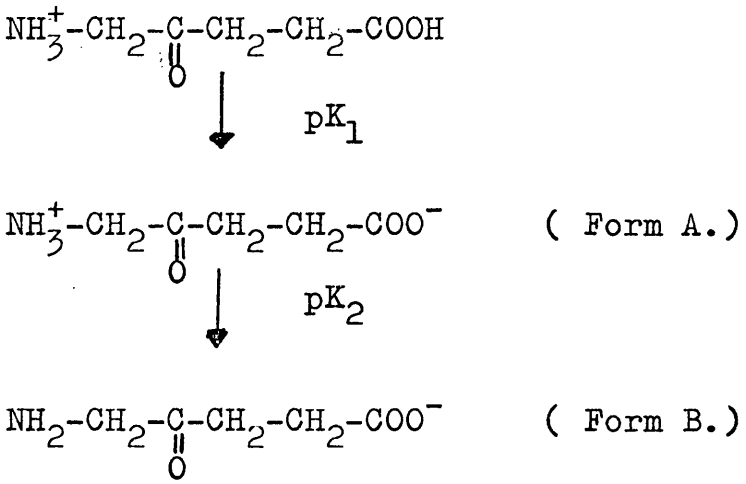
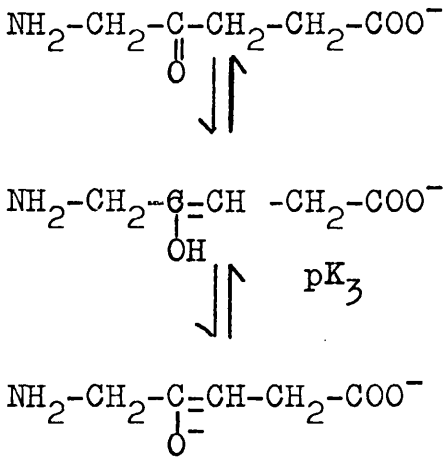


Fig.6.2. Keto-Enol Tautomerism of ALA.



significantly alter the position of the isoelectric point. With respect to the biological interaction of ALA therefore, it is evident that at physiological pH it will exist in two forms: both with a charged amino group, but one of which also has a charged carboxyl group.

It is notoriously difficult to predict any pharmacological activity of a substance by consideration of its chemical structure alone, and therefore no attempt will be made to do so. Two points, however, might be made:- the first being that the charged amino grouping is one present in many pharmacologically active substances; and the second is that in terms of membrane passage, the charge on the ALA molecule at physiological pH might be a hindrance to the movement of the molecule across lipid membranes.

DETERMINATION OF ALA

1) Blood

The method used for determination of blood ALA was based on that described by Haeger-Aronsen (1960). The principle is as follows:-

Thiol compounds, which would interfere with the subsequent reactions, are eliminated by the addition to the blood of iodoacetamide (Gibson et al (1958). It is then freed from proteins by precipitation with trichloroacetic acid (TCA) and centrifugation. The supernate which contains ALA is heated with acetylacetone which causes condensation of ALA with quantitative formation of a pyrrole (3-acetyl-2-methylpyrrole 4-(3') propionic acid) which gives a coloured complex in acid solution with p-di methyl amino benzaldehyde (DMAB).

0.2ml of freshly prepared iodacetamide (0.06M) was added to 3ml of blood. After two minutes 3ml of TCA (20%) was added and after mixing well this was centrifuged until clear. This usually required a spin of around 3,000 rpm for some ten minutes. 1.5ml of the supernate was taken and to this was added 1ml acetate buffer (pH 4.6) and 0.1ml sodium hydroxide (2.5N) and 0.05ml acetylacetone. This was then well mixed in a glass stoppered test tube. The tube was placed in a water bath containing boiling water for ten minutes after which it was removed and allowed to cool to room temperature. 2ml of

the resulting solution was taken and to this 2ml of Ehrlich's II reagent was added and the solution well mixed. The extinction of the solution was read exactly fifteen minutes after the addition of the Ehrlich's II reagent in a 1cm cuvette in a spectrophotometer (Pye Unicam SP 500) at 553 mu. As a blank the same blood was used being treated in the same way as the test but with the acetylacetone being added after heating.

2) Urine

The determination of ALA in the urine was usually associated with a simultaneous determination of urinary PBG. The method used was based on that described by Mauzerall and Granick (1956).

Principle

PBG is retained by the anion exchanger Dowex 2; in the acetate form ALA is not. ALA is however, retained by the cation exchanger Dowex 50 in the hydrogen form. After elution PBG can be demonstrated directly by means of p-DMAB, as can ALA after cyclisation with acetyl acetone as described above.

Method

A column was prepared with a 2cm layer of Dowex 2 resin. The column was then filled with water and this was allowed to run through until almost dry. Urine was then brought to pH 4-7 if necessary, using either HCl or sodium acetate. 1ml of urine was then run through the column and

collected in a test tube. The column was then washed through twice with 2ml distilled water and this was also collected. This fraction contained the ALA.

2ml of 1M acetic acid was then run through the column followed by 2ml of 0.2 M acetic acid. These fractions were collected, made up to 10ml with distilled water and mixed well. 2ml of this solution was mixed with 2ml Ehrlich's I solution and read after five minutes in a spectrophotometer at 555 mμ. 2ml distilled water + 2ml Ehrlich's I solution was used as a blank.

The first fraction obtained from this column was transferred to a column containing a 2cm layer of Dowex 50 which had been previously washed with a full column of distilled water. This was then washed free of urea by eluting with water until the eluate did not give a yellow coloration with Ehrlich's I reagent. 2ml M sodium acetate was then run through the column followed by 7ml M sodium acetate, this latter fraction being collected in a 10ml cylinder. To this was added 0.2ml acetylacetone and it was made up to 10ml with acetate buffer pH 4.6. It was then mixed and placed in boiling water for fifteen minutes. The ALA was determined colorimetrically as described above.

Reagents

Acetate Buffer pH 4.6. This was prepared by adding 136g of sodium acetate to 57ml of glacial

acetic acid, and making the resultant solution up to 1 litre with distilled water.

Ehrlich's II Reagent. 1g of p-DMAB was dissolved in about 30ml of glacial acetic acid and 8ml of 70% perchloric acid in a 50ml volumetric flask. This solution was then made up to the 50ml mark with glacial acetic acid. (This solution is active for only six hours).

Ehrlich's I Reagent. This was prepared by the addition of 2g of p-DMAB to 100ml of 6M hydrochloric acid.

Dowex 50 x 8 200-400 mesh. The finest particles were separated off by repeated suspension and sedimentation in water. The ion exchange was then converted to the sodium form by twenty hours storage in twice its volume of 2M sodium hydroxide, after which it was washed until the washings were a neutral pH. It was then reconverted to the acid form by alternate treatment with about 1 volume of 4M HCl and 6 volumes of 2M HCl. The exchanger was stored in a covered vessel in twice its volume of 1M HCl. (It will keep active in this state for 3-4 months at room temperature).

Dowex 2 x 8 200-400 mesh. This was placed in water and allowed to sediment after which the water was sucked off. The washing was repeated until the supernate was clear. The exchanger was then prepared on a column by conversion to the acetate form by washing with 3M sodium acetate until the eluate

was chloride free (no precipitate with silver nitrate). It was then washed with water until the washings were neutral. It was stored in twice its volume of water in a covered vessel. (It remains active in this form for 3-4 months at room temperature).

The other reagents were prepared in the usual manner.

PAPER CHROMATOGRAPHY OF ALA

The method used for the two dimensional chromatographic determination of ALA was based on that described by Haeger-Aronsen (1960)[2]. The chromatograms were run on Whatman No. 1 chromatography paper.

Phase 1 consisted of a mixture of -n butyl alcohol - glacial acetic acid - water in the ratio 63:27:10 v/v. It was run vertically and the time of migration allowed was four hours at 20°C.

Phase 11 consisted of a saturated aqueous solution of phenol (100g. phenol in 39ml water). This was also run vertically and the time of migration was eight hours, also at 20°C. The paper was then dried at room temperature and the spots located as follows:

A. The paper was sprayed with ninhydrin solution and dried in an oven at 60°C. The composition of the ninhydrin solution was:- 0.5% ninhydrin w/v in n butyl alcohol saturated with pyridine-glacial acetic acid - water in the ratio 10:1:95 v/v. ALA appeared as a yellow spot which turned to brown after a few hours.

B. The paper was sprayed with acetylacetone and then dried for ten minutes in an oven at 100°C. It was then sprayed with Ehrlich's I reagent (cf page 37) ALA appeared as a red-violet spot which turned blue-violet within a few hours.

The Rf values of ALA obtained were as shown in Table 2. These fall within the range described by Berlin et al (1956) for the same chromatographic phases.

TABLE 2

Rf Values of ALA

Phase	No. Determinations	Range
I	12	0.20-0.26
II	12	0.55-0,63

THE USE OF SODIUM PENTOBARBITONE AS AN ANAESTHETIC FOR 'IN VIVO' EXPERIMENTS.

In the majority of 'in vivo' experiments performed, the anaesthetic used was sodium pentobarbitone (Nembutal). This was obtained from Abbot Laboratories Ltd., as a solution with the following formulation per ml:- sodium pentobarbitone 60mg; alcohol 10% v/v and propylene glycol 20% v/v.

Nembutal was chosen as an anaesthetic agent for a variety of reasons: It is easily obtainable and simple to use and unlike, for example, a chloralose-urethane mixture, it does not require any preparation before use. Despite the fact that it does to some extent depress cardiovascular responses (Amundsen and Nustad 1965) most papers which describe animal experiments on the cardiovascular system reveal that Nembutal was the anaesthetic used. Thus results obtained from such experiments with ALA, could readily be compared with other results, where Nembutal was also employed as anaesthetic agent.

At the anaesthetic dose, Nembutal is porphyrinogenic, ie. it induces the synthesis of ALA and the other precursors of haem. As this is the basic biochemical abnormality in acute porphyria; it was thought that any effect of ALA seen in animals with their haem biosynthetic

pathways over-producing, might be relevant to the effects of ALA in the disease itself. Indeed it was felt that there may be some action of ALA which would only be apparent under such conditions, when there is an increased hepatic pool of the cofactors necessary for haem biosynthesis, with a consequent depletion elsewhere. The anaesthetic dose of Nembutal produces a maximal stimulation of ALA synthetase activity some six hours after injection (Moore et al, 1969). It is unlikely, however, that there would be any appreciable difference in circulating ALA due to stimulation of synthetase, as the activity of ALA dehydrase should be great enough to handle the increased ALA produced. This possibility was tested for however: Thus a rat was anaesthetised with Nembutal and its blood ALA content was determined six hours after injection by the method described on page 33 . The blood ALA of an unanaesthetised rat was determined at the same time. In neither case was any ALA ($<0.5\mu g/ml$) detectable by the methods used.

In almost every experiment performed, the dosage required to produce satisfactory anaesthesia for about three hours was 60mg/kg by the intra-peritoneal route. Full anaesthesia was assumed to have developed when the complete loss of righting, withdrawal and corneal reflexes was evident. This

normally occurred some twenty to thirty minutes after injection. If anaesthesia had not fully developed in this time, a further 3mg of Nembutal was administered by the same route; this was always found to be sufficient to produce anaesthesia.

In continuing experiments where the animal demonstrated the return of either the corneal or withdrawal reflexes a maxiumum of 3mg Nembutal was injected slowly by the intravenous route until the reflexes were absent once again.

It might be stressed that in all experiments performed, male Sprague Dawley rats were used. This strain required 60mg/kg Nembutal for the development of full anaesthesia, however such a dose would be lethal in other strains: thus in the hooded Norwegian strain a dose as low as 18mg/kg was lethal, yet this latter dose can produce satisfactory short-lasting anaesthesia in the albino Wistar strain (Payne and Chammings 1964).

ALA IN KREBS SOLUTION

In experiments 'in vitro' where it is required to have ALA present in a tissue fluid substitute such as Krebs solution, it is necessary to know if, during the time course of the experiment and at the temperature involved, ALA is changed chemically by the perfusing medium or bathing medium itself, into some other substance. In such an event of course, erroneous conclusions may be drawn from experimental results. Thus the concentration change with time of ALA in a solution of Krebs at 37°C was determined.

Method

1.5mg of ALA-HCl was dissolved in 1ml saline. This was added to 100ml of Krebs solution at 37°C giving a concentration of 11.8µg ALA/ml Krebs. The flask containing the Krebs was placed in a water bath at 37°C to maintain the temperature. Samples of the Krebs-ALA solution were removed at time intervals and assayed for ALA content as described on page 33. The results obtained are shown in fig. 7.

As can be seen from the figure, the concentration of ALA in the Krebs solution decreased in the first hour by about 17% and thereafter remained relatively constant. Such a decrease is to be expected as ALA is most likely hydrolysed to some extent initially. The reduction concentration however, is not too great over the time interval studied and the results indicate that ALA is relatively stable in Krebs solution at 37°C and does not require any stabilising additives.

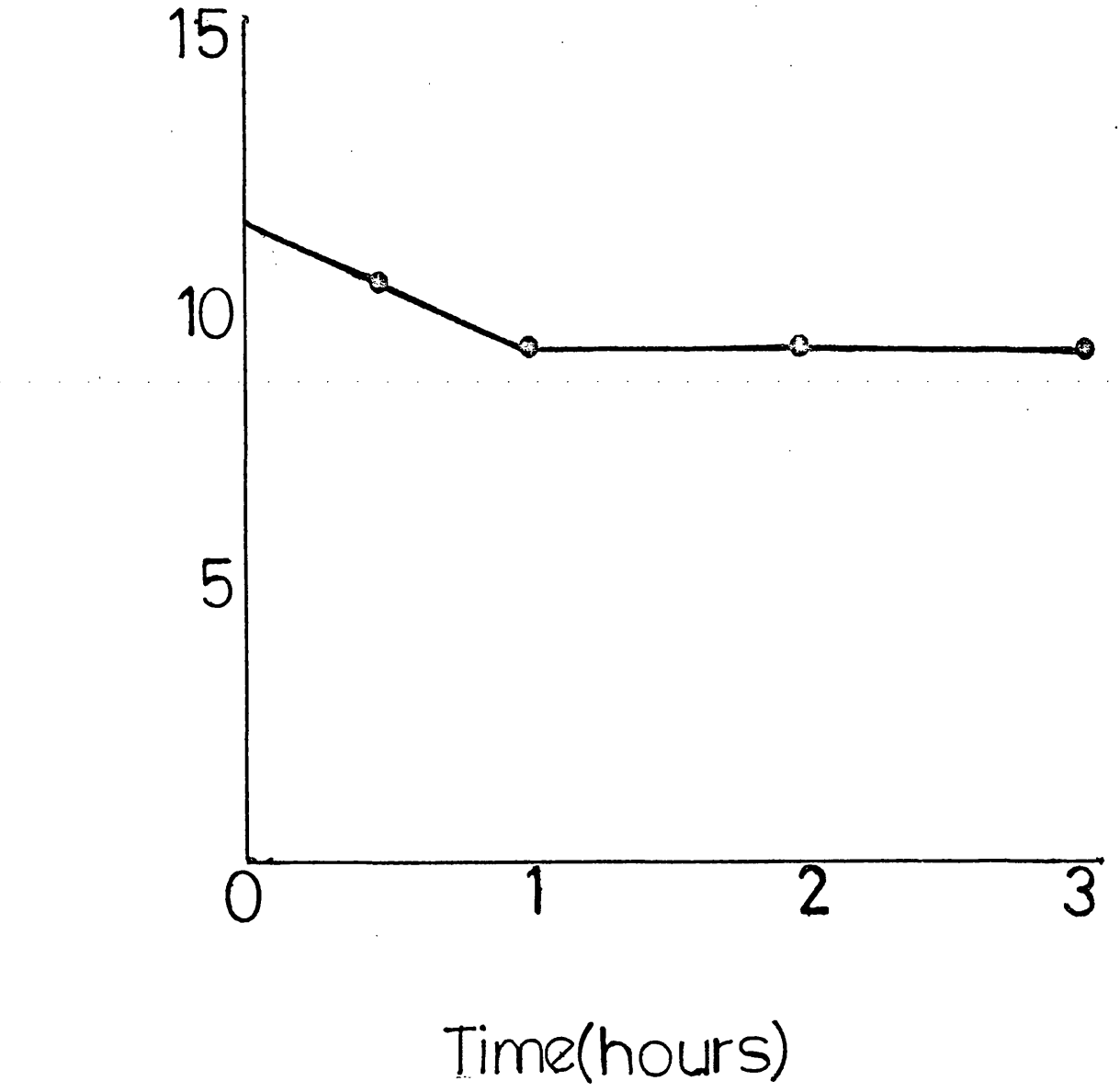


Figure 7. The change in ALA concentration with time in Krebs solution at 37°C.

EQUILIBRIUM ALA LEVEL IN THE RAT

In certain proposed experiments using rats, it was required to know when there was an equilibrium concentration of ALA in the blood after injection. A further consideration was that of attaining a blood equilibrium level of ALA similar to that found in an acute attack of AIP viz: around 24µg/ml (Sweeney et al, 1970). As the most convenient route of drug administration in the rat is by intraperitoneal injection it was decided to determine the blood concentration of ALA at given time intervals after an I.P. injection of ALA.

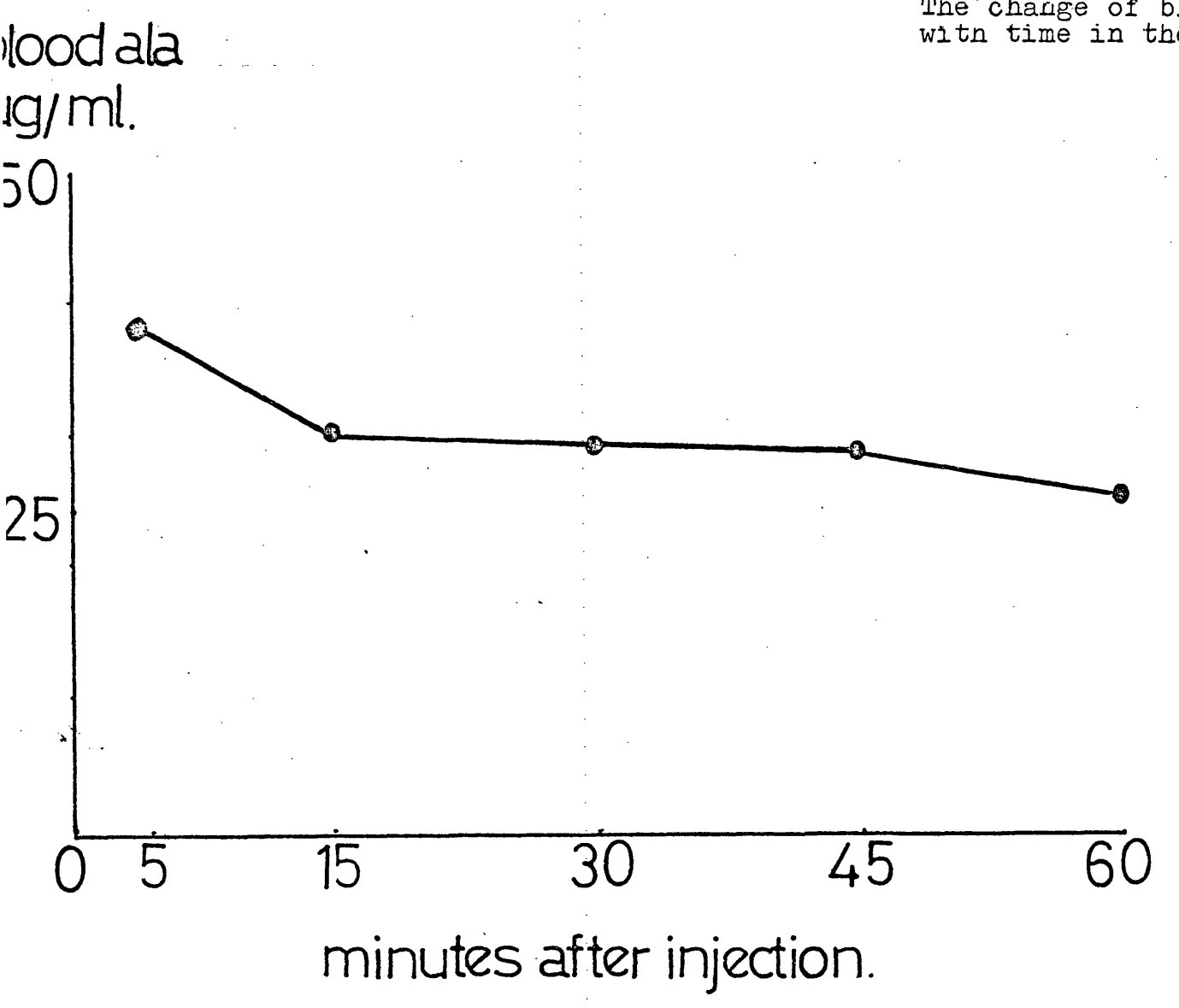
Method

A male Sprague Dawley rat (250g) which had been starved overnight was injected intraperitoneally with Nembutal 60mg/kg. After anaesthesia had developed an incision was made in the animal's neck and the trachea and one carotid artery exposed. The trachea was cannulated and artifical respiration maintained throughout the experiment by this route.

The carotid artery was also cannulated, the cannula being connected to a three way tap to which was attached a saline filled tube, leading to an electronic pressure transducer which displayed arterial pressure on a chart recorder, and two syringes, one containing heparinised saline, the other being empty. The rat was then injected I.P with 10mg ALA-HCl in saline brought to pH7 with bicarbonate, in

Figure 8.

The change of blood ALA concentration with time in the anaestnetised rat.



a total volume of 1ml. Five minutes after injection a sample of 0.1ml arterial blood was removed by the empty syringe. The tap was closed the syringe replaced and this time 0.2ml arterial blood was removed by again opening the tap and withdrawing the plunger of the empty syringe. The tap was then turned with the open position towards the syringe containing saline and 0.3ml heparinised saline was slowly injected down into the carotid artery. The subsequent procedure at 15, 30 and 60 minute intervals was similar viz: a) withdraw 0.3ml blood/saline from the carotid; b) withdraw 0.2ml arterial blood and c) inject 0.3ml heparinised saline into the carotid.

The blood samples thus removed (i.e. sample 'b') were assayed for ALA by the method described on page 33. and thus a function of blood ALA against time for an I.P injection of 40mg/kg ALA could be plotted (fig.8).

As can be seen from the figure an I.P. injection of this dose of ALA gave an equilibrium situation some fifteen minutes after injection which continued with only a slight decrease in concentration until the hour. Further this dose of ALA gave a blood concentration of ALA of a similar magnitude to that found in AIP.

As discussed on page 41 , the porphyrinogenic action of Nembutal, would have no significant effect on the blood ALA determinations.

BLOOD ALA LEVELS IN HUMAN SUBJECTS

The blood ALA concentrations of a number of porphyric subjects and of a number of normal subjects were determined by the method described above (p. 33). The porphyric subjects, who were all in remission, were classified clinically as follows:- acute intermittent porphyria, 4 cases: porphyria cutanea tarda, 3 cases: hereditary coproporphyria, 1 case.

In a single case of AIP, who was admitted to hospital in apparent attack, the blood ALA concentration was measured for ten consecutive days: a sample of cereborospural fluid was obtained from this patient, and the ALA concentration of this was also determined. (The normal subjects were eight laboratory staff, all in apparent good health).

Results

The mean normal value for the blood ALA concentrations was 0.38 ± 0.01 µg/ml. For the four subjects with AIP, the mean value was 0.78 ± 0.12 µg/ml. The difference between these two groups was significant when analysed by a students 't' test ($p < 0.01$). The mean ALA blood concentration of the three subjects with porphyria cutanea tarda was 0.27 ± 0.02µg/ml; and the case of hereditary coproporphyria gave a value of 0.33µg/ml.

In the single case of the AIP patient in apparent attack, the mean blood ALA concentration was 0.94 ± 0.27µg/ml, taking the values over ten days.

The maximum value reached was 1.40µg/ml, and as the C.S.F. concentration of ALA was determined on the same day, this gave a corresponding C.S.F. value of 0.33µg/ml.

Discussion

The results obtained indicate that in cases of AIP in remission, the blood concentration of ALA is significantly greater than in normal subjects. This would not appear to be the case in subjects with porphyria cutanea tarda in remission, and in the single case of hereditary coproporphyria investigated. It must be stressed with respect to these latter two, however, that only a small sample population was determined and the results are therefore inconclusive.

The significantly higher ALA concentrations in the blood of the AIP subjects, is a phenomenon almost certainly related to the increased urinary excretion of ALA by these subjects. It is likely that the increased biosynthesis of ALA results initially in an increased blood circulating level, with a consequent increase in renal excretion, which would account for the abnormally high concentrations of ALA in the urine of subjects with AIP. On this basis, it may be important that such patients have adequate renal function, as any renal damage could conceivably result in a decreased excretion of ALA and a resultant further

increase in its circulating concentration.

One notable observation is the fact that ALA was capable of being determined in the blood of normal subjects. This would suggest that ALA is capable of passing into, and reaching equilibrium in, various tissues in normal subjects, as well as in subjects with AIP (cf. Section IV (1).) It would also suggest that any pathological effect of ALA on the tissues, would only occur at concentrations somewhat greater than the upper limit of the normal values, a situation which most likely would be reached in a porphyric subject in attack.

The one subject in the present study who was admitted to hospital in an apparent attack of AIP was later diagnosed as <u>not</u> being in attack. This would account for the ALA_B blood concentration being much smaller than the values reported in true porphyric attack (Sweeney et al 1970). The detection of ALA in the C.S.F. of this subject is an observation of some interest. It may be that the presence of ALA in the C.S.F. is merely a reflection of the elevated blood concentration, as ALA can pass the blood-brain barrier (Section III (11)) and thereby enter the C.S.F. Alternatively it may be that there is an increased central synthesis of ALA in AIP, which might also be expected to result in the presence of ALA in the C.S.F. Whatever the mechanism, the fact that ALA <u>is</u> present centrally

is important in view of ALA's known pharmacological actions, and the central manifestations of AIP.

In summary then, ALA is present at significantly higher blood concentrations in subjects with AIP when compared with normal subjects: and, in one acute porphyric, ALA was detected in the C.S.F.

AIA INDUCED PORPHYRINOGENESIS AND EXOGENOUSLY ADMINISTERED ALA.

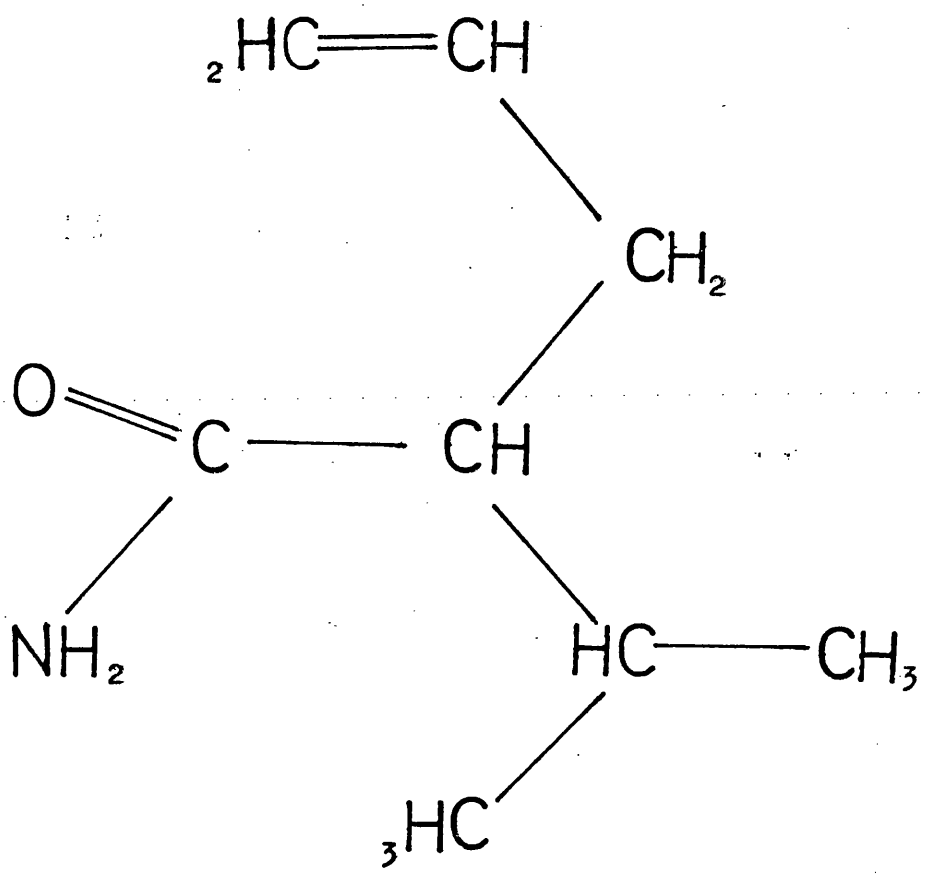
It is obviously important to know to what extent it is valid to compare the effects of increasing the tissue availability of ALA by exogenous administration of the salt, with the increase produced by AIA which is by stimulation of ALA's rate of synthesis.

It is also important to understand the consequences of these different methods of increasing the circulating levels of ALA, and how the action of AIA on haem biosynthesis is related to any other effects it may exhibit.

AIA was first introduced by Goldberg (1953) as an 'agent' which could simulate the porphyrogenic action of sedormid (allyl-isopropyl-acetylurea) without demonstrating the latter's hypnotic properties. Although it is generally agreed that AIA is a powerful porphyrinogenic agent, there are contradictory reports on AIA's 'non-hypnotic' action. Thus although Goldberg and Rimington (1954) demonstrated that AIA only caused 'dazing' in rats, other workers have reported rats to be in deep sleep after the same dose of AIA viz: 400-500mg/kg (Biemca et al, 1967).

The cause of this inconsistency is likely to be in the difference of the route of administration of AIA. Thus Goldberg and Rimington (1954) gave

The Chemical Structure of AIA.



their rats AIA by gastric intubation, whereas many of the subsequent workers who have reported sleep and other gross behavioural effects of AIA immediately after injection (Biemca et al 1967; Yuwiller et al 1970; Marcus et al 1970) have given AIA by intraperitoneal injection. Obviously administration by this latter route causes a much quicker and a much greater concentration of AIA in the plasma than the former, and hence overt toxic effects of AIA would be more in evidence. It is likely therefore that this fact alone accounts for the differences in these observations.

The disturbances of haem metabolism produced by AIA are analagous to those found in cases of acute porphyria (Tschudy, 1965). It is likely that AIA produced these effects on haem metabolism by stimulation of ALA synthetase activity, as it has been shown to stimulate this enzyme in embryo chick liver (Granick, 1966) and in rat liver (Marver et al 1966). Thus by increasing ALA and its subsequent metabolic products, AIA will cause other biochemical changes caused by the redistribution of the necessary cofactors of ALA synthetase from other tissues to the liver. Exogenous administration of ALA on the other hand, will produce qualitatively the same net effect on haem biosynthesis, without any associated depletion of the cofactors of synthetase.

If it is desired therefore, to see if any specific

change is produced in, e.g. animal behaviour, by increased circulating levels of porphyrins and their precursors per se, then it would be more informative to use exogenous ALA administration. This would increase haem synthesis without causing other effects due to 'redistribution' of cofactors to synthetase.

On the other hand, if it was desired to look for similar effects, as a result of an increased hepatic ALA synthetase activity - a situation more like acute porphyria where hepatic ALA synthetase activity is raised - then obviously it is better to use AIA. By doing this however, the results obtained cannot simply be related to the increase in haem biosynthesis, and the fact remains that any effects observed are not necessarily directly due to the elevated levels of haem and its precursors; but may be due to other changes produced as a result of synthetase activity being raised. For example as described below (III) the susceptibility of AIA treated animals to indirect convulsions could be a result of neural tissue depletion of the synthetase cofactor, pyridoxal phosphate. As well as an increased utilisation of pyridoxal phosphate, increased activity of hepatic ALA synthetase would lead to increased utilisation of other factors necessary for ALA synthesis, such as ferrous iron, glycine, and succinyl Co.A. Thus the effects produced by AIA may be due to the removal and possible depletion of

some of these substances from other metabolic pathways. It has, for example, been suggested that increased use of succinyl Co.A for haem synthesis could lead to a decrease in the formation of acetylcholine, as the latter is synthesised by a series of reactions which require the former.

There are however, certain effects of AIA which appear to be directly related to its ability to stimulate haem synthesis. Thus various workers have shown that a single injection of AIA is sufficient to increase the level of tryptophan oxygenase in rat liver (Feigelson and Greengard, 1961: Marver at al 1966 and Yuwiller et al (1970). It was postulated that tryptophan oxygenase activity would be elevated by an increased availability of haem (Feigelson and Greengard, 1961(b)) and hence the increase of the level of this enzyme might be a secondary result of AIA increasing haem synthesis. If so, this effect of AIA should be capable of being emulated by exogenous ALA administration.

A study testing this possibility was carried out by Wetterberg et al (1969) who gave rats acute injections of ALA, and when they measured hepatic tryptophan oxygenase found that indeed it was elevated. In this case therefore, it is reasonable to assume that both AIA and ALA produced this same result by a similar mechanism viz: an increased synthesis and availability of haem.

TABLE 3

DRUGS - FORM AND SOURCE

Acetylcholine Chloride .. (Koch Light)

l -Adrenaline .. (Koch Light)

Allylisopropylacetamide (AIA) .. (Roche)

$\triangle$ -Aminolaevulinic Acid Hydrochloride (ALA) (Koch Light or Sigma)

H^3 and C^{14} ALA .. (Radiochemical Centre, Amersham)

Atropine Sulphate Monohydrate .. (Koch Light)

α-Chloralase .. (Koch Light)

Choline Chloride .. (B.D.H.)

2-4-Dimethyl-3-Ethylpyrrole (Kryptopyrrole) (Emmanuel)

Ethanol .. (Burroughs)

Glycine .. (B.D.H.)

Heparin (Pularin) .. (Evans)

Hexamethonium .. (M. & B.)

Histamine Diphosphate .. (Koch Light)

Isonicotinyl Hydrazide (Isoniazid) .. (Koch Light)

Isoprenaline .. (I.C.I.)

Mepyramine Maleate (Anthisan) .. (May and Baker)

Morphine .. (B.P.)

Nicotine .. (Koch Light)

g -Strophantin (Ouabain) .. (Merck)

Pancuronium .. (Organon)

Pentobarbitone Sodium (Nembutal) .. (Abbot)

Phentolamine Mesylate (Rogitine) .. (C.I.B.A.)

Propranolol Hydrochloride (Inderal) .. (I.C.I.)

Trichloroethylene (Trilene) .. (I.C.I.)

Urethane .. (B.P.)

Vasopressin (Pitressin) .. (Parke-Davis)

SECTION III

CENTRAL STUDIES

CHEMICALLY INDUCED CONVULSIONS AND ALA

Introduction

Although previous workers (Goldberg, 1953; Gibson and Goldberg, 1955) found that animals made experimentally porphyric with AIA showed little signs of clinical change, Kosower and Rock (1968) demonstrated that such animals were significantly more susceptible than normals to chemically induced convulsions.

They found that rats pretreated with 400mg/kg AIA for 8 to 10 days convulsed much more readily than normals when challenged with isonicotinyl hydrazide (INH) or 4 methoxymethylpyridoxol. Further, they found that pyridoxol (2mg/kg) exerted a protective effect to convulsions of some 60% in the case of 4-methoxymethylpyridoxol (0.75mmoles/kg) though not to INH.

A continuation of this study would appear to indicate that the agent responsible for this decrease in convulsive threshold is ALA (Kosower et al, 1969). Thus these workers found that the substance methyl-5-diazo laevulinate, abolished the excessive urinary excretion of PBG in the AIA treated animals, without, however, significantly affecting the excessive ALA excretion. Further the convulsive threshold was unaffected, suggesting that ALA was the causative agent. An interesting

feature here was the fact that although urinary PBG decreased after methyl-5-diazo laevulinate, there was an associated increase in an unidentified substance which they term 'X'; almost identical amounts of this substance 'X' were excreted from normal rats similarly treated with methyl-5-diazo laevulinate. Assuming that ALA is responsible for this increased convulsive susceptibility two immediate possibilities present themselves. 1) It exerts such an action indirectly possibly by a mechanism related to the fact that increased synthesis of ALA under the influence of AIA causes depletion of pyridoxal phosphate from other tissues, particularly the brain and nervous tissues; 2) ALA exerts this action directly, probably by a central action.

As a simple pyridoxal deficiency was considered to be insufficient to explain this effect (Kosower and Rock, 1968) it was decided to investigate the second possibility.

Now if a direct central action of ALA is responsible it should be possible to produce increased susceptibility to convulsions by chronic ALA treatment alone and it would be expected that ALA should be capable of passing the blood-brain barrier. There is evidence for this latter possibility from human studies. Thus Sweeney et al (1970) have demonstrated that at a plasma

concentration of 24µg/ml, ALA could be detected in the CSF of a porphyric patient, whereas in normal circumstances ALA is undetectable in CSF.

It was decided therefore, to perform the following experiments:

(a) To repeat the investigations with AIA and see if AIA pretreated animals were more susceptible to convulsions.

(b) To investigate the possibility that chronic ALA treatment alone could elicit increased susceptibility to convulsions.

(a) AIA Pretreatment and Induced Convulsions.

Method

Two groups of eighteen male Sprague Dawley rats (200-250g) were used. The test group was given a daily intraperitoneal injection, for seven days, of 400mg/kg AIA. The volume of injection was 0.4-0.5ml. As the AIA was dissolved in propylene glycol, the control group were injected daily with an equivalent volume of this alone.

Twenty-four hours after the last dose of AIA the animals were taken and split into two sets (test and control) of three groups, each containing six animals. Each sub-group was then given an intraperitoneal injection of isonicotinyl hydrazide (INH). The dose given was either 0.8m. moles/kg; 1.12m. moles/kg or 1.68m. moles/kg.

The animals were then returned to their cages

and observed continuously for up to three hours. The number of animals convulsing in each sub-group was noted. A convulsion was recorded only when the animal showed complete tonic and clonic movements of the whole body. Isolated head jerks and other body tremors were ignored for the purpose of recording a convulsion.

Results

The results are shown in Table 4. No animal in either the test or control groups convulsed in response to the 0.8m. moles/kg dose of INH. The 1.12m. moles/kg dose however, caused convulsions of three animals in the test group, although none of the control animals convulsed. In response to the 1.68m. moles/kg dose of INH, four animals in the control group and six in the test group convulsed. All convulsions took place within 90 minutes of injection of INH.

The number of animals convulsing per sub-group, was expressed as a percentage of the total number of animals in that sub-group (viz. 6). This percentage was plotted against the dose of INH administered, resulting in a dose-response graph fig.(10).

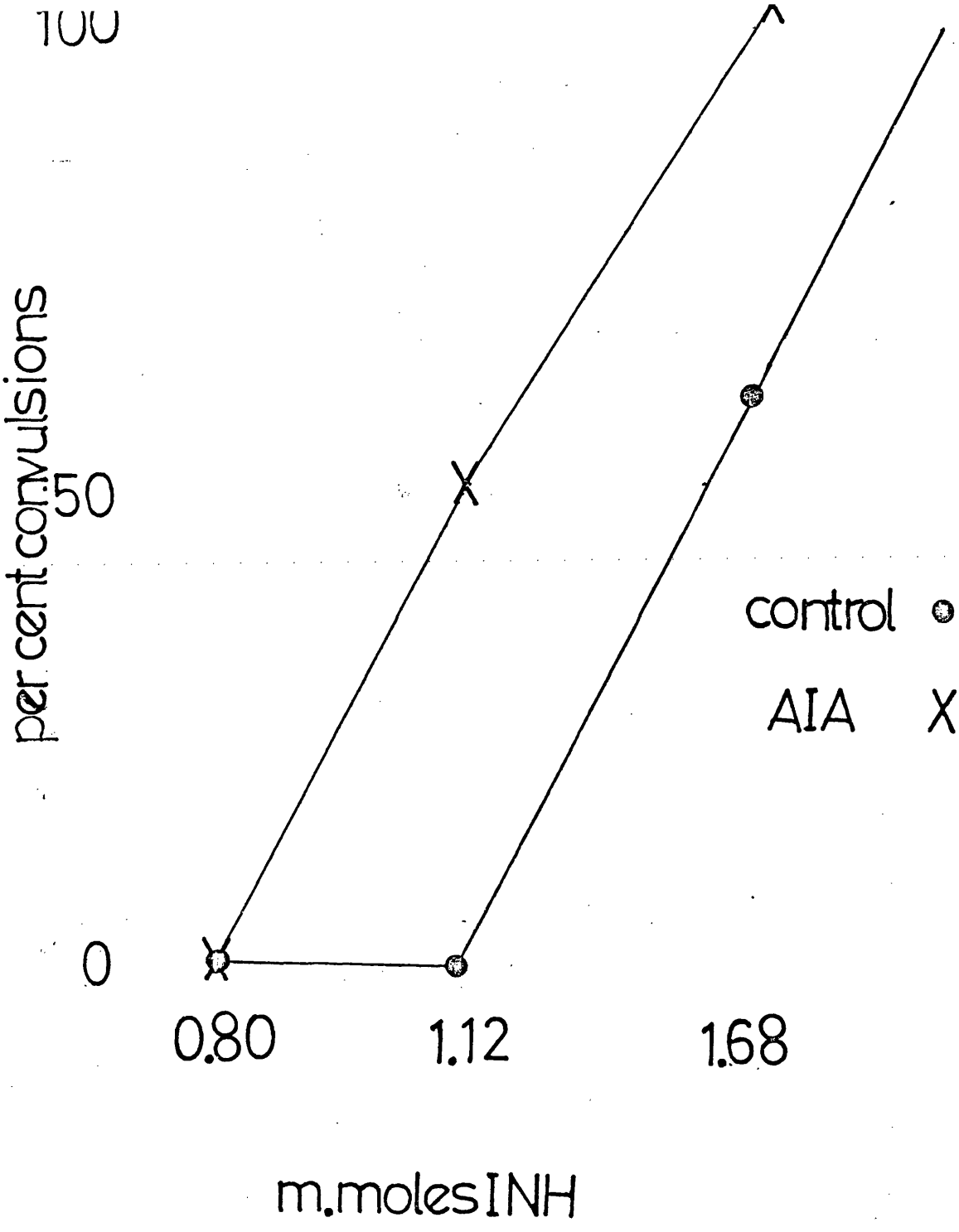
Analysis of the results by a 'Chi-squared' analysis, demonstrated that the dose response relationship for the AIA pretreated animals, is significantly shifted to the left of the control graph. ($p < 0.0005$) This shift indicates that the

AIA pretreated animals are significantly more susceptible to INH induced convulsions than are normal animals.

Dose INH (m.moles/kg)

	0.8	1.12	1.68	
Convulsions per sub-group	0/6	0/6	4/6	Control
	0/6	3/6	6/6	AIA
% Convulsions per sub-group	0	0	66.66	Control
	0	50	100	AIA

TABLE 4.



$X^2 = 28.6$ $P < 0.0005$

(b) Chronic ALA Pretreatment and Induced Convulsions

Methods

Two groups of seven male Sprague Dawley rats were injected every two days for four weeks, with either saline (control) or 40mg/kg ALA (test) in saline. The ALA was previously adjusted to pH 6-7 with molar sodium bicarbonate, and the volume of injection, which was by the intraperitoneal route, was $\ll$ 1ml.

The animals given ALA showed distinct behavioural changes after this time which are reported fully in the relevant section (III). One day after the last injection, the animals in both control and test groups were challenged with 1.12m. moles/kg INH. They were observed continuously for convulsive activity for the three hours immediately following this injection.

Results

None of the control or test animals convulsed in response to this dose of INH. In one control animal there was some degree of muscle twitching some fifty five minutes after injection, but no convulsion was forthcoming. No other animal showed evidence of any effect of INH.

Discussion

These results are in agreement with the findings of Kosower and Rock (1968) in that rats pretreated with AIA were more susceptible to INH induced convulsions; there were however, a few differences in the results obtained by these authors and those described above. Thus, the effective dose producing 50% convulsions (the ED_{50}) was found by Kosower and Rock to be in the region of0.6m.moles/kg for AIA treated animals and 1.6m.moles/kg for the control animals. In the present study the ED_{50}s were in the region of 1.1 and 1.5m.moles/kg for the AIA and control animals respectively. That is, in both the control and test situations these animals were less susceptible to INH than those reported by Kosower and Rock. Even allowing for the fact that the ED_{50}s are obtained in each case by interpoltation and thus liable to a relatively large error,the differences are great enough to be worthy of consideration.

There were differences in the experimental procedure which could account for the differences in the ED_{50}s found. Perhaps the main difference was the fact that while these authors used female Sprague Dawley rats, male Sprague Dawley rats were used in this study. Another factor is

that they used a solution of AIA dissolved in 0.15 molar sodium chloride, whereas the AIA in this study was dissolved in propylene glycol and injected intraperitoneally. This was so because it was found that AIA was difficult to dissolve in saline and that subcutaneous injection of AIA in propylene glycol led to development of scar tissue in the animals. So too the animals used in this study were injected with AIA for seven days, whereas some of those used by Kosower were injected for ten days.

These experimental differences may be sufficient to account for the variation in results obtained in these two studies. The important point however, is that both studies demonstrate that AIA pretreatment can increase the susceptibility of rats to chemically induced convulsions by INH.

This fact being established it is still uncertain how AIA effects this increase in susceptibility. As discussed above, experiments using methyl-5-diazo laevulinate (Kosower et al, 1969) suggest that ALA is the causal factor.

The chronic ALA studies have investigated this possibility, and the results suggest that elevated ALA levels alone are insufficient to account for the phenomenon. The dose of INH

used (1.12m.mole/kg) was sufficient to produce 50% convulsions in animals pretreated with AIA; yet it did not produce any convulsions in the animals pretreated with ALA.

This suggests, therefore, that if AIA does indeed decrease the convulsive threshold to INH by a mechanism which is in some way related to ALA, it is likely to be a result of its induction of ALA synthesis, rather than to any direct effect of the excessive ALA produced. Although Kosower and Rock (1968) found that pyridoxal phosphate did not protect against INH induced convulsions, in chemically induced porphyric animals, neural depletion of this substance could account for these results.

Thus increased hepatic synthesis of ALA is mediated by means of an increase in activity of ALA synthetase, of which pyridoxal phosphate is a cofactor. An increase in the hepatic pool of this cofactor could lead to a pyridoxal deficiency in other tissues, including nervous tissue, and such neurological changes as this produced could account for the decreased convulsive threshold to INH.

That AIA pretreatment and ALA pretreatment do not produce the same effects was also evident in another way. Although AIA has been reported as

being able to produce behavioural changes in rats (Marcus et al, 1970) there were distinct differences between these and the changes observed after ALA. This fact will be discussed in more detail in the section relating to ALA and animal behaviour; however the significance is that once more we are led to conclude that the effects of AIA are probably not related to its ability to increase ALA levels per se, but to the changes resulting from the mechanisms by which excessive ALA is produced.

ALA AND THE BLOOD-BRAIN BARRIER

Introduction

In view of the possible role of ALA in the clinical manifestations of acute porphyria, it is obviously important to determine whether or not ALA can cross the blood-brain barrier and thus enter brain tissue at the elevated blood concentrations occurring in the disease. This possibility has been investigated previously, but there were conflicting results. Thus Kramer et al, (1971) indicated that ALA was readily taken up by brain tissue, whereas Musyka (1969) indicated that it was not. Although the former studies were performed 'in vitro' and the latter 'in vivo' - a fact which may account for this discrepancy - the question remains - does ALA pass the blood-brain barrier?

Structurally, a small molecule like ALA might be expected to pass the blood-brain barrier readily: As however, the passage of even quite small molecules is often a relatively selective process, it was required to demonstrate this fact experimentally.

Ferguson et al, (1970) reported two cases of AIP where an attack was apparently precipitated by acute over-indulgence in ethanol. As ethanol is known to affect the permeability of the blood-brain barrier to certain substances such as

catecholamines (Hanig et al, 1973) it was decided to resolve whether or not acute ethanol intoxication had any effect on the permeability of the blood-brain barrier to ALA. In such an experiment there are a few possibilities which could give rise to misleading results and these must be accounted for before one can conclude that a substance does indeed pass from the blood across the blood-brain barrier into the C.S.F.

When the whole brain was assayed for ALA, allowance had to be made for the presence of ALA in the blood in the brain. This necessitated the measurement of the blood concentration of ALA and the blood volume of the brain. Further there are parts of the brain which are not served by the blood-brain barrier and, when present in the blood, ALA could reach an equilibrium situation with these by simple diffusion across capillary membranes, even though it may not penetrate into areas served by the blood-brain barrier. In such an event a total brain ALA estimation would not discriminate between those areas served by the blood-brain barrier and those not. To allow for this the ALA content of the brain was measured some time after injection of ALA, when the blood concentration was normal i.e. < 0.5 µg/ml, and presumably no such equilibrium state existed. ALA contents in the brains of animals not given ALA were also determined.

The ALA estimations were made from a sample of the supernate left after the tissue itself had been homogenised and spun down. Two assumptions were made at this point, which were experimentally tested for validity. The first was that all the radioactivity counted was due to radioactive ALA and not some metabolic or chemical product of it. The second was that all, or at least a very high percentage ($>$ 90%) of, the ALA passed from the tissue into the aqueous supernate. The methods by which the validity of both these assumptions were tested are described below under 'Subsidiary Experiments'.

Methods

Two groups of sixteen male Sprague Dawley rats (200-250g) were used. One group was given ethanol (5g/kg) as a 50% solution in water administered by gastric intubation, the other group was given an equivalent volume of water administered by the same route. The animals were then left for 30 minutes at which time the ethanol group showed obvious signs of intoxication, such as an inability to walk properly and a high frequency of falling over on their sides.

The animals were then injected intraperitoneally with ALA in saline in a dose of 40, 120 or 300mg/kg: Normal and ethanol treated animals were

injected alternately. The ALA had previously been adjusted to pH 6-7 with sodium bicarbonate and spiked with either uniformly labelled tritiated ALA or with C^{14} ALA. The specific activities of the radioactive sources injected were in the region of 100mCi/mM for H^3 ALA and 10mCi/mM for C^{14} ALA. The volume of injection was up to 1ml.

The animals were then left a further 30 minutes to allow the ALA to equilibrate, the equilibration time having been previously determined as described (page 45). Technecium labelled human serum albumin(Tc^{99}) was then injected into a tail vein (or in a few cases where this proved difficult into an exposed rear leg vein under light 'Trilene' anaesthesia) and allowed to circulate for two minutes, at which time the animal was stunned, exsanguinated by cutting the throat and the blood collected in an heparinised bottle. The skull was then cut open, the cranium broken away and the whole brain dissected out, blotted dry, weighed and then halved medially. One half of the brain was then homogenised in water (1:3 w/v) and the total resulting solution counted for Tc^{99} activity in a Wallac Gamma Sample Counter GTL 300-1000. The other half of the brain was homogenised in 20% TCA and the weight of total homogenate taken. This was then centrifuged at 3000rpm for 15 minutes. The resulting supernate was decanted into a fresh

tube and spun again at 3000rpm for a further period of 15 minutes. This supernate was stored for a period of not less than three days before counting for H^3 (C^{14}) activity.

The purpose in leaving the supernate for at least three days was to allow the Tc^{99} activity to decay. The energy of the gamma emission of Tc^{99} is 142.7 Kev, sufficient to produce secondary particles which would interfere with the H^3 (C^{14}) counting. However as the half-life of Tc^{99} is six hours, with the specific activity used, this period of time was sufficient to allow decay to occur to a level where a sample counted for gamma emission gave no more than the background reading, thus allowing a true reading of H^3 (C^{14}) activity in the sample.

A sample of 1ml supernate was taken and weighed in a counting vial, 9ml of 'Instagel' was then added to the vial and the solutions were well mixed. The vial was then closed and H^3 (C^{14}) activity counted in a Packard Tri Carb Liquid Scintillation Spectrometer. Insta-Gel (Packard) is a complete scintillator system, which allows immediate measurement of β-particle emission when an aqueous sample of the active source is added to it.

Tc^{99} and H^3 (C^{14}) activity was also determined in blood samples from the animals.

The procedure was identical to that of the brain except that dilutions for homogenisation were made on the basis of volume rather than weight. The concentration of whole blood ALA in µg/ml was also determined by the method of Haeger-Aronsen, described on page 33.

Calculation

The Tc^{99} counts in the brain per gram, divided by the Tc^{99} counts in 1ml of blood gave the blood content of the brain in mls per gram. This multiplied by the blood concentration of ALA in µg/ml, gave the ALA present in the brain tissue by virtue of its blood content.

The total brain ALA content was determined as follows: the H^3 (C^{14}) counts in a known weight of supernate was determined. This was multiplied by the weight of the total homogenate divided by the weight of the supernate, to give the number of counts in the total homogenate. This was then converted to number of H^3 (C^{14}) counts per gram brain tissue.

The number of H^3 (C^{14}) counts per ml of blood was divided by the concentration of ALA in blood in µg/ml. This gave the number of counts of H^3 (C^{14}) per µg ALA. The number of H^3 (C^{14}) counts per µg ALA was divided into the number of counts in the brain per gram. This gave a value

for µg ALA per gram brain tissue. From this value the ALA present in the brain due to its blood content was subtracted giving the value of ALA per gram true brain tissue.

Thus in summary:

$$\frac{^{3}H \text{ counts in Brain/gm}}{^{3}H \text{ counts/ug ALA}} \quad \frac{^{99}Tc \text{ counts in brain/g}}{^{99}Tc \text{ counts in blood/ml}} \quad X$$

blood ALA concentration (µg/ml)

Results

It was found that as the concentration of ALA in the blood increased there was a corresponding increase in the concentration of ALA in the brain. Except at the highest blood concentrations (100-120µg/ml) the concentration of ALA was higher in the brains of the animals untreated with ethanol; i.e. ethanol appeared to have an inhibitory effect on the passage of ALA from the blood into the brain tissue.

When the brain concentrations of ALA in µg/g were plotted against the blood concentrations in µg/ml, it was found that in both cases there were highly significant positive regression lines with the following equations:

1. Control:

   Brain ALA (µg/g) = 0.6 (blood ALA µg/ml)$^{1.27}$

2. Ethanol.

   Brain ALA (µg/g) = 0.02 (2.06 (blood ALA µg/ml))

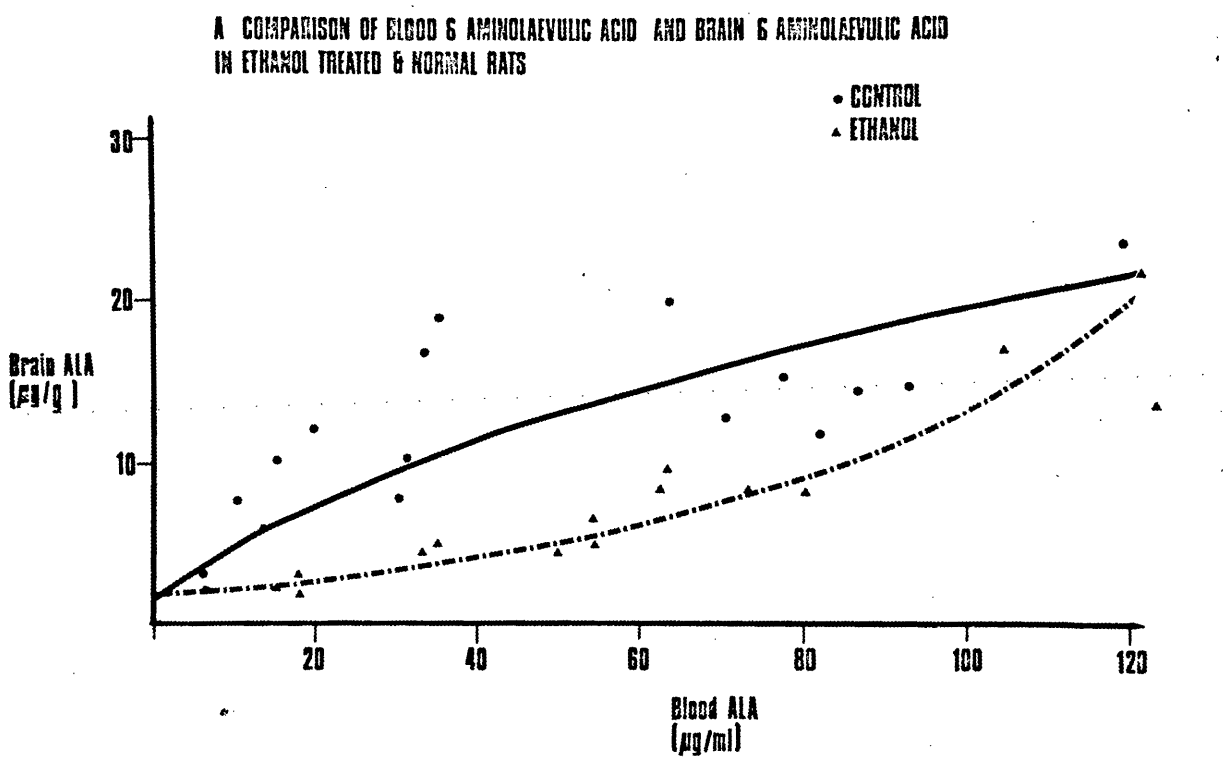
   The regression coefficient was 0.95

   These lines are shown graphically in fig.11.

TABLE 5

CONTROL		ETHANOL PRE-TREATED	
Blood ALA (µg/ml)	Brain ALA (µg/g)	Blood ALA (µg/ml)	Brain ALA (µg/g)
5.7	3.0	15.0	2.3
5.8	1.9	17.5	3.0
10.8	7.5	18.3	2.1
12.5	3.3	33.0	4.5
15.0	10.0	35.0	4.9
19.5	12.0	50.0	4.7
30.0	7.7	55.0	5.0
31.6	10.0	55.0	6.5
33.3	16.9	62.5	8.3
35.0	19.0	63.3	9.6
62.0	20.0	73.3	8.2
70.8	12.7	80.0	8.0
76.7	15.1	105.0	17.0
81.7	11.7	120.0	21.6
86.7	14.2	123.3	13.3
92.5	14.6	-	-

Figure 11.


A COMPARISON OF BLOOD & AMINOLAEVULIC ACID AND BRAIN & AMINOLAEVULIC ACID IN ETHANOL TREATED & NORMAL RATS
• CONTROL
▲ ETHANOL
30
20
10
Brain ALA (µg/g)
20
40
60
80
100
120
Blood ALA (µg/ml)

SUBSIDIARY EXPERIMENTS

a) The determination of brain ALA when the blood ALA concentration had returned to normal is described on page 101.

Results: ALA could be significantly detected in the brains of rats pretreated with ALA, but with normal blood ALA concentrations. Rats untreated with ALA demonstrated no significant ALA in the brain compared with the ALA treated rats.

b) To determine if all the radioactivity in the supernate was due to ALA the following procedure was carried out: A chromatogram was run (method p. 39.) of a sample of tissue supernate against a C^{14} ALA spiked standard of ALA. When the chromatogram was stained, it was divided into squares of side 1 cm, along the lines of migration of the spots. Each square was then eluted with water, and each eluate was counted for activity.

Result: It was found that all the radioactivity significantly above background was localised in the region of the supernate run which corresponded with the ALA spot on the standard run. The percent recovery of the radioactive ALA from the chromatographic procedure, was in the region of 90%.

c) To test if all, or at least a high percentage of the ALA had passed from the tissue on homogenisation into the aqueous supernate, a Hewlett Packard Sample Oxidiser was used. This machine oxidises the tissue

directly, giving off a vapour containing all organic carbon as carbon dioxide. This carbon dioxide is then automatically transferred into a solution which can be counted for C^{14} activity.

The brains of three rats which had been given C^{14} ALA, were halved medially and an equivalent corresponding portion of each half was determined for C^{14} activity either after oxidation in the Sample Oxidiser, or after homogenisation and centrifugation as described above.

Result: It was found that each corresponding sample showed no more than a 5% difference in ALA content whether C^{14} activity was counted after oxidation of the tissue, or after homogenisation and centrifugation.

These results indicate that it can be assumed that: a) ALA is not merely in a diffusion equilibrium with the parts of the brain not served by the blood-brain barrier: and that the normal brain ALA content is significantly less than that after administration of ALA.

b) The C^{14}/H^{3} activity counted, is due to labelled ALA alone and not to a metabolic or chemical product of it.

c) Almost all the ALA contained in the tissue passes into the aqueous supernate after homogenisation and centrifugation.

As a result any errorsin these procedures for

the measurement of ALA in brain tissue are likely to give an under-estimate of the amounts present, rather than an over-estimate.

Discussion

The results indicate that ALA can cross the blood-brain barrier, and that it can do so at blood concentrations known to occur in acute porphyria. As the relative distribution of ALA between the extracellular and intracellular fluids was not determined, the results only give the wet brain tissue content of ALA and not the subcellular nor cerebral distribution. There is little doubt that having passed the blood-brain barrier ALA can enter the brain cells, as cell membrane penetration by ALA has previously been established. (Scott et al, 1955) (Falk et al, 1956).

It is seen that the brain content of ALA increases with the blood concentration, and it is reasonable to assume that in AIP, an increase in circulating ALA results in a corresponding increase in passage into the C.N.S. If the above experiment is a representative animal model for the human condition, then with the circulating levels of ALA reported in AIP (up to 24/µg/ml) a brain concentration of some 10-15µg/g might be expected.

Such relationships between blood and brain ALA concentrations have in this case been studied acutely. In porphyria of course, the exposure to elevated

circulating ALA is over a prolonged period; thus depending on a number of factors, such as the nature of passage of ALA into the brain (i.e. whether active and/or passive) the equilibrium concentrations across the blood-brain barrier, and the rate of metabolism of ALA centrally, much higher control concentrations of ALA might occur. Further it is possible that ALA may be preferentially taken up at specific sites in the cells, e.g. nerve endings or myelin, where effectively very large concentrations may occur.

This central uptake of ALA is consistent with the finding of ALA in the C.S.F. of subjects in acute porphyric attack (Sweeney et al 1970). It also becomes relevant to the observations, referred to above, that ALA is capable of 'in vitro' inhibition of brain ATPase and transmembrane sodium transport.

A detailed account of the possible neurological effects of ALA due to its possession of these properties is given in Section IV. At this stage it is only necessary to say that as ATPase activity and membrane sodium transport are of critical importance in maintaining the integrity of neural function, their inhibition of these would lead to neurological disturbances. Further nerve ATPase activity is also thought to be a controlling factor in neurotransmitter release, and thus inhibition of ATPase by ALA could lead to inappropriate release of neurotransmitter with the resultant pathophysiological consequences.

Thus it is evident that ALA is potentially capable of effecting neurological changes which could conceivably account to a greater or lesser extent, for the neuropathology evident in AIP.

The question presents itself : to what extent can these 'in vitro' findings be related to the 'in vivo' findings? In terms of concentrations Becker et al (1971) found that ALA could inhibit ATPase in a concentration of 0.2m M. This concentration is about 26ug/ml. Similarly Eales et al (1971) produced inhibition of membrane sodium transport with an ALA concentration of 0.15 M, which is about 20ug/ml. The brain concentrations in this experiment corresponding with the ALA concentrations found in AIP, is not much less than these values, assuming a brain tissue density of just greater than 1g/cc, and could quite conceivably reach them in the event of chronic exposure to elevated levels of circulating ALA.

It would appear on the basis of these considerations therefore, that ALA could reach the required concentration in the brain 'in vivo' to exert similar actions to those found 'in vitro'. Evidently therefore, it would be of some interest to investigate the distribution, both at the gross anatomical and subcellular levels of central ALA, to resolve the likely results of such neurological effects of ALA in terms of known central neurophysiology and the possible relation between this and the manifestations of AIP.

BEHAVIOURAL STUDIES

Introduction

The fact that ALA can pass the blood-brain barrier together with the fact that it can influence brain ATPase (Becker et al, 1971) and membrane sodium transport (Eales et al, 1971) suggests that if given in a large enough acute dose, or over a period of time, ALA could produce behavioural changes in experimental animals.

A study of this nature was carried out by Marcus et al, (1970) who assessed the relationship between AIA and ALA induced changes in the biochemistry, behaviour and EEG of freely moving rats with chronically implanted cortical brain electrodes.

These workers found that AIA (400mg/kg) induced a reversible progression of EEG changes which were characteristic of central excitation. They observed however that repeated daily injections of AIA led to a progressive reduction in the duration and degree of the EEG changes and the behavioural changes which were initially observed viz: ataxia, intermittent propulsive movements and falling. They found however that although the peak behavioural and EEG effects occurred 2-4 hours after the first administration of AIA, the peak urinary excretion of ALA and PBG

did not occur until the second day after administration.

Two animals were given a similar series of injections of ALA (400mg/kg) but neither behavioural nor EEG changes were observed.

These experiments were carried out by measuring 'gross behaviour', i.e. the animals were merely observed in their cages for some distortion of normal activity. No specific index of behaviour was measured. Further few animals were used (4 given AIA, 2 ALA) and it is notoriously difficult to observe non-dramatic behavioural changes in small groups of animals. It was felt, therefore, that this evidence was not sufficient to conclusively say that ALA had no effect on animal behaviour.

To make an adequate appraisal of any potential behavioural effect of a drug it is necessary to look at the effect of the drug both acutely and chronically, using a defined parameter of normal behaviour, changes in which are capable of quantification.

The present study was designed to establish whether or not acute and prolonged ALA administration, could produce behavioural changes in experimental animals. The acute studies involved the use of mice and the index of behaviour used was spontaneous activity. As well as normal

mice, mice chronically pretreated with ethanol were also used. Ethanol is one of the drugs known to have an effect on haem biosynthesis. It can increase the activity of hepatic ALA synthetase (Shanley et al, 1968) and depress the activity of ALA dehydrase (Moore et al, 1970). It might be expected therefore, that chronic ethanol pretreatment would cause an increase in the circulating concentration of ALA; and if ALA had some effect on animal behaviour the effect of chronic ethanol pretreatment may be similar to this effect of ALA. Ethanol is also known to be capable of precipitating attack in AIP both when taken chronically and acutely (Ferguson et al, 1970). Thus it is possible that chronic ethanol pretreatment could affect the susceptibility of an experimental animal to any pharmacological action of ALA, presumably making any such action more evident.

The chronic ALA studies were carried out on rats, and three indices of behaviour were used viz: spontaneous activity, number of times the animal reared its forequarters and total time spent motionless. This latter parameter gave a double discrimination on total movement when used in conjunction with the spontaneous activity.

The activity cage used in the acute studies (fig. 12) consists of an enclosed metal box with

a removable lid, crisscrossed by two light beams which impinge on two photo-electric cells. The cells are electrically connected to a digital counter. When a mouse is placed in the box it moves about to explore and in so doing cuts the light beams. Each time a beam is cut a unit count is recorded on the digital counter. Over a given period of time the number of counts on the digital counter gives a quantitative representation of the spontaneous activity of the animal.

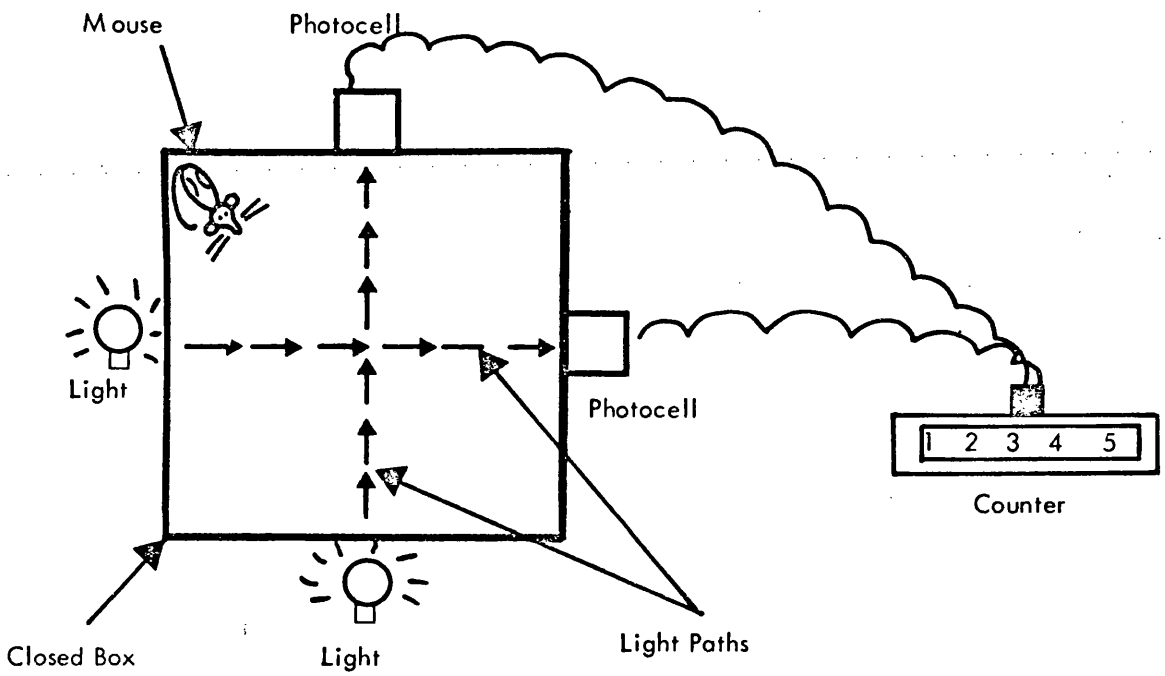
Methods

a) Acute Studies

Male albino mice (30-40g) were separated into two colonies of 82 and 32 animals, one of which was treated as normal and the other given ethanol (10% w/v) ad. lib. in the drinking water for six weeks prior to the experiment. After this time the spontaneous activities of the animals were determined by means of the activity cage.

The experimental procedure took the following form. A normal mouse, or one chronically pre-treated with ethanol, was injected intraperitoneally with either ALA 0.76m.moles/kg (in saline) or isotonic saline or isomolar glycine as controls. Saline was used as a control because it was the carrier for ALA, and glycine as it is an amino acid of similar structure to ALA. The ALA had

Figure 12.


ACTIVITY CAGE
Mouse
Photocell
Light
Photocell
1 2 3 4 5
Counter
Closed Box
Light
Light Paths

previously been brought to pH 6-7 using sodium bicarbonate and the volume of injection was $\leqslant$ 0.25ml. The mouse was allowed to recover for one minute after injection, and then placed in the activity cage and the counts recorded for five minutes. It was then removed and the next animal placed in the cage and so on. Five minute counts were taken from each animal at times 0, (namely 1 minute) 30, 60 and 90 minutes after injection. Some animals were counted at times 120 and 180 minutes after injection.

b) Chronic Studies

Two groups of seven male Sprague Dawley rats (200-250g) were used. One group was given ALA, 0.31m.moles/kg in saline, by intraperitoneal injection every two days for a period of four weeks. The ALA had previously been adjusted to pH-7 with molar sodium bicarbonate. The other group was given an equivalent volume of saline and served as a control group. The volume of injection was 1ml.

After four weeks three behavioural parameters were measured in these animals, two days after the last injection of ALA. The parameters measured were spontaneous activity, the number of times the animals reared, and the times the animals were 'motionless' (i.e. showed no movement such as scratching, head movements and so on.) In rats spontaneous activity was measured in an area (100 x 200cm) divided into 15cm squares. A rat was placed on

the central square and the number of times its hindquarters completely passed over the lines of an individual square was noted. This gave a quantitative value of the animal's activity. The number of times the animal reared, that is, raised its forequarters from the floor, was also noted as was the total time the animal spent 'motionless'. As with the acute studies in mice, each animal was investigated in this manner for a five minute interval; however, in this case only one time interval was used, viz. 0-5 minutes.

Results

a) Acute Studies

There was no difference in weight between the normal and ethanol groups and the ethanol treated animals showed no obvious signs of abnormal gross behaviour. As individual mice show a wide variation in spontaneous activity (some 16-90 counts per five minutes in the present study); for purposes of comparison the activities were expressed as a percentage of the original (zero time) activity. The zero time activities were expressed as number of counts/5 minutes.

Normal Mice

It was found that in the first five minutes after injection, the animals given ALA showed a significant depression in spontaneous activity compared with either the glycine or saline control

animals. (fig.13), (Table 6). There was no significant difference at any time between glycine and saline controls. 30 minutes after injection the control animals showed a decrease in activity compared with the zero time values; the ALA animals however, showed a significant increase in activity (fig.14). This increase was not only significantly greater than the corresponding control values, but also significantly greater than the zero time control values.

At 60 and 90 minutes after injection, this trend was continued, i.e. the animals given ALA were significantly more active than the controls. 120 minutes after injection the activities of the animals were not significantly different.

Mice Treated with Ethanol

The mice chronically treated with ethanol, demonstrated a similar response to ALA as did mice not treated with ethanol at times 0 and 30 minutes after injection. (fig.15) However at 60 and 90 minutes after injection there was no significant difference between the ALA and control groups ($p \leqslant 0.05$):

In no case was there evidence in any of the animals of limb weakness, or of abnormal withdrawal or righting reflexes.

TABLE 6

ABSOLUTE ACTIVITIES IN MICE/MIN AFTER I.P. INJECTION

	NORMAL		CHRONIC ETHANOL TREATMENT	
	No. of Animals	Activity (Movements/5 mins)	No. of Animals	Activity (Movements/5 mins)
Saline Treated	38	80.86 ± 6.62	16	86.8 ± 11.8
ALA Treated	33	51.39 ± 9.4	16	55.3 ± 7.4
Glycine Treated	11	87.82 ± 7.95	-	-
Significance with respect to controls		0.001		0.02

Results were analysed using a Student's 't' test, and expressed as Mean ± 2 SEM.

Figure 13.

The initial spontaneous activity of mice, expressed in absolute terms. Each result is expressed as a mean ± S.D. of a given number of observations.

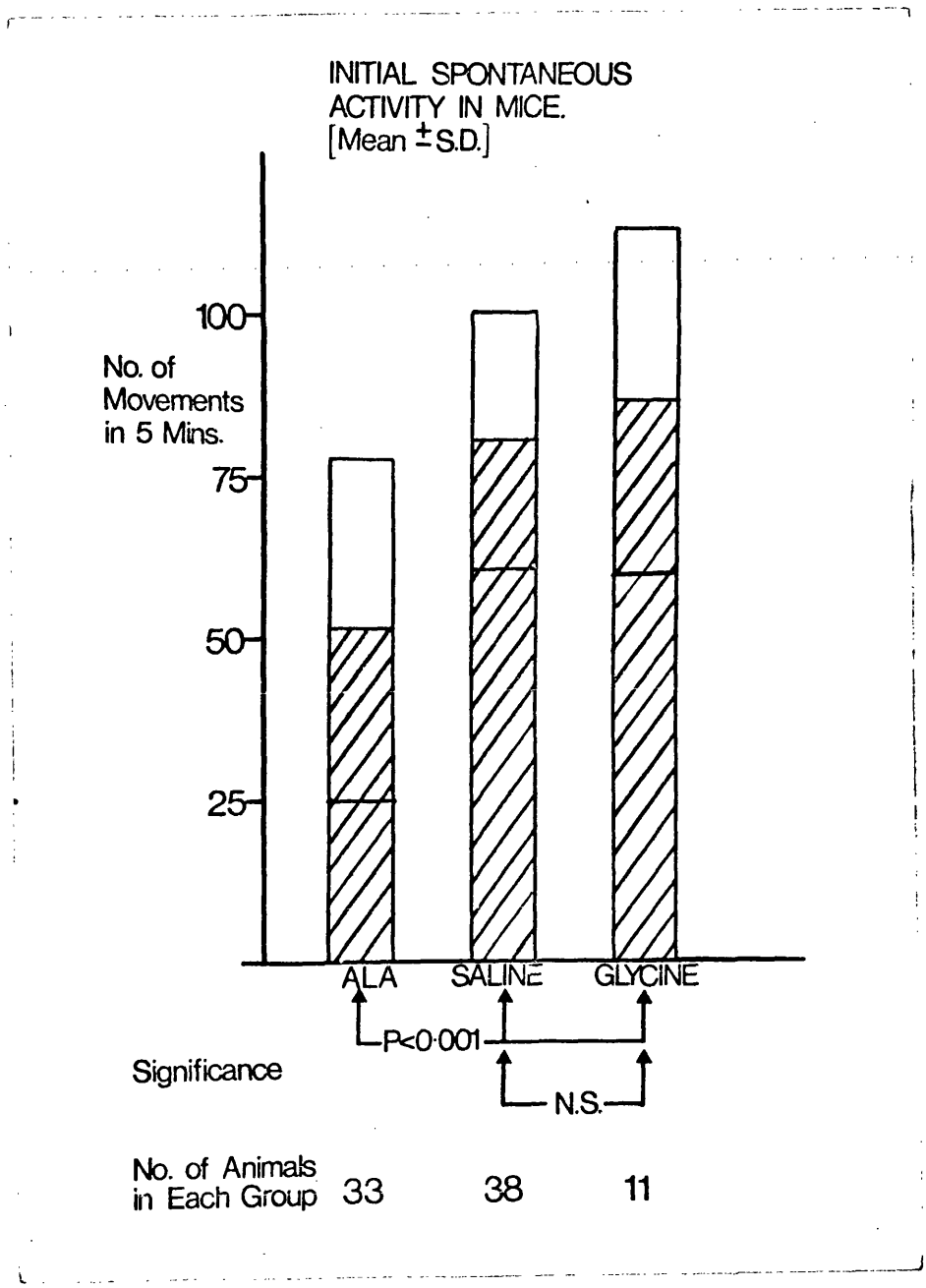


Figure 14.

Each point represents the mean of a number of experiments. The initial activity was taken to be 100%, and the subsequent results expressed as a percentage of the initial activity.

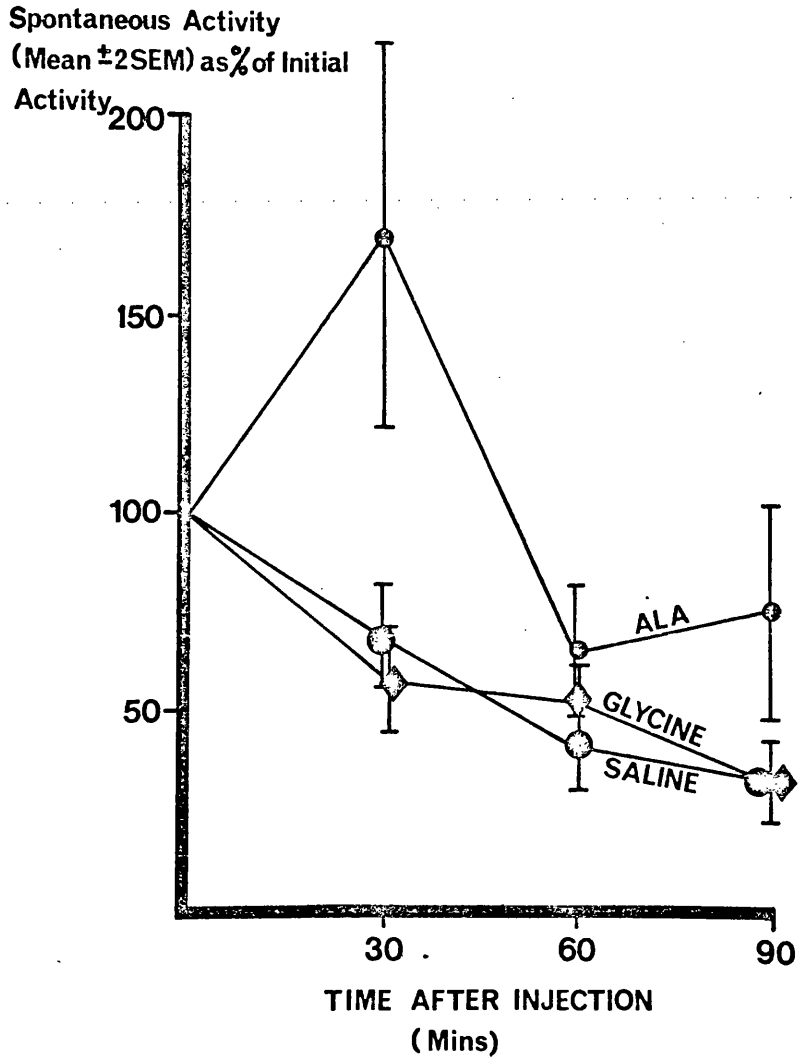
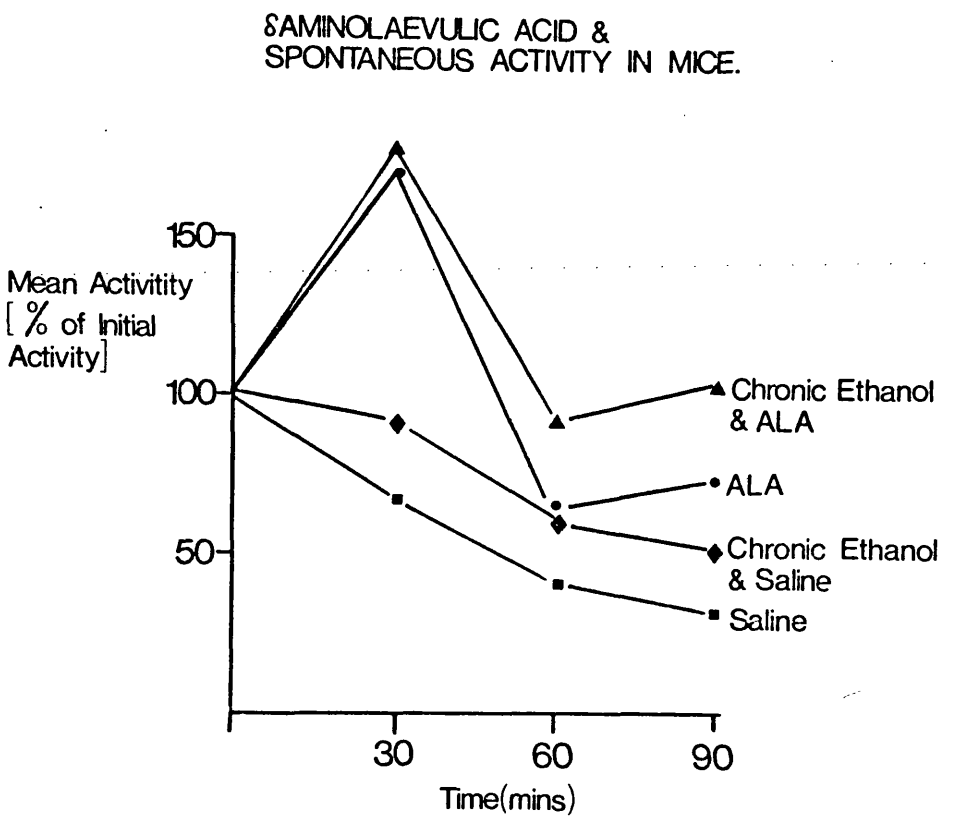


Figure 15.

This figure illustrates the finding that the chronic ethanol pretreated animals react to ALA in a similar manner to normals.



b) Chronic ALA Studies in Rats

There was no significant difference in weights between the ALA treated and control rats. The animals given ALA however, showed obvious signs of behavioural disturbances. In response to a stimulus such as clapping, where the control animals would respond by exploring and generally moving about their cage, the ALA animals would retreat to the back of the cage and huddle together, showing little movement. In general the animals given ALA appeared lethargic and apathetic compared with the controls. There were however, no obvious signs of limb weakness and withdrawal and righting reflexes appeared to be normal.

The results obtained from the quantitative studies are shown in Table 7. The animals given ALA showed a highly significant decrease in both spontaneous activity and in the number of times they reared in five minutes; they also spent a significantly greater time 'motionless' than did the controls, by the standards defined above.

Discussion

The results from the acute studies indicate that when given in a dose of 0.76m.moles/kg, ALA can significantly decrease the spontaneous activity of mice, compared with saline or glycine controls, for up to 30 minutes immediately following injection.

TABLE 7

ACTIVITY IN CHRONIC ALA TREATED RATS

	No. of Animals	Spontaneous Movement (square crossing)	Rearing (times)	Time Motionless (sec)
Saline	7	65.14 ± 11.48	37.43 ± 13.41	116.1 ± 23.4
ALA (10mg/kg) every 2 days for 4 weeks	7	20.0 ± 9.6	12.86 ± 4.59	207.0 ± 33.1
Significance ($p <$)		0.001	0.001	0.001

Results were analysed using a Student's 't' test

The mechanism by which this action is brought about is obscure, however it is possible that ALA causes a sudden, short lasting, fall in the blood pressure of these mice, which causes them to decrease their movements.

ALA has a short lasting hypotensive action on rats, at relative dose levels much less than those used in these acute studies. (McGillion and Goldberg, 1973). It is quite likely that it has a similar action on mice and such an effect could be responsible for the initial diminished activity observed in these animals.

It may be however, that this is a direct effect of ALA by some other mechanism. This initial acute response in mice, produced effects similar to those seen at the chronic ALA treatment of the rats; so too a depressant action on motivity has been observed after the ALA synthetase inducer AIA (Biempca et al, 1967). On observation of the animals immediately after ALA however, they showed none of the characteristic changes in general reaction shown by the chronically treated rats. If the mice were observed openly they certainly showed a decreased tendency to move, but otherwise appeared to be quite normal.

Thirty minutes after administration of ALA to the mice, the animals were seen to demonstrate a significant increase in activity, compared with the

controls - a trend which was significantly maintained for up to 90 minutes after injection. It may be that if the initial depression of activity is due to a hypotensive response, that the only true direct action of ALA on activity is excitatory; the initial excitation being masqued by the response to a fall in blood pressure. An important feature of this response was that the activity after 30 minutes was not only significantly greater than the corresponding same-time control values; but also greater than the zero time control values.

Thus the fact that ALA causes an initial depression in activity means that if the mice returned to normal activity after 30 minutes and this normal activity was compared with the expected decrease in the activity of the control animals at this time, then an apparent but unreal increase in activity of the ALA animals would occur. The fact however, that the ALA treated animals show greater activity after 30 minutes than the control animals at zero time indicates that this effect is a real action of ALA. At this time after injection, the animals appeared to have normal withdrawal and righting reflexes and showed no obvious signs of limb paralysis; thus it is possible that the effect seen here is a central one.

Once more the effect is seen to be relatively short lasting having totally disappeared 120 minutes after injection.

The similarity in the response to ALA by the normal animals and the animals pretreated with ethanol indicated that at least in this experimental system - there is little interaction between the effect of ALA and ethanol or the chronic effects of its administration. The main difference in response - viz: that the ALA-ethanol animals were not significantly different from the controls 60 and 90 minutes after injection - is probably accounted for by the small numbers of animals in these ethanol groups, compared with the normal groups.

The chronic ALA treatment in the rats can be seen to produce quite a definite decrease in the general activity of the animal as demonstrated by the high significance of the results of the quantitative studies. The significance of the 'time motionless' results, demonstrates that the total activity of the animal was decreased and not only locomotor activity. The low rate of rearing could be interpreted as a decrease in 'inquisitiveness' rather than as the result of muscle weakness or some related phenomenon.

As interesting as the effects of ALA which are expressed by the quantitative studies - was

the effect on the behaviour of the animals as seen when merely observed, or mildly stimulated. It is difficult to describe such empirical observations without invoking some element of anthropormorphism; however these animals showed signs which, if they were seen in a human subject, would be indicative of social withdrawal. Thus they were highly unreactive and unresponsive towards their environment yet they appeared to be totally aware of environmental stimuli as illustrated by their response to clapping. There were some similarities between the responses of these animals and the responses of those treated with AIA referred to in the introduction. Thus in each case ataxia is present. Marcus et al (1970) found that this occurred in rats a few minutes after injection of AIA, a finding consistent with that reported for the 'zero time' mice group above, but not consistent in terms of time, with the chronic ALA group nor in terms of response when compared with the reaction of the mice to ALA after about 30 minutes, when they were hyperactive.

So too the ataxia produced with AIA initially, disappeared after a few days, indicating the development of tolerance; whereas the effect of ALA in rats was not dramatically evident initially and gradually developed over the weeks the animals were exposed to it. The fact that Marcus et al (1970) observed no behavioural change with ALA, was probably

because they administered it only acutely, and the behavioural parameters used were not sufficiently sensitive to detect the changes which one would expect to occur on the basis of the acute studies on mice described above.

The difficulties involved in relating and comparing the effects of AIA and ALA - as well as any hypothetical considerations which may arise from such a comparison - have already been considered (Section I). Thus, although the similarities of certain aspects of the responses of experimental animals to these substances are of interest - inherent in any interpretation of the results on a 'similar action' basis is the acknowledgment that how they are exactly related is unknown.

A perhaps pertinent observation to this study with respect to the kryptopyrrole studies described below is the fact that Sohler et al (1970) have shown kryptopyrrole to have a central depressant action in experimental animals. If, as is suspected, ALA is biotransformed into an active substance like this it could account perhaps for the effects seen in the chronic ALA animals. This of course, must remain purely speculative at the moment.

By way of summary then: these experiments demonstrate that ALA is pharmacologically active in that it can alter the behaviour of experimental

animals. In acute experiments in mice, it has an initial depressant action on spontaneous activity immediately after injection, followed by enhancement of spontaneous activity which lasts until at least 90 minutes after injection. When chronically given to rats, parameters of activity, viz: spontaneous activity, time motionless and rearing, were all significantly depressed after administration of ALA for four weeks.

Introduction

As indicated in Section I, mental illness which is often severe enough to lead to the patient being certified is a relatively common characteristic of AIP. A correlation is likely between the central neuropathy described in porphyria (Gibson and Goldberg, 1956) and certain of these psychological aberrations. Recently however, more attention has been given to the study of the biochemical abnormalities occurring in mental illness and it is possible that some biochemical lesion in AIP could be responsible for at least some of the central manifestations of the disease. Invariably such a line of thought leads to speculations about a centrally active metabolite occurring in mental illness and if this was the case in AIP it might be expected that such a metabolite would have some link with haem biosynthesis.

In the past decade or so many attempts have been made to implicate specific biochemical findings in patients, with the schizophrenia found in these patients. The literature on this subject is voluminous and is succinctly reviewed by Boulton (1971). Of particular interest with respect to AIP however, is the

implication of a disturbed porphyrin metabolism in schizophrenia (Husak and Durko, 1966; Price et al, 1959).

In 1961 Irvine described an Ehrlich's positive factor in the urine of a high percentage of psychotic patients. This factor was termed the 'mauve factor'. This 'mauve factor' was identified as the substance 2,4-dimethyl-3-ethylpyrrole (Irvine et al, 1969) commonly called kryptopyrrole. (fig.16). Another factor commonly associated with schizophrenia is trans-3-methyl-2-hexenoic acid (TMHA) a substance contained in the sweat of chronic schizophrenics and said to produce the characteristic odour of these patients (Smith and Sines, 1960; Smith et al, 1969). The origin and role of these substances in mental illness however, is as yet ill-defined.

Krischer and Pfeiffer (1973) have put forward a scheme by which they suggest that TMHA and kryptopyrrole are formed from a common biochemical intermediate (2-aminomethyl-3-carboxymethyl-4-2'-carboxyethyl-5-carboxypyrrole). In terms of acute porphyria an interesting feature of this hypothesis is that this proposed common intermediate is a substance resulting from the condensation of a molecule of ALA with a molecule of its precursor α-amino-β-keto adipic acid.

Figure 16.
The Chemical Structure of Kryptopyrrole.

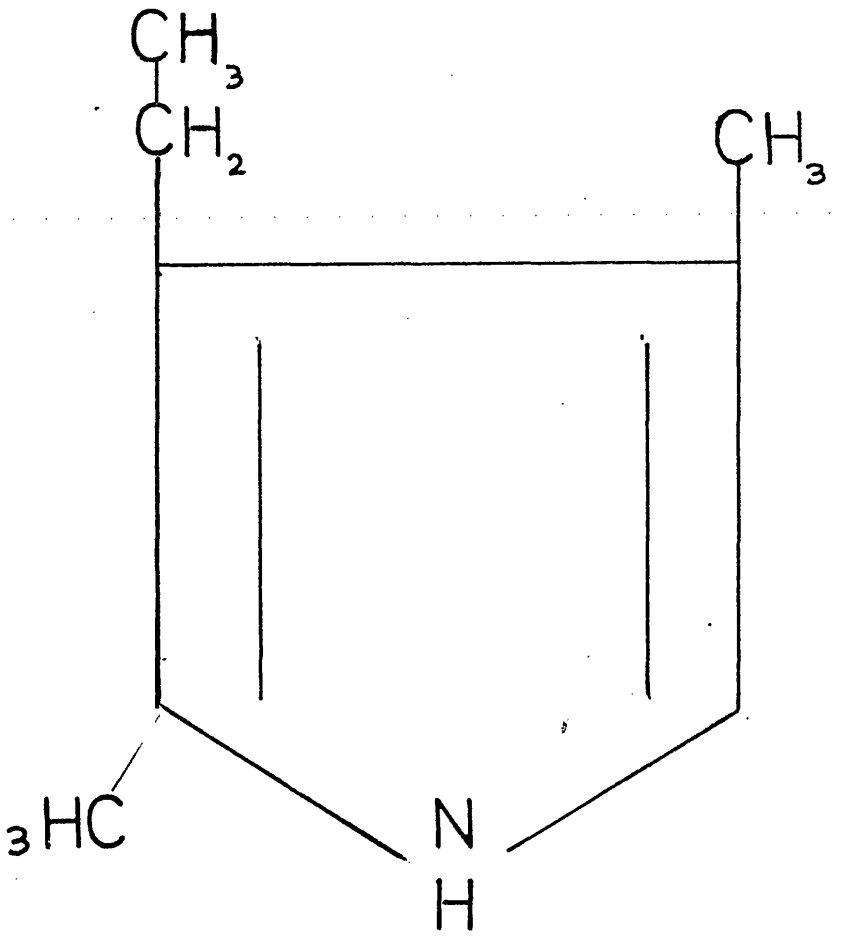
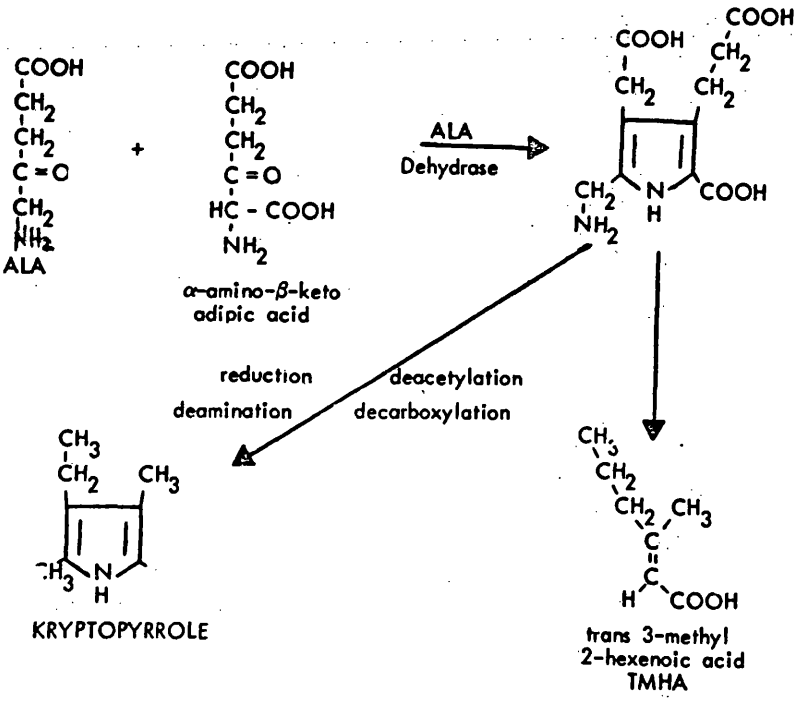


Figure 17.

A proposed mechanism by which krypto-pyrrole could be formed from ALA.
(After Krischer & Preiffer 1973.)



Possible formation of both KP and TMHA from delta-ALA

These workers propose that ALA synthetase does not function in the normal manner, leading to an accumulation of some of the proposed intermediate amino- -keto adipic acid. This then reacts with ALA under the influence of ALA dehydrase, to form the hypothesised precursor of kryptopyrrole and TMHA (Fig. 17). This then undergoes the reactions indicated forming these latter substances. Although Russel (1972) has proposed several schemes for the formation of kryptopyrrole from PBG by the action of an isomerase and a deaminase, Krischer and Pfeiffer point out that they believe ALA synthetase to be implicated in kryptopyrrole formation because they have observed that high doses of pyridoxine plus zinc decrease the output of kryptopyrrole in patients who normally excrete it. ALA synthetase of course, requires pyridoxal phosphate for decarboxylating activity.

Sohler et al (1970) demonstrated that kryptopyrrole had a central depressant action. Thus they produced a sedative effect in rabbits, by injecting kryptopyrrole intravenously. The relation between this action of the substance and its positive role in schizophrenia however, is obscure.

It was felt on the basis of these findings and considerations that it would be worthwhile to attempt to demonstrate the presence of urinary kryptopyrrole in AIP.

Methods: Estimation of Urinary Kryptopyrrole

The method used for the kryptopyrrole determination was based on that described by Irvine et al, (1961). 100ml of fresh urine was taken and mixed with 2g. activated charcoal (BDH). This was well stirred twice in the first five minutes after addition. This mixture was allowed to stand for two hours at room temperature and then suction filtered through a Buchner flask on two circles of Whatman paper containing the charcoal and then washed chromatographically with 80mls distilled water and the eluate discarded. It was then washed in a similar manner with 100ml reagent grade acetone, the eluate this time being collected and the charcoal discarded. The acetone was then evaporated to dryness 'in vacuo' at 30°C and the residue taken up in 3.3ml water as a suspension. This was then extracted three times, each time with 3.5ml ether (peroxide free reagent grade). This resulted in an aqueous phase and the combined ether phases ($\overset{\text{Fraction}}{\text{E + I}}$). To the aqueous phase (Aq.1) 2.2ml acetone was added and this was then extracted twice each time with 6.6ml of ether. This gave further aqueous phase (Aq.11) and combined ether-acetone phase (E + II). The fractions E + I, E + II and Aq.11 were flash evaporated at 30°C. Each residue was then taken up in 2ml acetone. One tenth of each resulting solution was applied separately to

Whatman 3mm chromatography paper, the spot size being restricted as far as possible to 10mm diameter. The spots were dried in cool air. A standard was run of a sample of kryptopyrrole. The solvent used for chromatography was IpRAM and the chromatography was run vertically for twelve hours. After thoroughly drying in still air at room temperature the high Rf part of each chromatogram was inspected under u.v. light. The paper was then dipped in Ehrlich's Reagent and the excess moisture blotted with filter paper. The colours, intensities and time of development of the spots was noted periodically during heating with hot air at around 55°C. Kryptopyrrole appeared as a well-defined spot with an Rf of 0.899 giving a purple to bluish mauve colour with Ehrlich's with perhaps a transient rose or blue colour. It was present in fractions E 1 and/or E11.

Reagents IpRAM:- This had the following composition:- 20 parts isopropanol, 1 part conc. ammonium hydroxide and 2 parts water v/v.

Ehrlich's Reagent:- 1g. of PABA was dissolved in a 1 : 9 mixture v/v of concentrated hydrochlorich acid and methanol.

Urinary kryptopyrrole estimations were made on three subjects with acute porphyria who were in remission: and in four normal, apparently healthy

subjects. In all cases the following estimations were also made:- urinary ALA and PBG concentrations, from a twenty-four hour sample of urine; and blood ALA concentration.

Results

The results obtained are shown in Table 8 . As can be seen from the Table, the chromatograms of the normal subjects gave no spot corresponding with the kryptopyrrole standard when stained. On the other hand all the chromatograms of the porphyric subjects showed a spot which corresponded with, and stained similarly to the kryptopyrrole standard. The Rf values of these spots and of the kryptopyrrole spot were 0.899; the same value as given by Irvine (1961).

	Mean Urinary ALA (mg.%) ± S.D.	Mean Urinary PBG (mg.%) ± S.D.	Mean Blood ALA ± S.D. (ug/ml)	Krypto-pyrrole Estimation
Normals	0.18 ± 0.10	0.11 ± 0.02	0.38 ± 0.05	All negative (4 subjects)
Porphyrics	2.17 ± 0.60	3.43 ± 1.84	0.70 ± 0.31	All positive (3 subjects)

TABLE 8

Discussion

The results obtained clearly demonstrate that in the samples studied, kryptopyrrole could be detected only in those subjects with acute porphyria. It is important to stress that these subjects were in remission, and showed no evidence of psychological abnormality: and none of them were on drugs of any kind. Although these studies used only a small sample of subjects, the findings are in agreement with those recently announced by Husak et al (1973). These workers investigated the appearance of kryptopyrrole in acute schizophrenics with positive porphyrin screening tests and in patients with acute porphyria and porphyria cutanea tarda. They found that not every porphyrin excreting schizophrenic was a kryptopyrrole excreter, but that all true porphyrics were.

These findings are evidence in favour of the postulate outlined above, that kryptopyrrole may be formed from a biochemical reaction involving ALA, and this could possibly occur when there is excess ALA available, as in AIP. The other obvious possibility is that kryptopyrrole might be formed by a biotransformation of PBG. Their chemical structures are similar, and such a reaction could quite conceivably occur when there is excessive production of PBG.

It is difficult, at the present stage of knowledge, to directly relate the detection and presence of kryptopyrrole to the psychological

manifestations of AIP. All that can be reasonably stated is that here is a biochemical factor, known to be present in a high incidence of schizophrenics, which is also present in subjects with AIP. This difficulty, however, is one encountered by all workers who are attempting to relate specific biochemical changes to changes in mood states.

In the case of porphyria, the problem is further complicated by the fact that there are other biochemical abnormalities in the disease, which are also present in mental illness. For example Price et al, (1959) have found a similar type of abnormal tryptophan metabolism in patients with porphyria, and patients with a variety of psychoses; while patients with neurological abnormalities demonstrated a tryptophan metabolism which was essentially normal. So too, changes in globulin patterns (Fessel et al, 1964) and other biochemical features, are common to both mental illness and AIP.

Much interest, however, has recently been given to the abnormalities in indole and pyrrole metabolism occurring in these diseases and it is with respect to the latter particularly, that the occurrence of kryptopyrrole in AIP subjects is of interest.

The fact that the subjects in the experiment described above, showed no signs of mental abnormality demonstrates that the presence of kryptopyrrole does not per se, elicit a schizophrenic reaction.

Indeed the substance has been found in completely normal subjects (Sohler et al, 1967). However the general consenus of informed opinion is that kryptopyrrole is, in many cases, a significant biochemical feature of schizophrenia and as such it is a substance the biochemistry of which should be elucidated. The fact that it is pyrrolic in nature and considering the psychological abnormalities and the abnormalities of the haem biosynthetic pathway in AIP, indicates that further studies on the production, metabolism and general properties of this substance in porphyric subjects, both when in remission and attack, might well contribute to our knowledge of the relationship between biochemical abnormalities and associated mental illness.

SECTION IV

PERIPHERAL STUDIES

TISSUE UPTAKE OF ALA

As well as determining the brain uptake of ALA, it was thought worth while to use the same techniques to determine ALA uptake into other tissues. These experiments were designed primarily to see if at similar concentrations to those known to occur in the plasma of acute porphyrics, ALA could penetrate into the various tissues. It was also decided to see how long ALA would remain in specific tissues, if it indeed did penetrate, after the blood concentration had returned to normal.

Methods

Fifteen male Sprague Dawley rats (200-250g) were given I.P. injections of 40mg/kg ALA in saline. The ALA had previously been spiked with C^{14} ALA and adjusted to pH 6-7 with molar sodium bicarbonate. The volume of injection was $\leqslant$0.5ml. As controls a further three rats were given I.P. injections of C^{14}-ALA only; this allowed ALA determination by isotopic methods but represented the injection of $<$10µg ALA.

Thirty minutes after injection three of the animals given ALA and the three control animals were injected into a tail vein with technecium (Tc99) labelled serum albumin. This was allowed to circulate for two minutes at which time the animals were stunned, exsanguinated from the throat, and samples of the following taken: heart, lung,

liver, kidney, spleen, gut (ileum), brain, mesenteric fat and blood. The blood ALA content was determined chemically by the method described by Haeger-Aronsen and the ALA content of the other tissues, independent of their blood ALA contents, was determined as described above for brain.

Using the same methods, ALA was determined in the heart, liver, brain and blood of three animals at times 3, 24, 72 and 192 hours after injection.

Results

The results obtained 30 minutes after injection are shown in Table 9. In the control animals it can be seen that the tissue ALA contents were either undetectable or very low. The highest was found to be in the blood where a mean content of 0.25µg/ml was obtained. After ALA was injected however, all the tissues had high ALA contents compared with their control levels. The relatively greatest increase was seen in the liver, and the lowest in the brain.

The change of ALA tissue content with time is shown in Table 10. The blood concentration of ALA remained elevated above normal for up to 24 hours after injection; at 72 hours it had returned to normal. The liver, heart and brain tissues however, maintained ALA levels greater than normal even after the blood level had returned to normal. Thus at 72 hours all these tissues showed ALA levels greater than the

TABLE 9

MEAN TISSUE ALA CONTENT (μg/g) 30 MINUTES AFTER I.P. INJECTION

	Control (C^{14}ALA) only	Test (40mg/kg ALA I.P.)
Blood	0.25 ± 0.15	26.66 ± 7.43
Brain	0.046 ± 0.04	1.07 ± 0.41
Heart	0.00	23.53 ± 3.16
Liver	0.06 ± 0.04	162.53 ± 60.71
Kidney	0.21 ± 0.10	23.33 ± 3.70
Spleen	0.00	74.10 ± 17.26
Gut	0.00	72.97 ± 3.48
Fat	0.00	54.70 ± 10.30

Each result is expressed as the mean of 3 experiments. ± S.D.

TABLE 10

Tissue ALA (µg/g)	TIME AFTER INJECTION IN HOURS 3	24	72	192
Blood	3.80 ± 0.51	0.83 ± 0.18	0.30 ± 0.10	0.27 ± 0.06
Brain	0.49 ± 0.10	0.14 ± 0.05	0.12 ± 0.05	0.04 ± 0.01
Heart	2.62 ± 0.54	1.03 ± 0.10	1.22 ± 0.35	0.50 ± 0.10
Liver	82.73 ± 7.16	3.49 ± 0.57	3.18 ± 0.40	0.38 ± 0.91

Each result is expressed as the mean of 3 experiments ± S.D.

control values, and at 192 hours after injection, although the brain content was normal, the heart and liver continued to do so.

Discussion

These results show that in rats exposed to an equilibrium blood concentration of ALA similar to that found in an acute porphyric attack, there is passage of ALA in to the various tissues studied. The fact that the ALA content of the liver, heart and brain can remain greater than the control contents, even when the blood concentration has returned to the control values, suggests that in these tissues at least ALA is not in a diffusion equilibrium with the blood, but is either bound or actively maintained there.

As with the studies of the passage of ALA into the brain, the tissues were exposed to ALA acutely. In acute porphyria however, the tissues will be exposed to elevated concentrations of ALA which although they may be less in magnitude, will certainly persist for a greater time than those used here. Thus it seems fair to conclude that in AIP, the ALA content of the tissues will be elevated.

The studies on the persistence of ALA in the tissues with time did not indicate whether the decrease with time was due to metabolism or diffusion out of the tissue of ALA. As with the

brain ALA experiments chromatograms were run to demonstrate that the C^{14} activity was due to ALA and not to metabolic product of it, and indeed a high percentage of the activity was found which corresponded with the ALA standard. Irrespective of the nature of the fate of the ALA in the tissues, the important fact is that it can remain there against a concentration gradient and is therefore actively maintained there, or as is more likely, bound to some other molecule; the free ALA being released by the experimental precedures for determination.

It has been suggested that ALA can chemically bind to certain intracellular substances, the resultant complexes being capable of enzyme inhibition (Kosower and Rock, 1968). This possibility is of interest when one considers that these results demonstrate that ALA is taken up and probably bound, in the main tissues which show signs of clinical abnormality in AIP viz: gut, heart, liver and brain.

It is possible that an action of some complex of ALA or one of its in vitro actions referred to previously, either in the tissues themselves or on the nerves, which serve them, could account to some extent for the abnormalities shown in the disease.

CARDIOVASCULAR STUDIES

Introduction

The cardiovascular abnormalities of AIP have been described above Section I. Although there is occasional hypotension the most common cardiovascular features of the disease are tachycardia and hypertension. Several theories have been advanced to account for the aetiology of these manifestations, such as direct toxic involvement of the cardiovascular system (Waldenstrom, 1937); stimulation of the sympathetic nervous system or of thyroid secretion (Vannoti, 1937) and 'escape' hypertension caused by polyneuritis of haemodynamic controlling nerves (Kezedi, 1963).

A most informative and illuminating study on the pathogenesis of the cardiovascular manifestations of AIP has been carried out by Schley et al, (1970) who investigated hormonal and renal factors in four female patients during the acute and latent stages of the disease. Their findings can be summarised as follows: During the acute episodes all subjects showed systolic and diastolic hypertension with tachycardia and a distinct elevation of catecholamine (16% adrenaline; 84% noradrenaline) excretion. There was also a transient increase in vanillinmandelic acid excretion. A positive correlation existed between hypertension, heart rate and catecholamine excretion.

Administration of the β- receptor blockers

Transicor or Inderal reduced the blood pressure and heart rate to normal. On withdrawal of these drugs however, these parameters increased once more.

At no time during the period of study was aldosterone excretion or plasma renin concentration elevated. All four patients showed a reversible impairment of glomerular filtration, PAH clearance, and phenol red excretion during the acute phase, with or without retention of substances normally excreted in urine; there was however, no clear association between these changes and the severity of the cardiovascular signs. The authors conclude from this that it is unlikely that the hypertension associated with the acute phase of AIP is of a renal origin and point out that tachycardia is not a clinical feature of renal hypertension.

In all the patients it was found that there was excessive urinary excretion of total porphyrins, PBG and ALA. These levels of ALA and PBG vastly exceeded the normal values during the acute phase, and persisted above the norm during the latent phase. The total porphyrins however, were either normal or only slightly greater than normal during the latent phase.

The authors conclude that the increase in catecholamine excretion in these subjects is due to enhanced sympathetic tone which causes release of catecholamine from the sympathetic nerves and the adrenal medulla. They stress however, that it is not certain that the excessive catecholamines themselves cause the

hypertension and tachycardia.

These findings certainly suggest that there is the likelihood of a causal relationship between sympathetic nerve function and/or catecholamine secretion and metabolism, and the cardiovascular manifestations of acute porphyria. Again in the above studies there is found an elevated urinary excretion of ALA and PBG and the question presents itself - does ALA have any effect on sympathetic nerve activity or catecholamines? The possibility also exists of course that ALA is itself active on the cardiovascular system directly, or that it can affect it in some way unassociated with the sympathetic nervous system.

It was decided therefore to investigate the effect of ALA on various pharmacological preparations which give information of a cardiovascular nature. Three preparations were used: The anaesthetised rat blood pressure preparation, the pithed rat blood pressure preparation and the 'in vitro' isolated perfused rabbit ear artery preparation.

Anaesthetised Rat Preparation - a)

Male Sprague Dawley rats (200-250g) were used in these studies. In almost every case the anaesthetic used was sodium pentobarbitone (Nembutal, Abbot Laboratories). In a few experiments, however, other anaesthetics were used in order to ascertain whether or not the anaesthetic used affected the results obtained with ALA. It was found that none of the following changed the response to ALA which was initially found under Nembutal anaesthesia:- urethane 1.5g/kg αchloralose-urethane mixture, $\frac{50}{500}$mg/kg α chloralose 100mg/kg.

Method

The animal was given an intraperitoneal injection of sodium pentobarbitone 60mg/kg in a volume of 0.2-0.25ml. Anaesthesia was tested for by observing the absence of withdrawal and corneal reflexes. This usually occurred twenty to thirty minutes after injection. If anaesthesia had not developed in this time a further 3mg of anaesthetic was administered. With the dose of pentobarbitone used there was rarely any evidence of cyanosis or severe respiratory depression.

When anaesthesia was developed the rat was laid on its back and pinned through the limbs on to a dissecting board on a small heated operating table. A piece of skin was cut from the neck and the trachea exposed by blunt dissection. A small

cut was made in the trachea and a tracheal cannula inserted and tied in place. Any tracheal mucous was removed by means of a thin plastic tube attached to a syringe.

A carotid artery was then dissected out, freed from its associated nerve and connective tissue and tied off towards the head end. A bulldog clip was then clipped on the artery as far towards the heart as possible. A thread was pulled beneath the artery between the tie and the clips. On this part of the artery a slight cut was made sufficient to allow the entry of the end of a fine pair of forceps. The tip of the forceps was entered into the lumen of the artery and this was used to guide in a fine heparin/saline filled arterial cannula. When this was entered the forceps were removed carefully and the cannula tied in place. The free end of the cannula was then attached via saline filled rubber tubing to a pressure transducer (Devices type 2ST) which displayed blood pressure on a Devices M2 chart recorder. The bulldog clip was then removed and the resting blood pressure recorded. The tracheal cannula was then attached to a respiratory pump and artificial respiration effected. This was maintained throughout the experiment.

A piece of skin was cut from a hind leg and the femoral vein exposed by blunt dissection. This was dissected free from the associated artery,

nerve and connective tissue. Two threads were pulled underneath and left free. A bulldog clip was clipped in position at the body end of the vein which was then engorged with blood by stroking gently towards the clip. A thread was then tied round the foot end of the vein. In a similar manner to the artery the vein was snipped and a venous cannula inserted and tied in place with the remaining thread. Attached to the venous cannula was a saline filled rubber tube which ran through an injection block to a constant volume burette filled with saline. By this means drugs could be injected into the rubber tubing held rigid in the injection block and hence washed with saline into and through the venous cannula into the vein.

The volume of injected drug plus saline was kept constant as far as possible to 0.3ml total. In most animals such an injected volume had little effect on blood volume as observed by the effect on blood pressure. So too, where possible, the drugs were injected at regular intervals usually every three minutes. Where the response of the drug was greater than this, however, the interval was accordingly altered. When it was desired to infuse a drug constantly while injecting also, the other femoral vein was also dissected free and cannulated, the cannula in this case being attached to a slow injection apparatus containing a

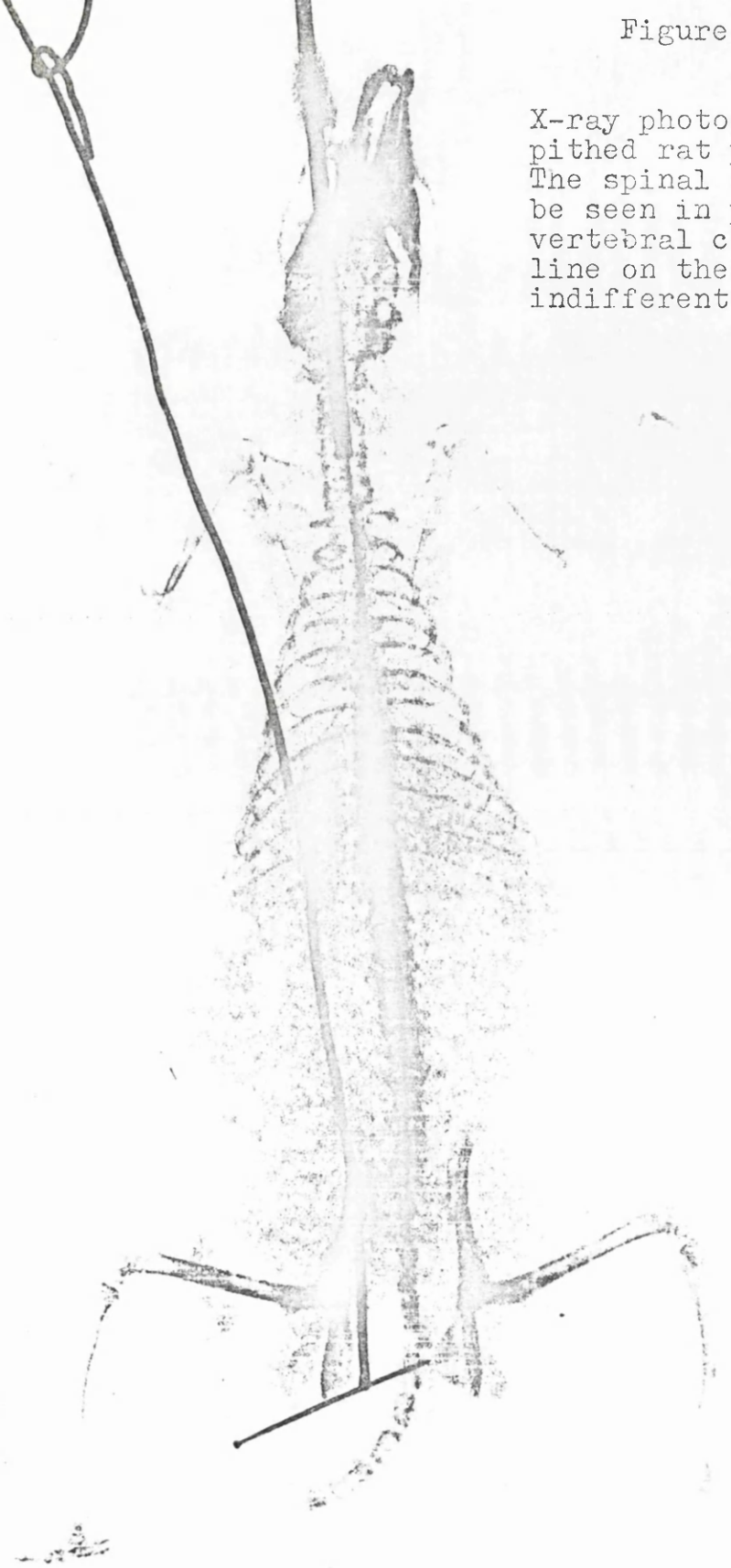
syringe of the required solution. In four rats, ECG traces were run during the experiment.

The Pithed Rat Preparation (Fig.18).

The method used in preparing the pithed rat was basically that described by Gillespie et al, (1970). A male Sprague Dawley rat (250-200g) was anaesthetised with trichloroethylene (Trilene), a trachea cannula was inserted and the animal was then pithed by inserting a short hollow steel tube (13 S.W.G.) through the orbit and the foramen magnum and down into the spinal column to the level of the sixth cervical vertebra. Through this tube was passed a Teflon tube (0.16mm O.D.) which acted as an insulator and through this a fine steel tube (26 S.W.G.) which was extruded at the sacral end and so completed the pithing. A steel rod (13 S.W.G.) was inserted behind the skull and pushed down between the vertebral column and the skin to act as an indifferent electrode.

The level of stimulation was determined by varying the depth of insertion of the Teflon tube. The number of segments stimulated was determined by varying the length of steel electrode exposed in the vertebral canal. According to Gillespie et al, (1970) the length of the vertebral column varies little, and so in pithing the animals a standard length of Teflon tubing and steel electrode was used, the position and length of the stimulating

Figure 18.



X-ray photograph of the pithed rat preparation. The spinal electrode can be seen in place in the vertebral column. The dark line on the left is the indifferent electrode.

electrode exposed in the column being determined from the length of Teflon and steel protruding from the skull.

Immediately after pithing, the tracheal cannula was attached to a respiratory pump and artificial respiration effected. The animal was then prepared for blood pressure recording, and injection of drugs as described above for the anaesthetised animal. An injection of pancuronium (1mg/kg) was given to abolish any muscle twitching resulting from stimulation.

Stimulation of the sympathetic outflow opposite T7-9 was carried out to give an increase in blood pressure which was specifically a result of vaso-constriction. Similarly stimulation of the sympathetic outflow opposite C7-T1 was used to give an almost specific cardio-accelerator response.

Histamine Release

ALA was tested for potential histamine releasing ability by two methods. The first was self-administration of 0.2mg ALA in 0.02ml saline by subcutaneous injection, and observing for the 'Triple response' characteristic of histamine release (Lewis and Grant 1924). As a control, 0.02ml saline was administered simultaneously.

The second method was based on that described by Fawcett (1954) in which a solution of the suspected histamine releaser is injected into the

peritoneal cavity of an animal. This is then left for sufficient time for the suspected drug to act on the histamine containing mesenteric mast cells, and then the solution is removed and assayed for histamine. In this case three groups of four rats were injected intraperitoneally with either 20ml saline, 20ml of saline containing 1µg/ml of the histamine releaser 48/80, or 20ml saline containing 1mg/ml ALA. The rats were then left for thirty minutes at which time they were killed by a blow on the head. The abdomen was cut open, and the remaining intraperitoneal fluid carefully removed by syringe. The fluid was then assayed for histamine content on the isolated guinea pig ileum preparation.

Capillary Permeability

To see if ALA increased capillary permeability, the method described by Feldberg & Miles (1953) was adopted. Three groups of two rats were anaesthetised with Nembutal. Each rat was then given an intravenous injection of Evans blue. This was allowed to circulate and then a further intravenous injection was given of either saline, histamine (100µg) or ALA (10mg). An increase of capillary permeability was looked for by examining the various tissues for blueing, where the dye had escaped through the capillary walls.

TABLE 11

BLOCKING AGENTS USED IN THE ANAESTHETISED RAT PREPARATION

Blocker	Normal Effective Dose	How Block Was Established
Phentolamine	1mg/kg	Abolished pressor response to injected catecholamines.
Propranolol	0.5mg/kg	Block of cardiac response to isoprenaline.
Atropine	1.2mg/kg	Block of depressor response to acetylcholine.
Hexamethonium	1.2mg/kg	Abolished pressor response to injected nicotine.
Mepyramine	200-500µg/kg	Abolished depressor response to injected histamine.

Perfused Rabbit Ear Artery

An angora rabbit of either sex ($1\frac{1}{2}$-3kg) was killed by an injection via an ear vein of a lethal dose of sodium pentobarbitone. This normally required an injection of some 120mg pentobarbitone for the smaller animals, with a correspondingly greater dose for the larger ones. The animals died by this method quietly and without obvious excitement.

Immediately after death the ears were removed and shaved. The layer of skin above the central ear artery was slit down each side of the artery and cut away by stroking gently with a scalpel blade, while gently pulling it away from the surface of the ear. This exposed the artery surrounded by connective tissue. Next, using a binocular dissection microscope, the artery was cleared of surrounding connective tissue, cannulated with a Krebs-filled cannula, and the cannula tied in place.

By means of the microscope the artery was followed up its length and any branch arteries were either tied off, or if they were small enough, sealed by pulling between two pairs of forceps. This completed, the required length of artery was cut off and removed with the cannula intact, to an artery bath containing Krebs solution at 37°C being

TABLE 12

COMPOSITION OF KREBS SOLUTION

	milli molar	Grams per litre
Sodium chloride	113.00	6.60
Potassium chloride	4.70	0.35
Calcium chloride (dihydrate)	2.50	0.49
Potassium dihydrogen phosphate	1.20	0.16
Magnesium sulphate (septahydrate)	1.20	0.59
Sodium hydrogen carbonate	25.00	2.10
Glucose	11.50	2.10

continuously gassed with 5% CO_2 in O_2. The tissue was kept moist with warmed Krebs solution during the whole of the dissection procedure.

The artery was perfused with Krebs at a pressure of 80-100mm.Hg. by means of a 'Watson Marlow' constant flow inducer. Perfusion pressure was monitored and displayed in a similar manner to the rat blood pressure. Stimulation of the periarterial sympathetic nerves was by means of ring platinum electrodes surrounding the artery. The method of stimulation was as follows:- using a frequency of 10 or 20 Hertz, a voltage response relationship was determined. A suitable supramaximal voltage was obtained, this usually being two times plus 50% of the voltage giving a threshold response. Using this supramaximal voltage a frequency response relationship was determined.

When noradrenaline was injected, it was injected directly into the bath: and ALA was both injected into the bath and present in the perfusing medium. At the concentrations of ALA used, the Krebs was found to be an adequate buffer.

In the majority of cases the experiment took the following form:- A frequency response relationship was obtained by stimulation, for fifteen seconds every three minutes, at different frequencies using a supramaximal voltage. A dose response relationship to noradrenaline was then established. These

frequency response and dose response relationships were repeated until at least two sets of reproducible responses were obtained. ALA was then added to the bath, and the tissue perfused with Krebs containing ALA. The dose and frequency responses were then repeated.

Results

a) Anaesthetised Rat

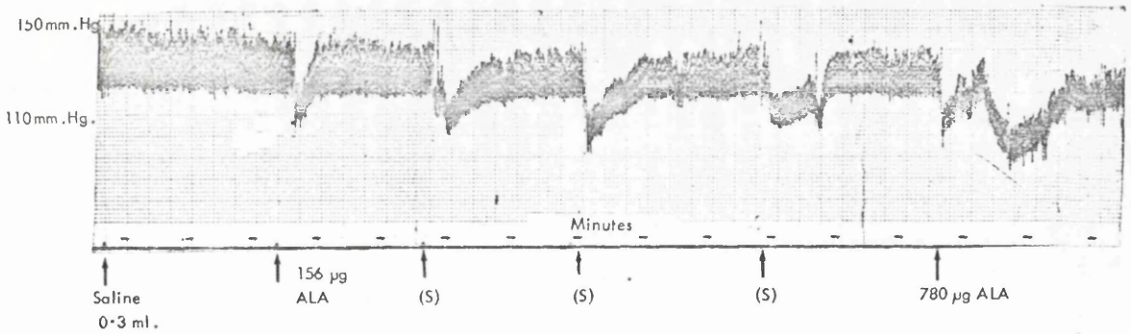
A single dose of ALA was found to produce a fall in blood pressure in the anaesthetised animal (fig. 19). This response was usually elicited with a dose of 500-1,000µg ALA salt; this produced a fall of some 10-15mm Hg, which gradually returned to normal over 1-5 minutes. Smaller doses were, however, effective, e.g. a dose of 125µg elicited a fall of 8mm Hg in one animal, the pressure returning to normal after about one minute.

The fall in blood pressure in response to ALA was usually immediate, and if associated with a decrease in heart rate, this occurred only in the initial stages. At the new pressure, and as the pressure returned to normal, the heart rate was normal. It was not associated with any abnormalities on the ECG traces which were run on four separate occasions.

In response to a single injection of ALA,

Figure 19.

This trace shows the effect of ALA on the anaesthetised rat blood pressure. On the trace 'S' stands for same dose i.e. 156 ug. of ALA. This animal was particularily sensitive to the action of ALA.

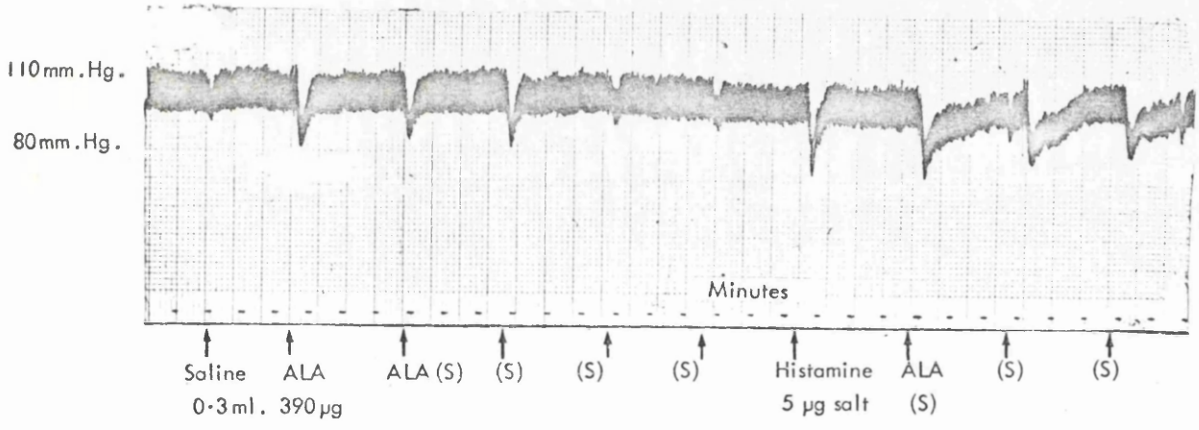


EFFECT OF ALA ON RAT B.P.

Figure 20.

In this figure it can be seen that the response to ALA is decreased with continuous administration. This process, however, is seen to be reversed by histamine.

On the trace ' S ' represents same dose, i.e. 390ug., ALA.



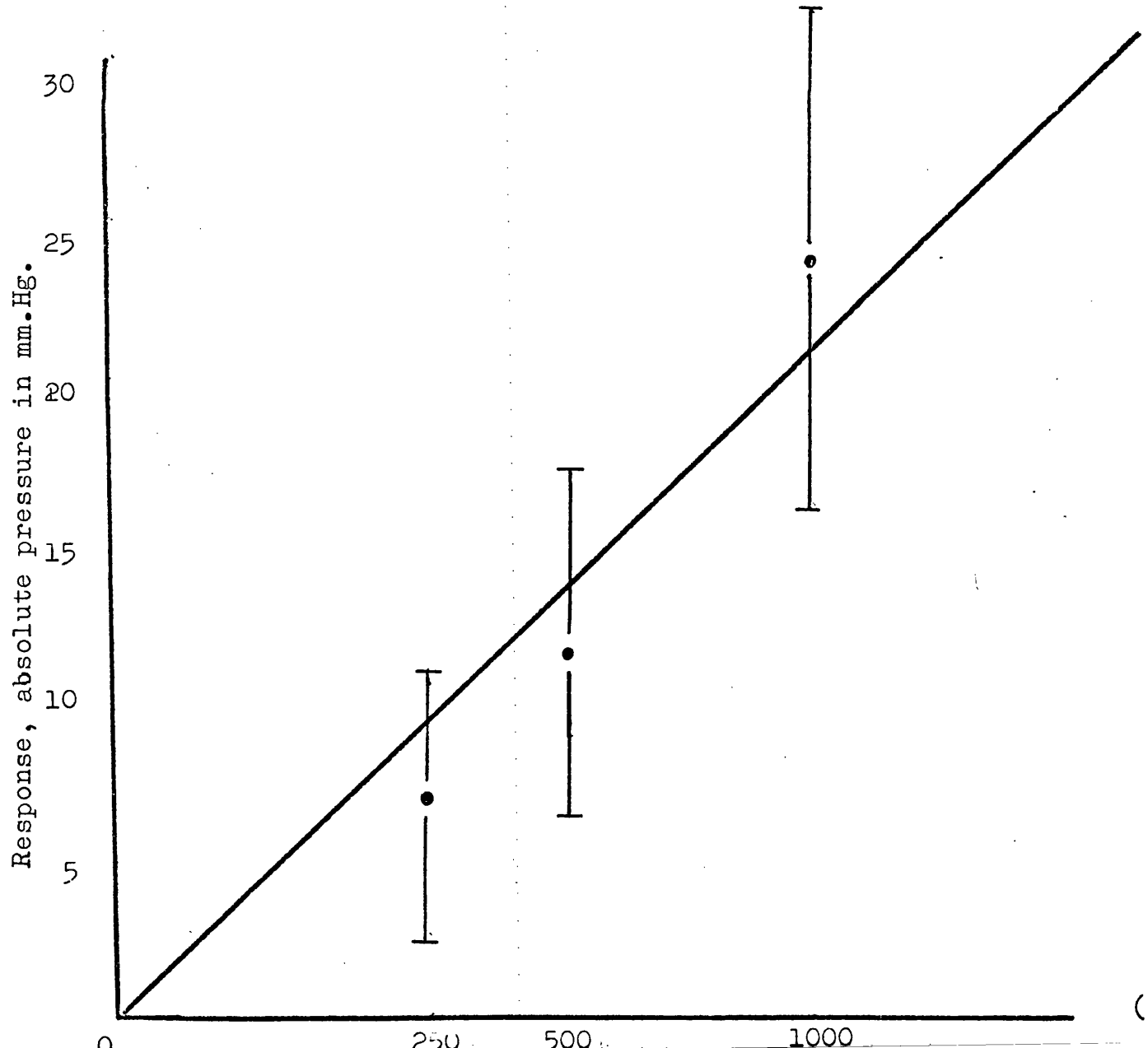


Figure 21.
Dose response relationship for ALA on the rat blood pressure. Each point is the mean ± S.D. of 12 initial responses, in 12 different animals.

there was a great deal of variation within individual animals. Thus although all animals showed a decrease in blood pressure in response to ALA, the response to a repeated dose in any given animal was often inconsistent. The usual finding was that the first few doses produced responses of increasing magnitude. Repetitive administration however, often produced a decreased response to ALA, and occasionally no response at all. (fig.20). To prepare a dose-response curve, therefore, the means of the different responses produced by doses of ALA in a number of animals was taken (fig.21).

When ALA was given continuously by intravenous infusion, a maintained fall in pressure was evident, which gradually returned to normal when the infusion was discontinued. Thus, for example, an infusion of 100ug ALA/min. produced a fall in blood pressure of 15mm Hg after $1\frac{1}{2}$ mins. This was maintained, and the infusion was stopped after ten minutes. The pressure remained at this level for a further five minutes, and then gradually rose and returned to normal over the next five minutes.

If a suitable acute response was obtained to ALA and then either adrenaline or noradrenaline were administered, there was an immediate reversal

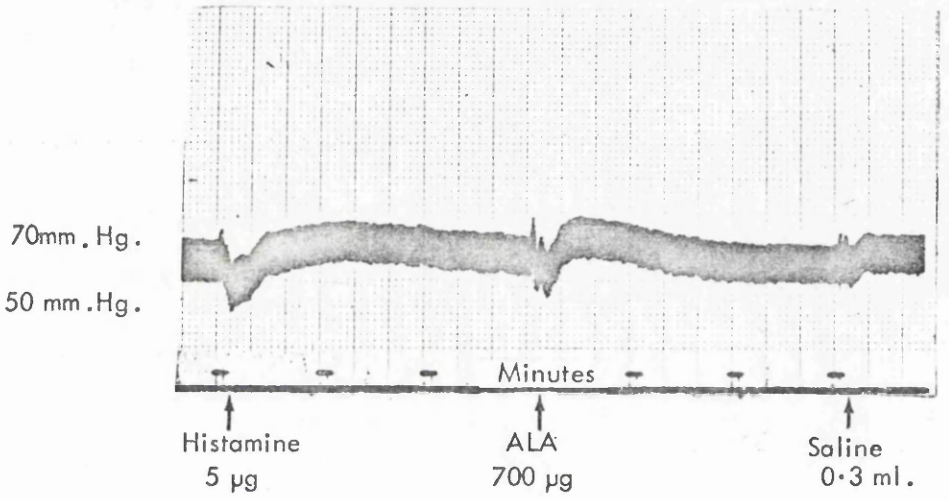
of the ALA response and, after the pressor response of the catecholamines, the blood pressure returned to normal.

Dose-response relationships were determined for noradrenaline in the absence and presence of ALA in the same animals, and no significant difference was found. Similarly intravenous infusions of catecholamines were given before and after ALA, and again no significant differences were found. The response to injected nicotine after atropine was similarly unaffected by the presence of ALA.

The hypotensive effect of ALA was apparent after blocking doses of the following: hexamethonium, propranolol, phentolamine and atropine. (Fig.22). The response was decreased after these drugs, but so too was the resting pressure. Qualitatively the response to ALA was similar to that of histamine, although histamine was more effective by a factor of about one hundred. A dose of mepyramine, sufficient to block a response of histamine of similar magnitude to that produced by ALA, decreased the latter by 20% but did not block it. (Fig. 23).

The tests described above, designed to test for endogenous histamine release by ALA, were negative. Thus ALA did not produce a 'triple response' when self-administered subcutaneously, nor was there any significant histamine release from the intraperitoneal

Figure 22.
In this trace it can be seen that ALA can still produce a fall in B.P. after administration of these blocking agents.



HISTAMINE AND ALA AFTER PHENTOLAMINE
PROPRANOLOL AND HEXAMETHONIUM

Figure 23.

On this line drawing it can be seen how, after blockade of the response to histamine by administration of mepyramine, ALA could still cause a fall in B.P.

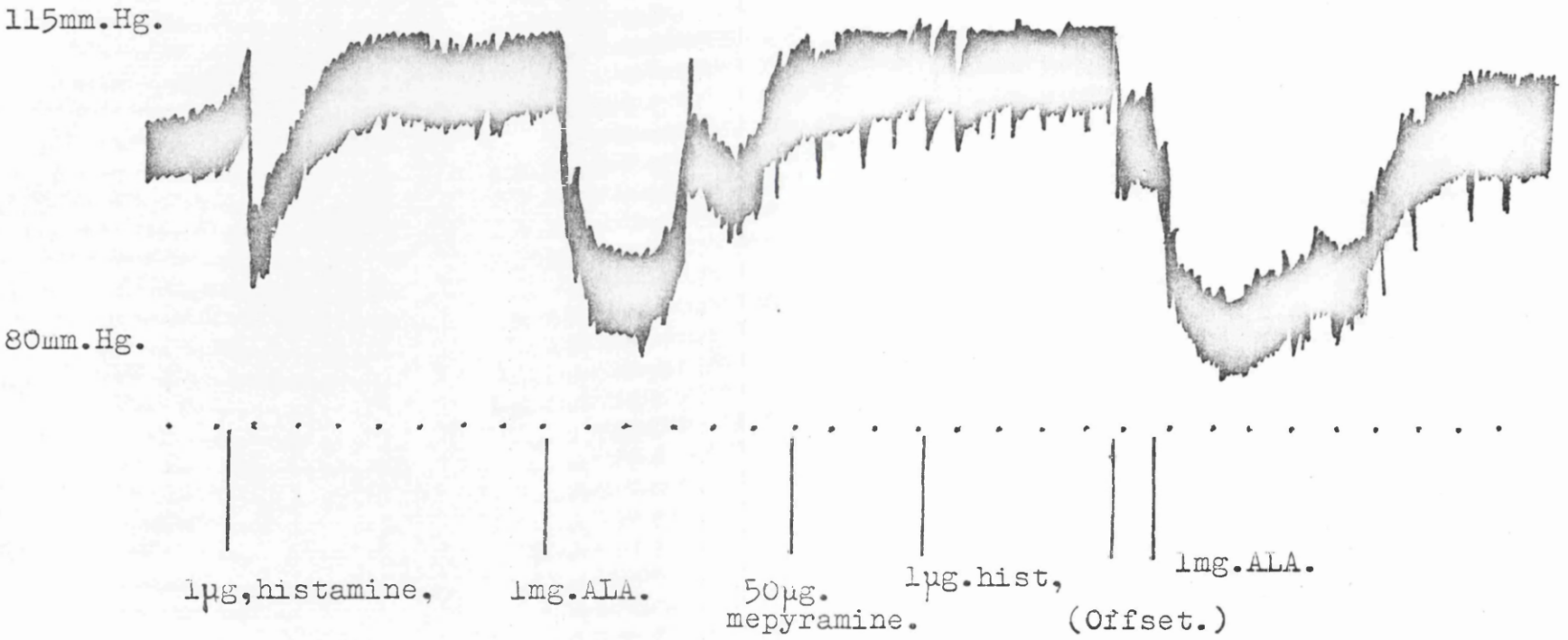
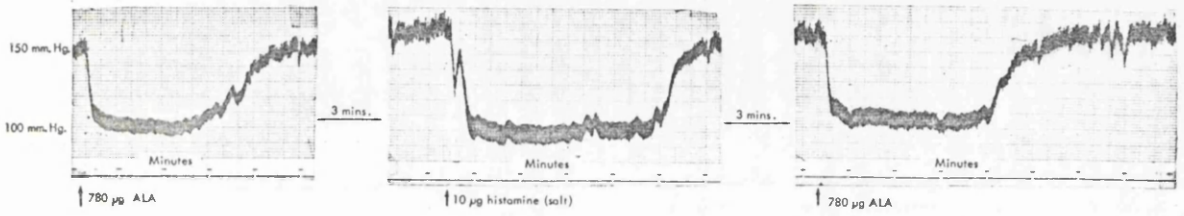


Figure 24.

This figure demonstrates the similarity in response to ALAand histamine. This animal was particularly sensitive to the actions of both these agents.



cavities of rats by ALA, although 48/80 did produce massive histamine release.

An interesting observation was that histamine appeared to potentiate the response to ALA, and indeed appeared capable of making the animal responsive again to ALA, after the response had decreased. Thus on most occasions the response to ALA was considerably enhanced, by a preceding injection of histamine. (fig. 20). So too, animals which were particularly responsive to the vasodepressor action of histamine, were also particularly responsive to ALA (fig. 24).

When anaesthetised animals were made hypersensitive to vasopressin by a previous injection of hexamethonium, the pressure response of vasopressin was antagonised by ALA. A similar antagonism was evident in animals not given hexamethonium; however in this case the response to vasopressin was slight and the antagonism by ALA consequently smaller and less dramatic. *

b) Pithed Rat

In the pithed animal, a decrease in blood pressure could be ascertained after ALA. Due to the low resting pressure in these animals the fall was small in magnitude; it was however, similar to that produced by an equi-potent dose of histamine and easily discriminated from a 'saline' response. (fig. 25) This response to ALA was also evident

*The capillary permeability experiments were positive and are described below.

Figure 25.

The effect ofALA on the pithed rat blood pressure. It can be seen from this figure, that ALA can decrease theB.P. in the pithed animal.

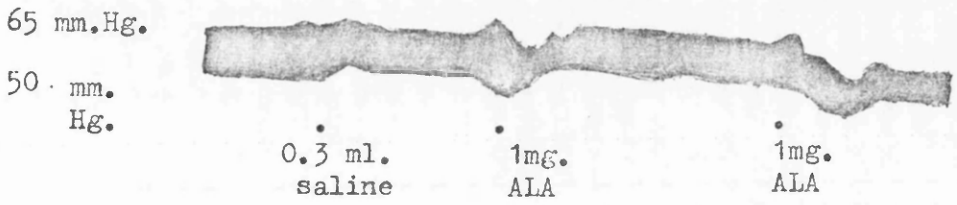
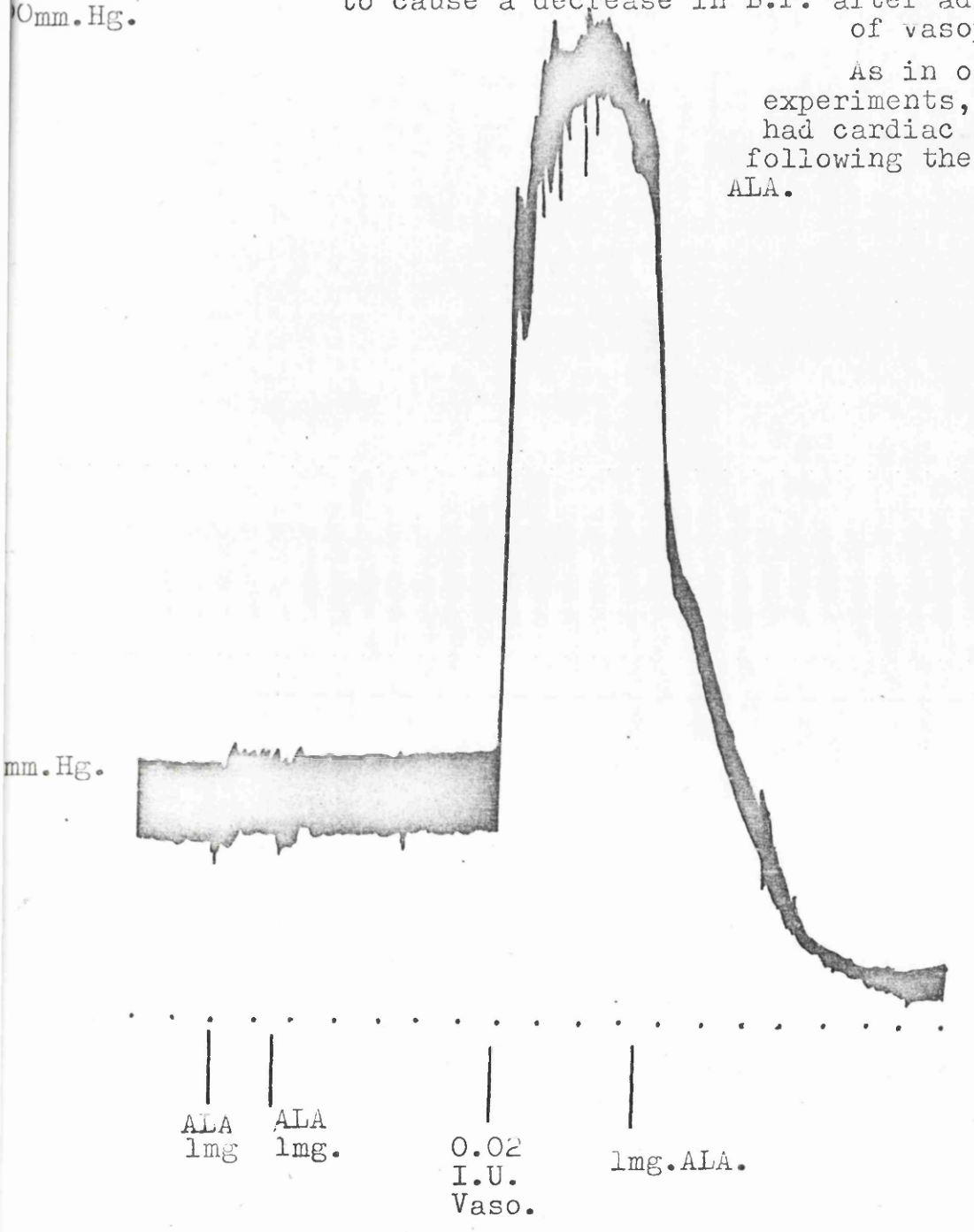


Figure 26.

In the pithed animal, ALA is seen to cause a decrease in B.P. after administration of vasopressin.

As in other similar experiments, the animal had cardiac arrest following the response to ALA.



(N.B. In this experiment the pressure was displayed on a ' Servoscribe' chart recorder.)

after α receptor block by phentolamine.

Stimulation of the spinal electrode in the region of C7-T1 caused an increase in heart rate as measured by increasing the speed of the chart recorder and counting the pulse rate. Thus in response to stimulation of 10 Hertz the heart rate increased from four beats per second to six beats per second. This observation was repeated, and then ALA was given (4mg/kg). When stimulation was again carried out the heart responded as before, with no change in the presence of ALA.

Similarly the blood pressure was increased by almost specific vasoconstriction by stimulation of the spinal electrode in the T7-9 region. No cardio-accelerator response was evident and the blood pressure increased from 42mm Hg to 115mm Hg after stimulation of 20 Hertz. Again this response was reproduced and then ALA (4mg/kg) was given. Once more no change in the response to stimulation occurred after ALA. In three separate pithed animals an injection of vaso-pressin was given. This produced a large response as these animals are particularly sensitive to the pressor action of the drug (Goodman and Gilman 1971). This response to vasopressin was reproduced and then during the progress of a further response ALA was given. ALA reversed the elevated pressure produced by vasopressin, and in two animals out of the three,

this effect was followed by cardiac arrest (fig. 26).

If ALA was given before an injection of vasopressin, no obvious effect on the response was apparent.

The response of the pithed animal to nicotine in the absence and presence of ALA was also determined, and again ALA had no significant effect.

Discussion (a and b)

Blood Pressure Experiments

The outstanding effect of ALA in the rat is to lower the blood pressure an effect inconsistent with a role for this porphyrin precursor in the hypertension of AIP. An elucidation of the exact mechanism of ALA in lowering blood pressure was made more difficult to analyse by the inconsistency of the size of the response.

Nevertheless it was clear that as ALA was still capable of producing this response after hexamethonium in the anaesthetised animal, and in the pithed animal, that at least part of its action is not mediated either by affecting ganglionic transmission or centrally.

So too the presence of its effect after propranolol suggests that it does not act by an inhibitory action on β adreno receptors; similarly the persistence of the ALA effect after phentolamine, and the absence of any effect of ALA on the nicotine response and the responses to stimulation of the sympathetic outflows to the blood vessels, suggest that it does not act by

inhibition of α adrenoreceptors, whether the latter are stimulated by sympathetic innervation or only by circulating catecholamines. That ALA is active after atropine indicates that its hypotensive action is not mediated by a cholinergic mechanism or by an effect on/or like acetylcholine directly.

The experiments with the catecholamine infusions in the presence and absence of ALA, indicate that even at maintained elevated levels of catecholamine, as occur in AIP (Sohley et al, 1970) ALA has little observable interaction. On a few occasions ALA appeared to potentiate the effect of the catecholamines, however similar responses were seen in the control situation.

The absence of any effect of ALA on the response of the heart to stimulation of its sympathetic airflow and the absence of any ECG abnormalities, after injection of ALA, indicate that this is not the site of action of ALA. The initial decrease in heart rate seen in most responses would be consistent with a transient change in rate associated with the heart changing size to accommodate a decreased venous return.

There is a striking resemblance between the response of both anaesthetised and pithed animals to histamine and to ALA. Qualitatively the responses are very similar and when animals appeared to be hypersensitive to histamine, the same was found to be the

case with ALA (cf. Fig. 24). This could of course, have represented only that these animals were especially sensitive to hypotensive agents. Nevertheless, the similarity in response to these two drugs was evident on many occasions. ALA did indeed show properties which would be consistent with a drug being an endogenous histamine releaser, (Paton 1957). However the experiments designed to investigate this possibility were negative.

The possibility that ALA has a histamine like action on H_1 receptors was also deemed unlikely as mepyramine did not block the effect of ALA at a dose sufficient to block an equivalent hypotensive action of histamine. An H_2 action would, of course, have resulted in a pressor response.

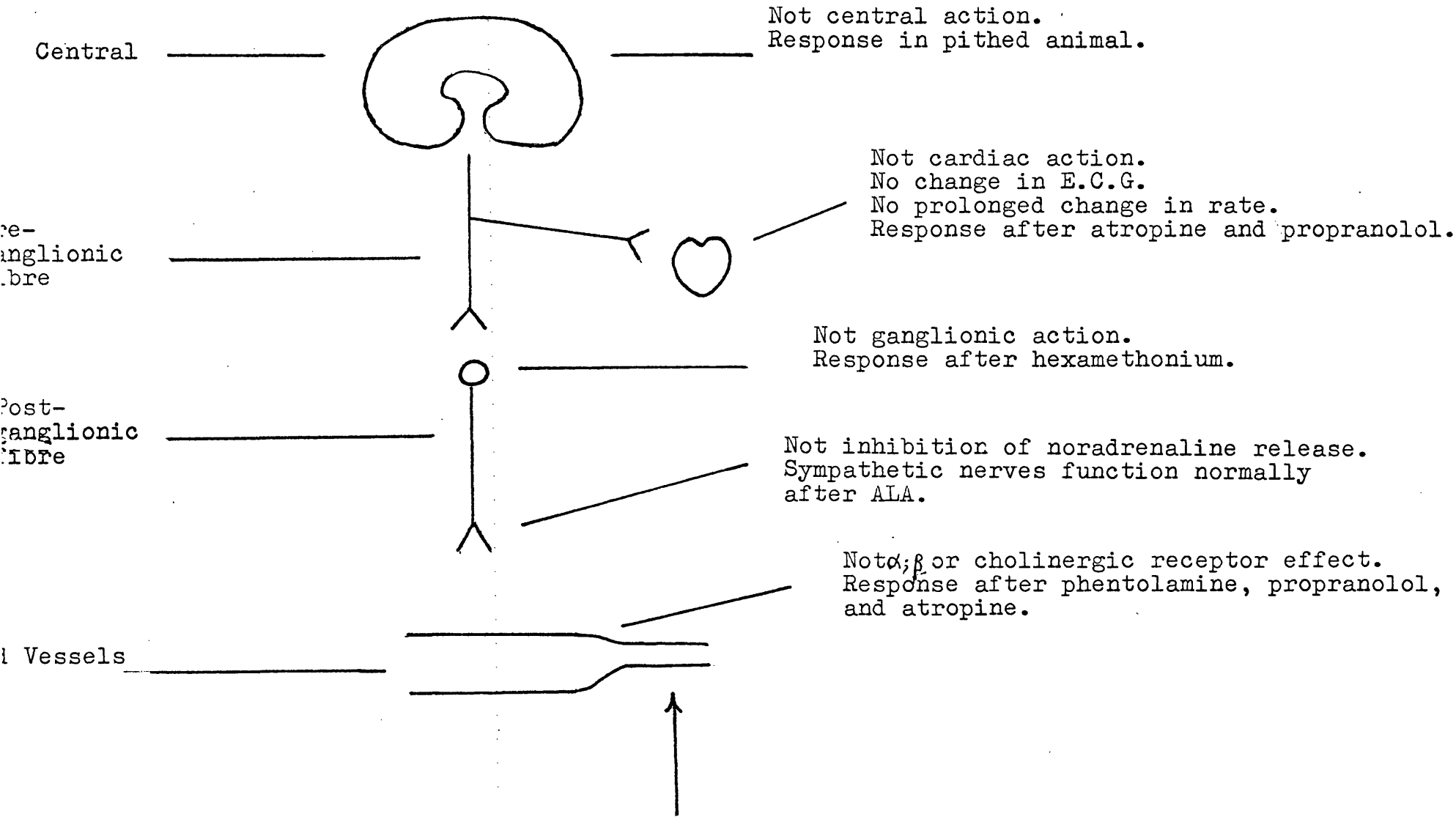
The apparent potentiation of the response to ALA by a previous injection of histamine is an observation, the basis of which is obscure. It may have been relevant to a role of ALA as an endogenous histamine releaser; however, as we have said, ALA does not appear to act in this way. It is possible that histamine given before ALA 'primes' the receptor site at which ALA acts, making the ALA interaction more efficient. Such a possibility is however purely speculative.

The effect of ALA on reversing the pressor response to vasopressin is informative. Thus it demonstrates that when the pressure in the pithed animal for example, is raised by a direct vasoconstriction, ALA can exert an effect as large as, or greater than, its effect in the normal anaesthetised animal. This is evidence that the whole effect of ALA is mediated by a mechanism not involving the central or peripheral nervous systems.

The reversal of the effect of vasopressin by ALA is likely to be an effect present because ALA produces the physiologically opposite action to vasopressin, as opposed to a pharmacological antagonism of vasopressin at the site of vasopressin's action. If the latter was the case, a previous injection of ALA would be expected to antagonise subsequent responses to vasopressin: an observation which was not evident.

The question remaining therefore, is what is the site of action of ALA? Consideration of the results obtained and described above indicate that it is possible ALA exerts a hypotensive effect by increasing the permeability of or dilation of the capillary bed in a manner similar to the production of hypotension by histamine. (Fig.27)

Fig.27. Potential Sites of Hypotensive Action of ALA.



From the capillary permeability experiments evidence was obtained that ALA may act at this site. Thus on objective observation it was found that there was an increase in blueing around the blood vessels in a skin flap of both the ALA and histamine treated animals, compared with the controls. This, however was not nearly so dramatic nor evident as is seen with other substances (suchas $^{48}/_{80}$) and this suggests that the action of ALA was relatively weak in this respect.

It must be borne in mind that the word 'capillaries' used here with respect to the above experiments refers not only to the morphologically distinct vessels properly called capillaries, but also to small diameter arterials. It seems probable, therefore, that ALA may exert its action at this site possibly by both increasing the permeability of, and possibly also by dilating, these 'capillaries'. It is felt, however, that such an action of ALA would not assume any significant degree of importance in acute porphyria, except perhaps in occasional circumstances, as referred to.

It is possible that this hypotensive effect of ALA is evident in subjects with AIP. Thus, although the vast majority of acute porphyrics with abnormal blood pressures exhibit hypertension, cases of hypotension do occur. It is quite conceivable that in these situations, an increase in the circulating levels of ALA would produce a hypotensive response similar to that seen in the rat.

An immediate apparent objection to such a hypothesis, however, is that it would be expected that all porphyrics with elevated circulating ALA concentrations should show this effect if it was due to ALA. This is not so. As the animal experiments demonstrated, the hypotensive action of ALA was readily reversible by injected catecholamines. Further, Schley et al, (1970) have demonstrated a significant elevation of catecholamines in acute porphyrics <u>with hypertension</u>. Thus it is possible that in most porphyrics, there is increased circulating catecholamine during attack, (and possibly to an extent during remission) with a consequent hypertensive response, and a masking of the hypotensive effect of ALA. In those porphyrics who demonstrate hypotension, however, there is unlikely to be any such increase in circulating catecholamines, and hence, as there

would be no antagonism, the effect of ALA as a hypotensive agent becomes evident.

In this respect it would be of interest to see if porphyrics showing hypotension exhibited the other signs of porphyria such as constipation and tachycardia, which could be attributable to increased sympathetic tone or increased circulating catecholamines.

With respect to the hypertensive episodes of AIP therefore, it seems unlikely from these animal studies, that a direct action of ALA is a contributory factor. More likely is the possibility that these episodes are related to abnormalities of catecholamine production and/or excretion.

c) Rabbit Ear Artery

In almost every experiment performed there was no immediately apparent effect of ALA on the response of the artery to injected noradrenaline or electrical stimulation. As can be seen from the relevant dose-response and frequency response curves figs. 28 and 29 , there is no significant difference between the pre and post ALA situations. Each of these curves expresses the response as a percentage of the maximum. This was a valid means of expression as there was no significant difference between the maximum absolute responses in the pre and post ALA situations. Each of the points in these curves represents the mean $\pm$ S.D. of a single reproducible pre and post ALA dose, or frequency, response from a number of separate experiments.

Despite the fact that when taken as a whole these experiments indicate that ALA has no effect on the rabbit ear artery in the given situation; there were single experiments in which it did appear to have an effect. Thus in four cases out of thirty, the response to sympathetic stimulation and injected noradrenaline was enhanced after ALA fig.30 . Similarly in two experiments out of six, where the tissue was equilibrated with normal Krebs and then perfused with ALA Krebs,

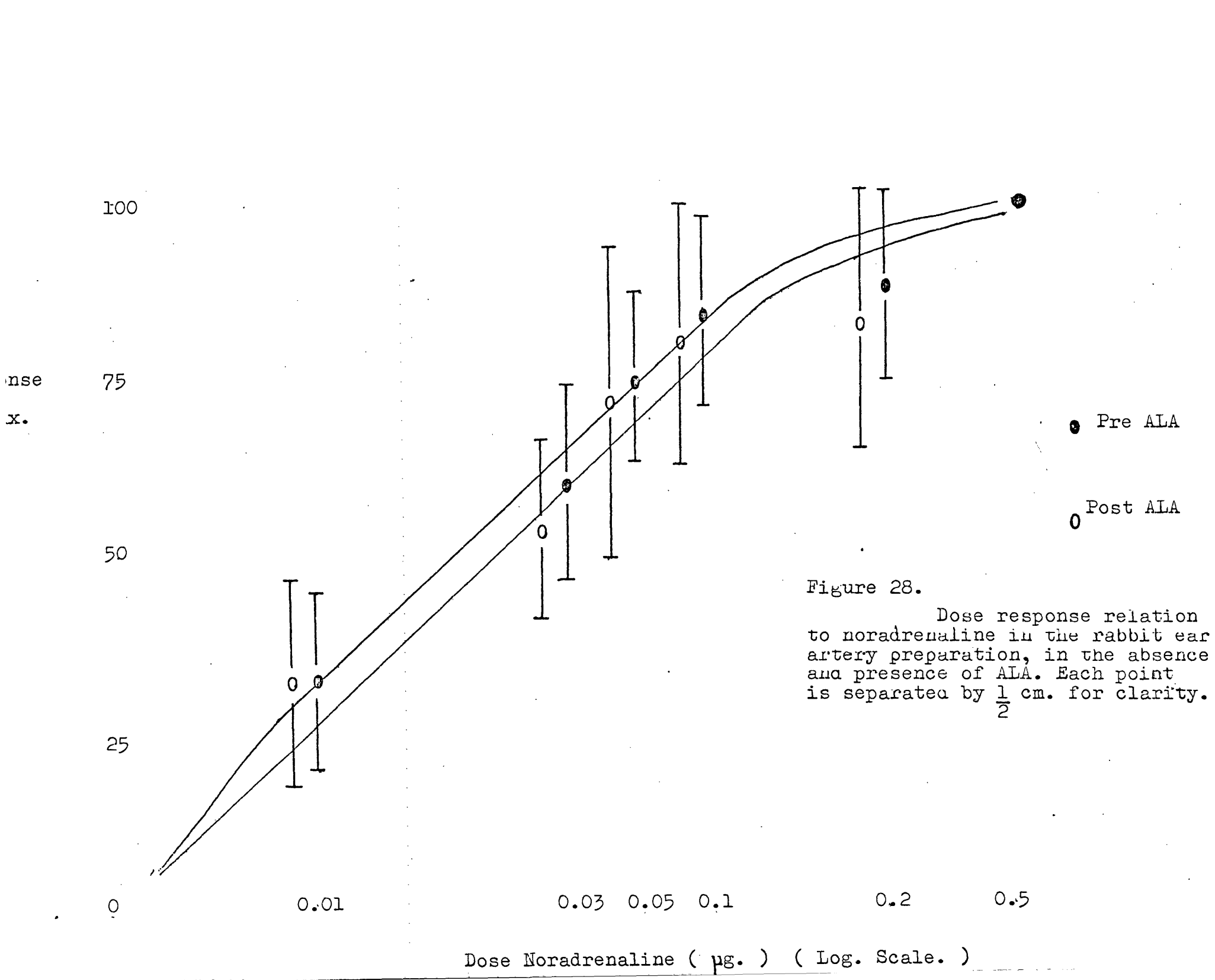


Figure 28.

Dose response relation to noradrenaline in the rabbit ear artery preparation, in the absence and presence of ALA. Each point is separated by $\frac{1}{2}$ cm. for clarity.

Figure 29.

Each point in this figure represents the mean ± S.D. of 30 experiments. The points are separated slightly for the sake of clarity. The curve showing the response in the presence of ALA is seen to be shifted to the right, but the difference was not significant.

The response was measured as the perfusion pressure in mm.Hg., and expressed as a per-centage of the maximum response.

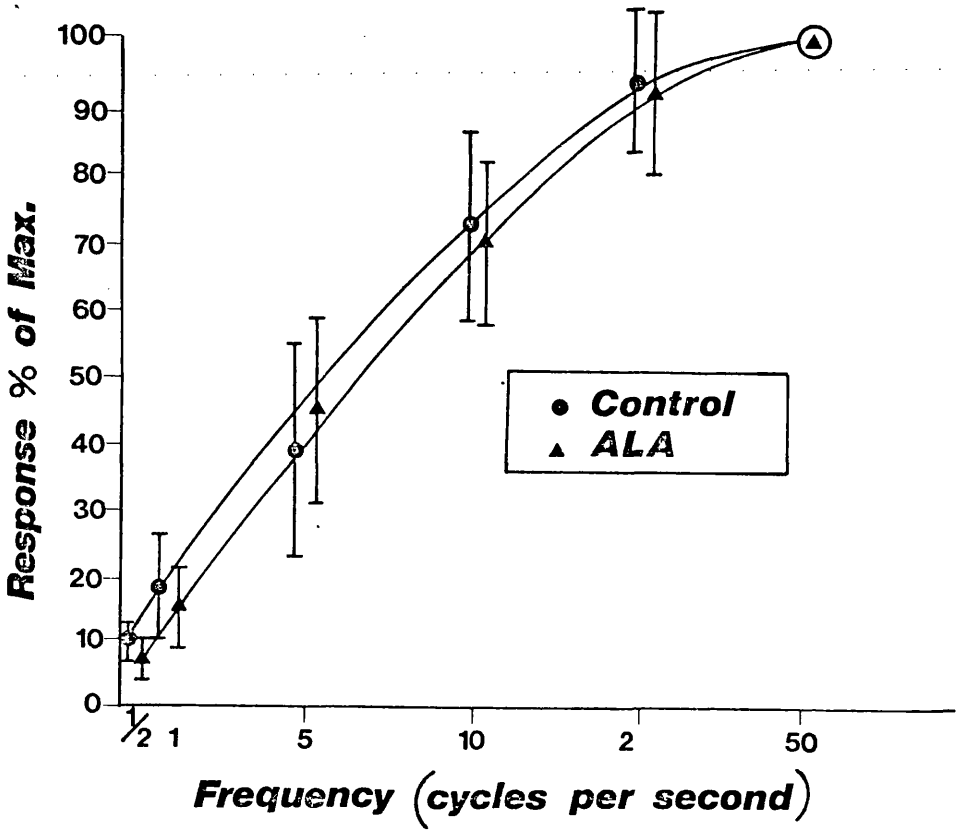
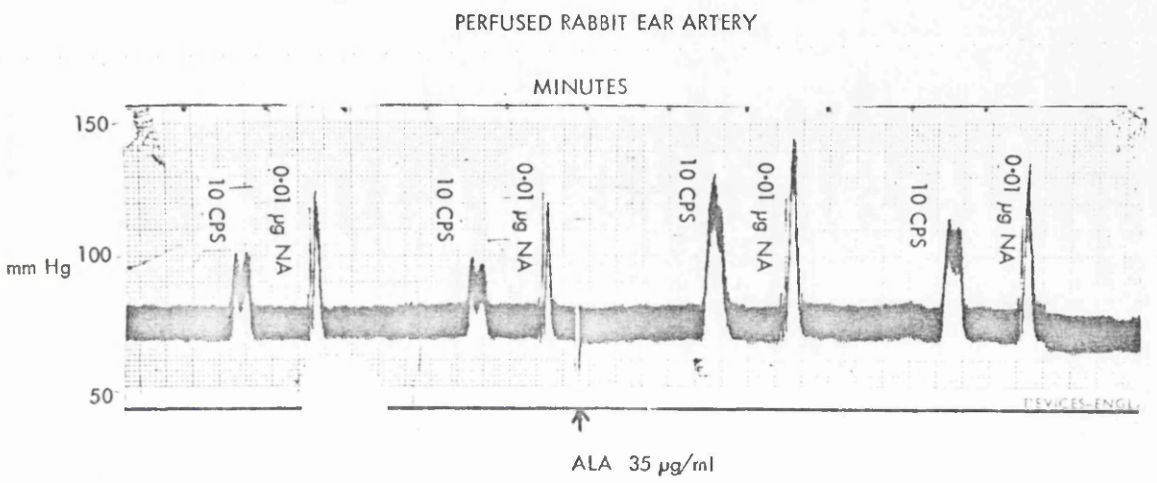


Figure 30.

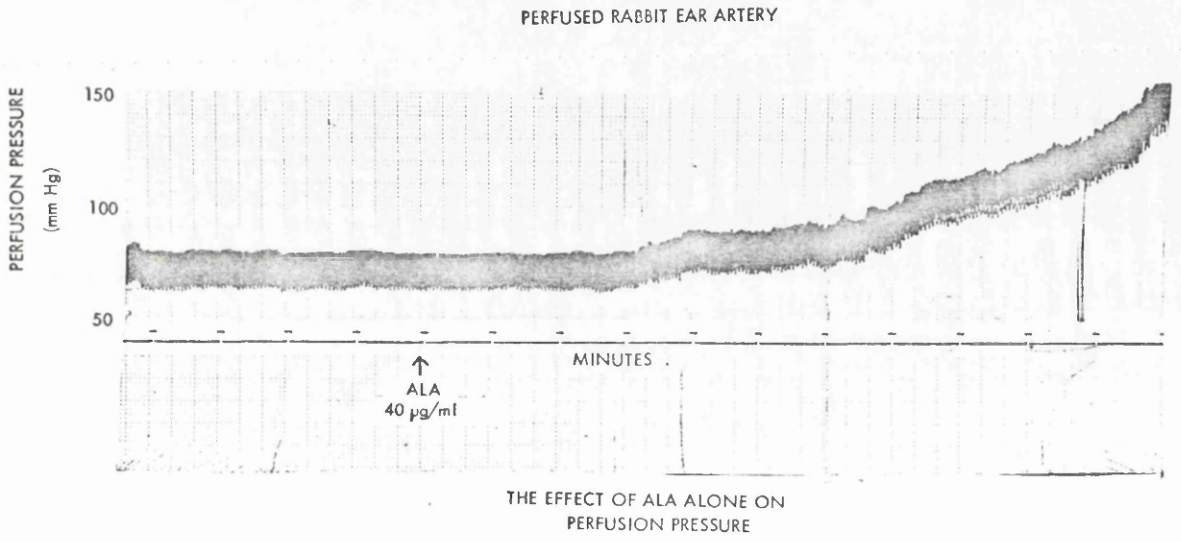
This trace shows that immediately after the addition of ALA to the perfusing medium, there was an increase in the response of the artery to both sympathetic nerve stimulation and exogenous noradrenaline. Such an effect was found in only 4 cases out of 30.



POTENTIATION OF THE RESPONSE TO NERVE STIMULATION AND INJECTED NORADRENALINE BY ALA.

Figure 31.

In this experiment there was no injection of noradrenaline, or sympathetic stimulation. In 2 cases out of 6, ALA alone was found to increase the perfusion pressure.



THE EFFECT OF ALA ALONE ON PERFUSION PRESSURE

there was a large delayed increase in perfusion pressure after the onset of ALA perfusion. In the case illustrated (fig. 31), this occurred some twenty minutes after the initial contact of the tissue with 20µg/ml. ALA, and some five minutes after the concentration was increased to 40µg/ml. When perfused again with ALA-free Krebs, the pressure returned to normal, rising again about five minutes after once more being perfused with the higher concentration of ALA. The increase in pressure was found to be antagonised by phentolamine (20µg/ml). In these six experiments there was no sympathetic stimulation or injection of noradrenaline.

Discussion

For the most part the results obtained from these experiments are negative. When taken together the means of the dose and frequency response relationships are not significantly different in the absence or presence of ALA. This would suggest that, at least under the given experimental conditions, ALA had no effect on the response of the artery to noradrenaline or sympathetic stimulation. The occasional instances, however, where ALA did appear to produce an effect are worthy of consideration.

The fundamental problem here is - why did ALA appear to have an effect in certain cases but not

in the majority of cases? There are perhaps, three main possibilities: the first is that there was some gross experimental or procedural error in these cases, after ALA was perfused into the artery. The second is that, due to some biological process unrelated to the action of ALA, the tissues showed a sudden increase in sensitivity to noradrenaline and/or sympathetic nerve stimulation, which just happened to coincide with the addition of ALA to the perfusing medium. The third possibility is that in certain cases, ALA did have an effect on the responsiveness of the tissue, and it did so in these cases specifically, because the tissues were in some undefined way, predisposed to, or more susceptible to, this action.

When one considers that the experimental procedure was largely routine and carried out in a similar manner in most cases, it seems unlikely that some experimental error is the explanation of these results: particularly as the electrical stimulation in one case where potentiation occurred, was kept constant. (cf.Fig 30) In terms of chance, the second possibility would appear to be much too coincidental, and hence the third possibility would appear to be the most likely explanation.

Thus we may assume that in certain cases, the tissue became more responsive to noradrenaline and

sympathetic stimulation after ALA, by some mechanism which is as yet obscure, and which did not occur in the majority of tissues studied. In support of this postulate, were the results obtained from the experiments where ALA was perfused into the artery and the perfusion pressure rose. Again this occurred in only two cases out of six, but it was both reproducible and reversible, indicating that it was a real effect of ALA. (Fig.31)

With respect to these anomalous results it might be said that in the tissues where ALA did appear to be active, there were no obvious differences in the animals used; or experimental procedure. Thus the ALA sensitive tissues did not come from animals of a different strain, sex or weight than the others, nor from animals that were otherwise obviously different from the rest. Nor were the tissues themselves exposed to different physical or chemical conditions from the rest. Finally, and perhaps most important, there were cases where two arteries from the same animal were used simultaneously and treated to similar experimental procedures, yet only oneof them showed evidence of an effect of ALA on their responsiveness.

Accepting the fact that ALA had no statistically significant effect on either injected noradrenaline

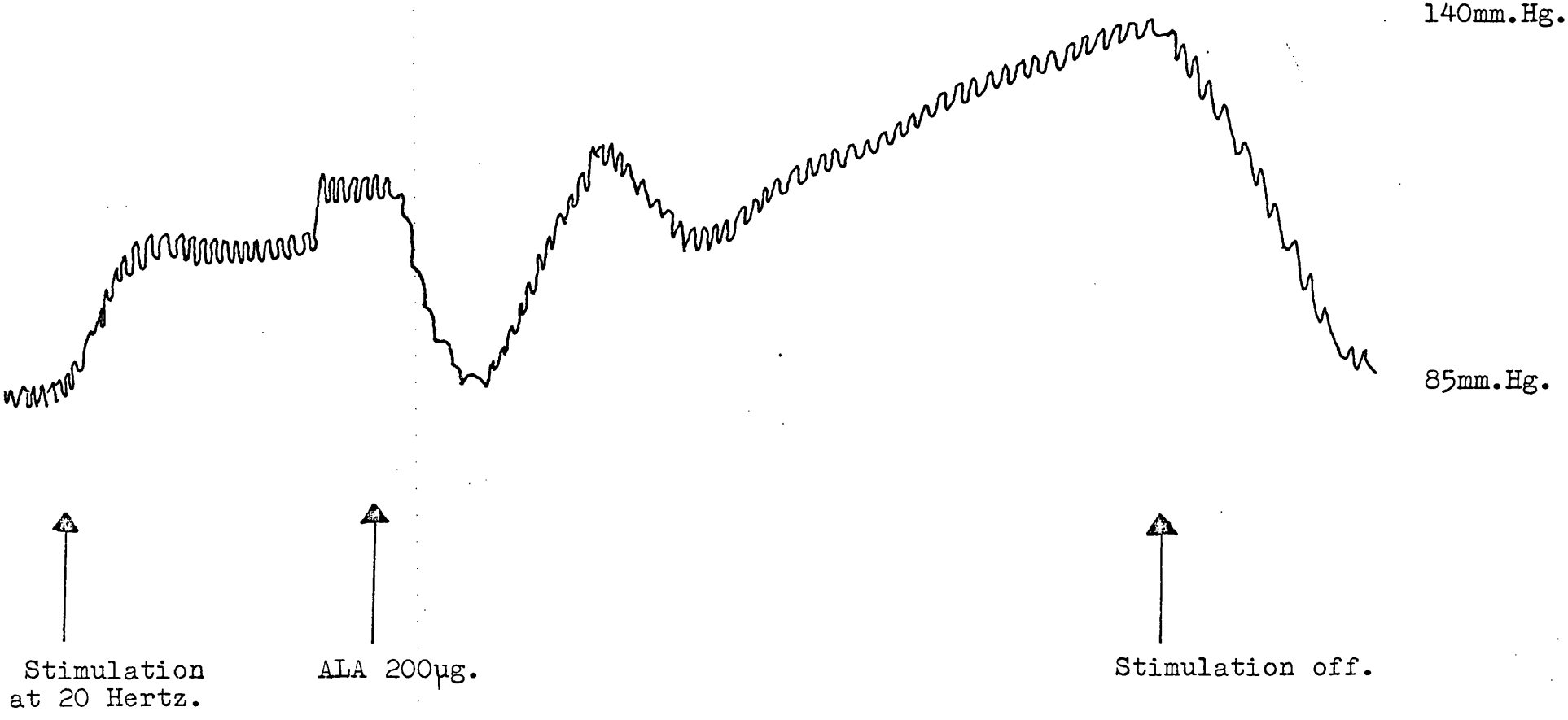
nor on periarterial sympathetic nerve stimulation, there is no evidence from these studies that would link ALA with the postulated abnormalities in sympathetic nerves which occur in acute porphyria. If, however, it could be shown that ALA could affect the response to sympathetic stimulation in humans, in the manner demonstrated in a few of the experiments in this series, then this would be highly informative. For the moment however, this must remain purely speculative until there is more information available on the factors which control the biological actions of ALA.

In both the frequency and dose-response curves, it can be seen that in the presence of ALA the curve shifted to the right in the presence of ALA. Although this shift was in no case statistically significant when analysed at each dose or frequency by a 't' test: it does suggest a possible mild inhibitory action of ALA on the response of the artery to noradrenaline and electrical stimulation.

This may appear to be paradoxical in view of the findings referred to above of an occasional apparent potentiation by ALA on these stimulii. However it is of interest that in a preliminary experiment on the artery, - where the perfusion pressure was monitored by a mercury manometer,

Figure 32.

On this line drawing of a fast moving soot trace of perfusion pressure, two effects of ALA are seen on the response to sympathetic stimulation. Thus there is an initial antagonism of the response, followed by an apparent potentiation. In this tissue the same effect of ALA was apparent on injected noradrenaline.



and displayed on a kymograph trace - both of these effects were evident in response to a high dose of ALA. fig.(32)

Thus it is possible that ALA may occasionally potentiate the response of certain tissues to catecholamine and sympathetic nerve output (as discussed above), and it may also exert a very weak inhibitory action on these. These contradictory effects could be rationalised by assuming that in the former case ALA has an effect on the uptake or metabolism of catecholamines, and in the latter a weak inhibitory on the muscle adrenergic receptors. Such suggestions, however, are only mentioned in terms of the results of some isolated experiments, and are no more than speculative.

In summary then, it can be seen that, with the exception of a few specific experiments where ALA appeared to have an effect, there is no significant evidence from the above experiments to suggest that ALA is active on the response of the isolated artery to injected noradrenaline or sympathetic nerve stimulation.

GASTRO-INTESTINAL STUDIES

Introduction

As discussed above Section I, there is a high incidence of gastro-intestinal manifestations in acute porphyria, including abdominal pain, constipation, vomiting and, occasionally, diarhoea. As is the case with the other features of the disease the pathogenesis of those gastro-intestinal features is unknown.

An observation of some interest in this respect is the successful use of the anticholinesterase, neostigamine, in treating certain aspects of the porphyric attack (Gillhespie and Smith 1954). Berlin and Cotton (1950) have observed by X-ray examination that neostigmine caused an improvement in the peristaltic activity of the stomach and small intestine in porphyric subjects in attack. Similarly Goldberg and Rimington (1962) have reported the alleviation of constipation and urinary retention by neostigmine in one porphyric subject, and in another case they found that a dramatic improvement in the neurological and psychological state coincided with the administration of a course of neostigmine.

Neostigmine does, of course, exert its pharmacological action by inhibiting acetylcholinesterase, the enzyme which metabolises neurally

released acetylcholine. As a result of cholinesterase inhibition, acetylcholine can act for a greater time at its site of action, and can reach greater concentrations there than normal.

The use of neostigmine in successfully treating certain aspects of AIP, such as constipation, immediately suggest possible mechanisms by which this constipation may be produced. Thus one could postulate one or more of the following:

a) In AIP there is a circulating anticholinergic substance which can reversibly antagonise the actions of neurally released acetylcholine at the muscarinic (smooth muscle) receptor sites.

b) In AIP there is a significantly diminished output of neural acetylcholine in the gut, either in the extrinsic parasympathetic innervation to the gut, or in the intrinsic innervation of the longitudinal muscle layer of the gut.

c) In AIP the autonomic inflow to the gut is unnaturally dominated by the sympathetic system.

Either of the above conditions would account for the 'neostigmine sensitive' decrease in tone in the gut, which would give rise to constipation and associated signs of parasympathetic dominance in the clinical situation. There are other possibilities of course, but the above are the most obvious.

It was decided therefore, to investigate the effect of ALA on the response of the isolated guinea pig ileum to acetylcholine, to see if ALA exerted any cholinergic receptor blocking activity. The effect of ALA on the response to histamine was also observed. The results of this experiment would give evidence either in favour or against postulate 'a' above.

Postulates 'b' and 'c' are more difficult to investigate experimentally. There is experimental evidence however, suggesting that ALA could interfere with neural action and lead to alterations in transmitter output. Such changes in neurotransmittor release could account not only for the constipation present in AIP, but for other manifestations as well. The theoretical and experimental basis for suggesting such an action of ALA is as follows:

Becker et al, (1971) demonstrated that ALA could inhibit the Na^{+}-K^{+} fraction of red cell and brain ATPase 'in vitro'; although no inhibition of the Mg^{2+} fraction was evident. They concluded from this that ALA could block nerve conduction and hence cause paralysis. It is possible however, that if Paton et al are correct in assuming that acetylcholine release can be increased by ATPase inhibition, that ALA could cause increased output

of acetylcholine by ATPase inhibition.

Such a link between neural membrane ATPase activity and neuro-transmitter release applies not only to cholinergic nerves but to other nerves as well. Thus it was thought worthwhile to investigate the effect of ALA on the output of neuro-transmitter. The system used was the isolated longitudinal muscle strip of the guinea pig ileum.

Methods

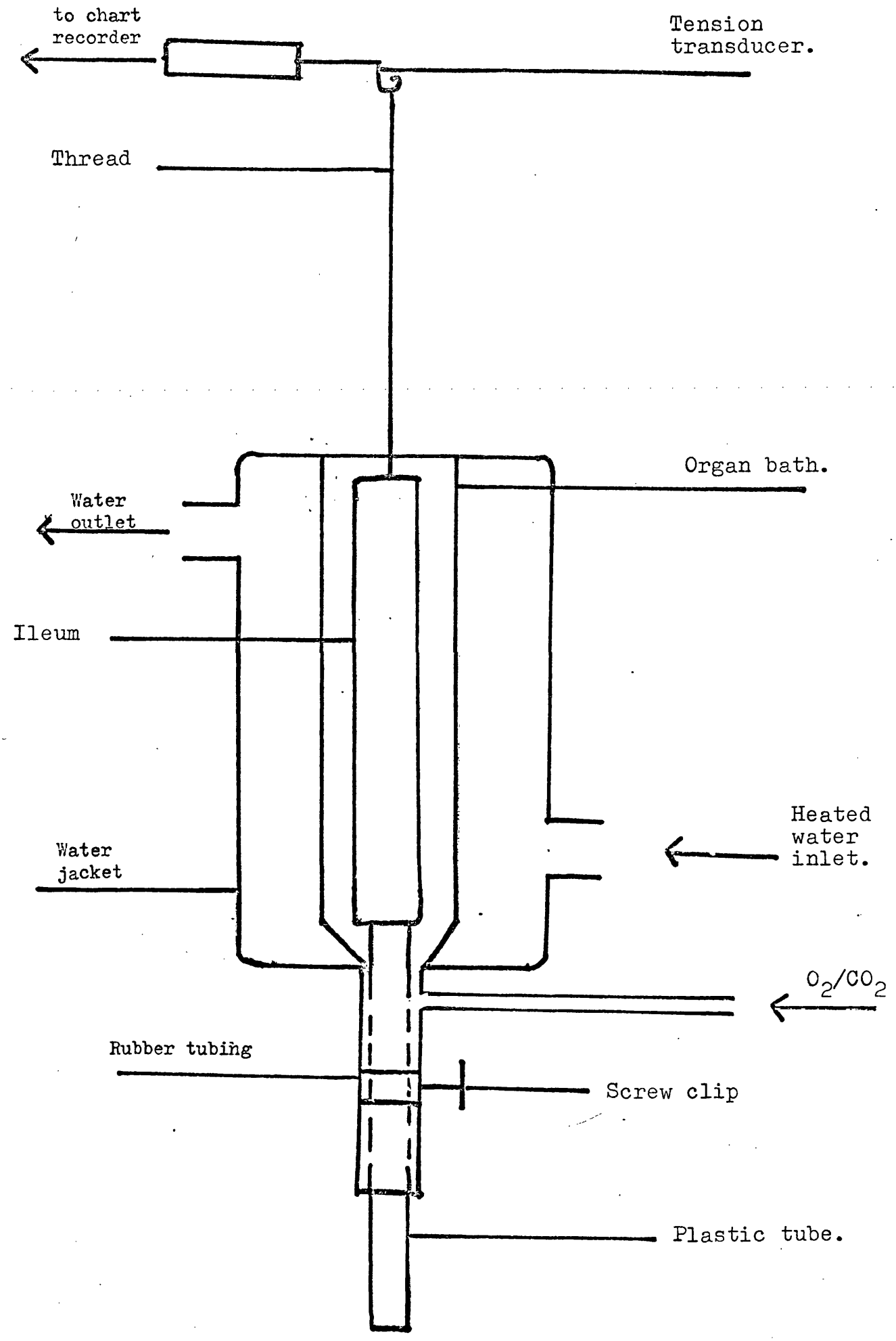
(a) Guinea Pig Ileum:

A guinea pig of either sex was stunned by a blow on the head and exsanguinated by cutting the throat. The abdominal wall was cut open and the caecum turned back to expose the ileum. The latter was cut at the ileo-caecal junction and a length of ileum was dissected free of mesentery. 8-10cm of the terminal ileum was discarded and a further length of 8-10cm was then taken and placed immediately into warm Krebs solution in a petri dish. A piece of small diameter plastic tubing was then inserted into the lumen at the aboral end of this piece of ileum and this was tied in place. A thread was then tied round the oral end of the tissue, one end of which was left long for attachment to a tension transducer.

The plastic tube with the piece of ileum attached was fed down through a piece of rubber

Figure 33.

Apparatus for Guinea Pig Ileum Preparation.



tubing at the base of an organ bath and firmly fixed in place with a screw clip (cf.fig.33). The organ bath was then filled with Krebs solution containing 10µg/l morphine and 5µg/l eserine, and the thread at the oral end of the ileum was attached to a movable auxotonic tension transducer. The transducer was connected to a Servoscribe chart recorder which monitored the tension of the ileum. An initial resting tension of around 1g was maintained.

The Krebs solution was of the composition shown in Table 12. It was fed into the organ bath by way of a heating coil at 37°C. The organ bath itself was contained in a water jacket in which water at 37°C continuously circulated. The Krebs solution in the reservoir, in the heating coil and in the organ bath was gassed with 5% CO_2 in O_2 throughout the experiment.

Any intralumenal contents of the ileum were forced by peristalsis down into the plastic tube attached to the aboral end. This device dispensed with the necessity of 'pipette washing' of the tissue before mounting in the organ bath, and thus reduced the possibility of tissue damage.

Drugs were injected into the organ bath either manually or by an automatic injection apparatus. Where necessary allowance was made for the volume of injected drug by removing an equivalent

volume of Krebs from the bath before injection. Dilution of drugs was made initially in distilled water, the final dilutions being made in Krebs.

Stimulation of the ileum was by means of two platinum electrodes at either end of the bath. A supramaximal voltage was used and the pulses had the following characteristics:- A ten second stimulation at a pulse width of 0.5m.sec. at a frequency of 2-4 Hertz. The time cycle normally employed was as follows:- 0 minutes: inject drug, leave 15 seconds, wash.1 minute: stimulate for 15 seconds, wash.2 minutes: inject drug:- and so on.

Results

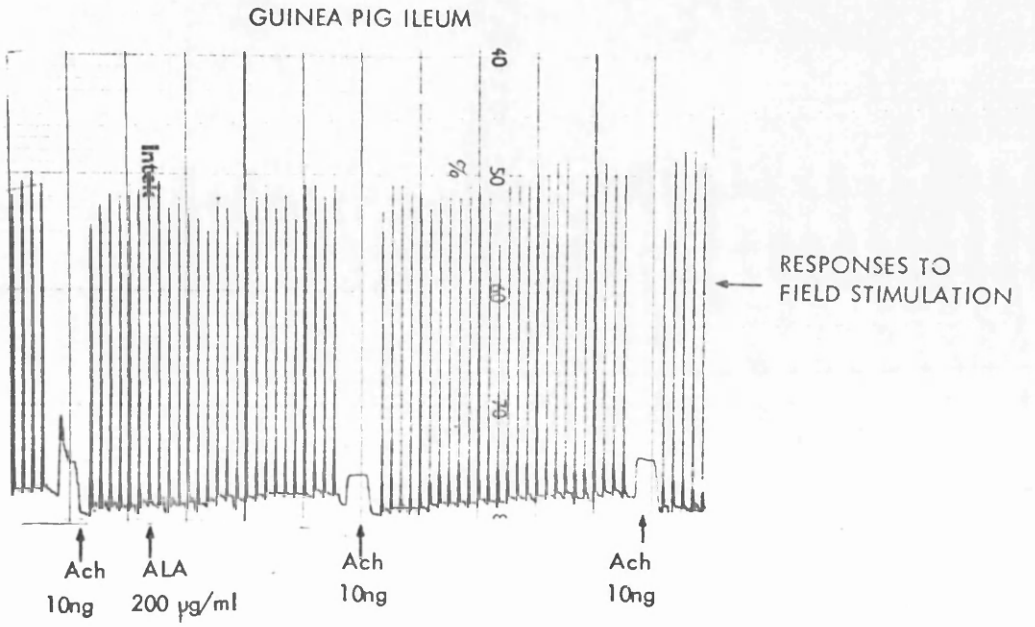
At a concentration in the Krebs solution of up to 300µg/ml, ALA had no apparent effect on the response of the ileum to electrical field stimulation.

The means of twelve dose-response curves to acetylcholine, carried out on separate tissues, were not significantly different whether determined with ALA absent or present (200µg/ml) in the Krebs. In specific instances, however, the presence of ALA in the solution appeared to have an effect on the response to acetylcholine. Thus in two cases the form of the response to acetylcholine changed after addition of 200µg/ml ALA to the Krebs solution. The response lost its initial fast component and flattened out, the duration of the response remaining the same. (fig. 34) When the ALA was washed out of the Krebs, the response returned to its former shape. On another two occasions, ALA appeared to potentiate the response to acetylcholine. In most experiments however, as the statistical evaluation shows, ALA had no apparent effect on the parameters measured.

As was the case with acetylcholine, there was no significant difference in the response of the ileum to histamine, before or after addition

Figure 34.

In this experiment ALA was seen to change the form of the response of the ileum to acetylcholine. The response of the ileum to field stimulation was not affected.



CHANGE IN FORM OF RESPONSE
TO ACETYLCHOLINE AFTER ALA

of ALA (200 µg/ml) to the Krebs solution. As a further control the response of the ileum to acetylcholine, histamine and to electrical field stimulation was determined in normal Krebs and then after addition of γ amino-butyric acid (200µg/ml). As was the case with ALA, γ amino-butyrate produced no significant changes in the responses of the ileum to these agents.

Discussion

These results indicate that under these experimental conditions, ALA is relatively inactive on the ileum, and on the contractile responses of the ileum produced by electrical field stimulation, or injection of acetylcholine or histamine. The occasional positive effect of ALA on the response to acetylcholine, indicated that there may have been some action, however this did not occur frequently enough to give a statistically significant effect.

In this respect the ileum's response to acetylcholine after ALA, paralleled somewhat the response of the rabbit ear artery to noradrenaline after ALA. In each case there were occasional experiments where there appeared to be an effect of ALA on the neurotransmitter being studied (acetylcholine or noradrenaline) which at its most dramatic appeared as a large potentiation of the

response to the transmitter. Over a number of experiments however, in each case, this effect became insignificant.

It might be considered that these effects were not merely experimental artefacts, but real effects of ALA. If this was the case then it would indicate that some tissues are more susceptible to the actions of ALA than others; however there is no information from the present study, to suggest why this might be so. It is unlikely, for example, to be related to tissue viability as all the tissues were similarly responsive to either field stimulation or injected agonists: yet only four out of twelve showed any effect of ALA.

It must be concluded from the present study therefore, that there are no apparent effects of ALA on the guinea pig ileum, which could account for any of the gastro-intestinal features of acute porphyria. In terms of the postulates considered above, there is no evidence from these studies that ALA has any anticholinergic activity at the muscarinic site, nor is there any evidence that it affects parasympathetic transmission. Thus if the neostigmine sensitive constipation of acute porphyria is a result of an over-dominance of sympathetic activity, the evidence

from these studies suggests that this would be likely to be the result of overactive sympathetic nerve activity, rather than the result of an over-dominant sympathetic system produced by virtue of an ALA induced underactivity of the parasympathetic system.

As with all similar 'in vitro' and 'in vivo' studies with ALA, it must be borne in mind that the ileum, in these experiments, was in contact with ALA for a very short time compared with what would be the case in acute porphyria. Even allowing for the fact that a dose of ALA was used which was a factor often greater than the blood concentration in acute porphyria; we are still left to interpret the results of an acute experiment with ALA, and attempt to relate it to a human situation where there is chronic exposure to elevated ALA concentrations.

Methods

(b) ALA and ATPase

The assay of total ileum ATPase was based on the method described by Becker et al (1971). The enzyme activity was determined by the amount of free phosphate released by the hydrolysis of ATP.

A length of ileum was removed from a guinea pig which had been killed by stunning and exsanguination. The ileum was then weighed and homogenised in 0.25M sucrose/imidiazole-histidine buffer at pH 7.1. The homogenate strength was 1:3 $^{w}/_{v}$. 1ml aliquots of the homogenate were added to tubes containing the following:- 0.5ml imidiazole-histidine (pH 7.1); 0.1ml (5µ moles) magnesium chloride, 1ml ATP (5µ moles), potassium and sodium were added to a final concentration of 0.08 and 0.03M respectively each in 0.1ml volumes. A further 0.3ml distilled water was then added.

To two tubes 2ml of 10% perchloric acid was added before addition of ATP; these samples gave the zero time phosphate values. Of the remaining tubes, six were incubated at 37°C for one hour with no ALA present (control) and a further four groups of 6 tubes were similarly incubated for one hour with ALA present. The ALA concentrations were 15, 30, 120 or 180µg/ml of the total volume of the contents of the tubes.

After one hour's incubation, at 40°C, the reactions were stopped by the addition of 2ml of 10% perchloric acid. The resulting precipitate was centrifuged at 3000 r.p.m. for ten minutes, and the supernate decanted into fresh tubes. The phosphate content of 1ml supernate in each sample was determined by the method of Mozersky et al (1968).

Results

(b) ALA and ATPase

The activity of ATPase was measured by the determination of the free phosphate produced by ATP hydrolysis. The results are expressed in u.moles phosphate/hour, at an incubation of 40°C. From each test and control value, the zero time value of free phosphate was subtracted.

The results are shown in Table 13. As can be seen from the Table, 15 or 30µg/ml ALA caused no significant change in total ATPase activity, however the 120 and 180µg/ml concentrations, caused a significant inhibition of activity: with 'p' values of < 0.05 and < 0.01 respectively when analysed by a 'Student's t test'.

TABLE 13

THE EFFECT OF ALA ON TOTAL ATPASE ACTIVITY OF THE GUINEA PIG ILEUM

Concentration of ALA (µg/ml)		Free Phosphate µ.moles/hour at 40°C
(Zero Time)	0	2.38 ± 0.15
(Control)	0	4.59 ± 0.24
	15	4.43 ± 0.21
	30	4.49 ± 0.31
	120	4.20 ± 0.24
	180	4.02 ± 0.24

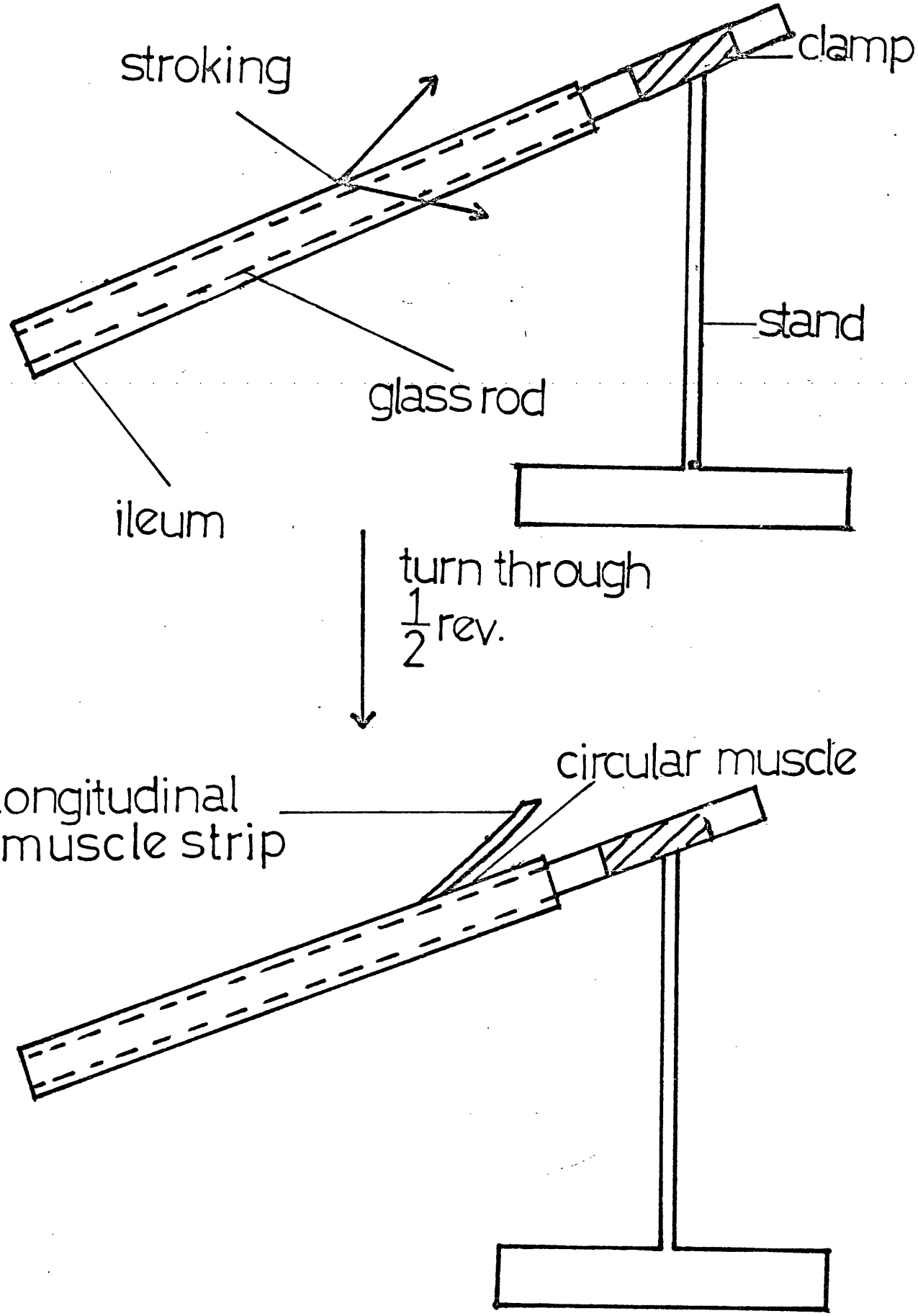
Each result of free phosphate is expressed as the mean of six experiments minus the mean zero time value ± standard deviation.

(c) Acetylcholine Release

Measurements of acetylcholine output were made on longitudinal muscle strips of the guinea pig ileum. The strips were obtained by the method described by Paton et al, (1968). A length of ileum was dissected out as described above. In this case most of the ileum was taken and placed in a beaker with warm Krebs solution. 25ml warm Krebs solution was run from a pipette through the lumen of the intestine to dislodge any faecal matter present. The end of the ileum was then gently pulled up and stretched over a glass rod inserted into the lumen (cf.fig. 35). The mesentry was then carefully cut away. By stroking tangentially with a piece of cotton wool, the longitudinal muscle was separated from the underlying circular muscle. The end of the longitudinal muscle was then tied with a thread, and a strip of muscle was gently pulled off. Care was taken to keep the tissue moist with Krebs throughout this whole procedure.

Strips thus obtained were mounted in an organ bath with Krebs solution containing 1mg/l. choline chloride, and 2mg/l. eserine. The temperature was maintained at 37°C by continuous heated water circulation and the Krebs was continuously gassed with 5% Co_2 in O_2. The

Preparation of muscle strips



apparatus was similar to that described above for the isolated ileum, except that in this case the contraction of the strips was not recorded.

After a period of equilibration of up to one and a half hours, the Krebs was replaced and the strips were left in contact with the fresh Krebs for ten minutes. At the end of this time this Krebs was collected in a small measuring cylinder, the strips were washed with two bath volumes of fresh Krebs and finally left again in contact with fresh Krebs for another given time interval. The Krebs in the measuring cylinder was then assayed for acetylcholine content on the isolated ileum preparation described above.

The assay procedure was as follows:-
A dose response relationship was determined on the ileum for acetylcholine. A given volume of the test sample was injected and the response obtained was noted. The response was then increased, or decreased by altering the volume of test solution injected, so that a response of some 70% of the maximum was obtained. A response to acetylcholine just less than, just greater than and equal to, this response was then obtained. This was then repeated using another test sample.

To prove that it was acetylcholine that was being released by the muscle strips, an acetylcholine

block was produced by atropine and all the test samples were then tested once more for activity.

The assaying procedure was repeated until the tissue gave out a constant concentration of acetylcholine for three consecutive assays. ALA, up to 300µg/ml, was then added to the Krebs and the assay procedure was repeated. In this way an internal control situation for acetylcholine release before and after ALA was obtained.

The calculation of the amount of acetylcholine released per minute by the tissue was made as follows:-

Time of Incubation: 10 mins.

Total volume of Krebs in organ bath: Xml.

Volume of Krebs assayed for acetylcholine: Yml.

Acetylcholine content of Yml Krebs (from assay): Zng.

Total concentration of acetylcholine released from muscle strip in ng/ml/min: $= \frac{x}{y} \times \frac{z}{10}$ ng/ml/min.

For each experiment, the mean acetylcholine output before the addition of ALA to the organ bath, was compared with the mean output after ALA. The results were then analysed by a 'paired t test'. For a total number of 28 observations, the mean output of acetylcholine before ALA was 1.4ng/ml/min $\pm$ 0.2.S.E.M. The mean output after ALA was 1.8ng/ml/min. $\pm$ 0.4.S.E.M. Thus although the mean acetylcholine output was greater after ALA, the difference was not statistically significant. ($p < 0.2$)

It may be pointed out that as each piece of tissue served as its own control, there was no need to express the results in terms of the weight of the tissue.

Discussion

The finding that ALA is capable of inhibiting total ATPase activity in the guinea pig ileum is consistent with the findings of Becker et al, (1971) that ALA can inhibit the sodium-potassium dependant fraction of red cell and brain ATPase.

The experiment was not elaborated in order to determine the source of, or which fraction of, the enzyme was inhibited: or what the mechanism of action of ALA was in causing such an inhibition. The sole purpose in this experiment was to see if ALA had a similar effect on ATPase activity in the ileum, as it undoubtedly has in brain and red cell 'in vitro'. These findings suggest that it has,

It was important to see if ALA inhibited ATPase in the ileum as this tissue was used to test the possibility that ALA could affect cholinergic transmission. The hypothetical basis of this latter investigation depended on ALA having such an action on ATPase, and so the effect of ALA on ATPase in the ileum was determined.

A number of substances, and certain changes in the ionic compposition of biological fluids, can both inhibit ATPase activity in various tissues, and

increase neural output of acetylcholine. Thus sodium, potassium or calcium deprivation (Skou 1957), the presence of ouabain (Skou 1957); or p-hydroxymercuribenzoale (Skou 1963), are all capable of both inhibition of ATPase, and of increasing the release of acetylcholine from the longitudinal muscle strip of the guinea pig ileum (Paton et al, 1971). The acetylcholine thus released is of a neurological origin (Paton et al, 1968).

These observations have prompted the suggestion that there is a link between an axonal membrane ATPase and transmitter release, such that inhibition of the former increases the neuronal output of the latter (Paton 1971).

As ALA can inhibit certain ATPase activity, (Becker 1971) and as in the experiment described above it was shown to be capable of such an action in the guinea pig ileum, it was felt that it might show this duality of action of both inhibiting ATPase and increasing acetylcholine output.

The results from the acetylcholine output studies did not confirm this hypothesis. Although the mean acetylcholine output did increase after ALA; the change was not significant.

Once more the possibility remains that in acute porphyria the concentration of ALA in the

vicinity of the axonal membrane, plus the prolonged period of exposure to ALA, could lead to ATPase inhibition with the consequent changes in transmitter release. Such a course of events could neatly explain certain manifestations of the disease. For example, if both sympathetic and parasympathetic nerves were similarly affected, the fact that acetylcholine is more efficiently metabolised than noradrenaline could lead to a sympathetic dominance, and hence the explanation of many of the signs and symptoms of AIP.

Speculative as these considerations are, it is felt that the likelihood exists that some such progress occurs in AIP. ALA <u>is</u> capable of ATPase inhibition, and Paton et al, (1971) did favour the possibility that all agents which inhibit ATPase, also increased neurotransmitter release. The fact that this was shown not to be significantly so in these experiments, does not mean that under other experimental conditions the effect would not be apparent.

In the experimental system used to test for acetylcholine release, the concentration of ALA used for incubation of the muscle strips was more than a factor of two times the concentration found to inhibit ATPase in the ileum. This higher concentration was used because Paton et al (1971) found that it required

a greater concentration to increase the transmitter output, than was effective in inhibiting ATPase.

It may be that much higher concentrations of ALA would have had to be used to produce an effect on transmitter release. At the time of the experiments, however, it was felt that even 300µg/ml was more than might be reasonably expected to be reached in nervous tissue in acute porphyria. Depending on the bio-compartmentalisation of ATPase and ALA 'in vivo', however, it may be that such higher concentrations are effectively attained.

It may also be the case that the tissue was not in contact with the ALA for a sufficiently long time for an effect to develop. In most cases, however, the tissue was in contact with ALA for at least one hour, and this should have been more than sufficient for an effect to develop Paton (1971).

In conclusion, therefore, while it is evident that ALA can inhibit the total ATPase activity of the guinea pig ileum, it does not appear to significantly alter the output of acetylcholine from longitudinal muscle strips of the ileum at a concentration of 300µg/ml.

It is evident, that as such an effect might be expected to occur on theoretical grounds, a more comprehensive pursual of this possibility

would be worthwhile. This would invariably be an extensive project in terms of the relationship of the experiments to the biological abnormalities of acute porphyria: It is felt however, that such a study may well significantly contribute to our understanding of the relationship between ALA and the neurological disturbances of the disease.

SECTION V

GENERAL DISCUSSION

GENERAL DISCUSSION

The most significant findings of this study are that at blood concentrations known to occur in acute porphyria, ALA penetrates into various organs and tissues, and exhibits pharmacological activity. These findings alone provide a basis from which further research can be carried out with a view to elucidating the exact role which ALA may play in the production of the signs and symptoms of acute porphyria.

There is now ample evidence that ALA can demonstrate varied pharmacological activity. Thus there are the observations obtained and reported in this thesis, plus those made by such as Becker et al, (1971); Eales et al, (1971) and Feldman et al, (1968). The previous negative observations made in this respect by other workers, were likely due to the fact that they had no purified ALA available (Goldberg et al, 1954) or due to the fact that the doses of ALA used were too small to elicit a response capable of measurement. For example, Jarret et al, (1956) found no change in the blood pressure of an anaesthetised cat after administration of ALA. The dose of ALA used, however, was 100µg/kg which would roughly correspond with a blood concentration of $\sim$ 2µg/ml.

As described above, to evoke a blood pressure response in the rat, a blood concentration in the region of 80µg/ml was normally required. (Assuming a blood volume of 50ml/kg in each case.)

It is evident, however, that the manifestations of acute porphyria are not merely acute responses to excessive circulating concentrations of ALA. Thus porphyric subjects can excrete large quantities of ALA and show no evidence of clinical manifestations of the disease (Haeger 1958), and ALA can be administered by mouth to subjects who demonstrate no subsequent symptoms resembling those of AIP (Berlin et al, 1954).

It is much more likely that prolonged exposure of the tissues to elevated ALA concentrations brings about changes in them which make them susceptible to whatever unknown agencies precipitate the porphyric attack. This would appear to be, theoretically at least, particularly applicable to the nervous systems. Whether such an effect can be mediated directly by ALA, or whether it is mediated by a biotransformation product of it (such as kryptopyrrole) or by the changes resulting from its increased synthesis, must remain speculative at present. It is possible that all these factors are involved.

In retrospect it is obvious that a great deal of potentially useful information might be obtained

from a series of pharmacological studies carried out on animals chronically pretreated with ALA. It was felt with many of the studies described above, that a comparison of normal and chronic ALA treated animals, would have been informative. In the experiments where this was carried out (convulsions and behavioural studies in rats) the effects of ALA were quite dramatic. It was felt, however, even in these cases, that a more steady and constant exposure to ALA was desirable to emulate the human condition in AIP.

It is possible that an ALA implant of some sort, or an intramuscular or subcutaneous bolus of ALA might achieve this end: where there would be a situation closer to the steady state of production and excretion which likely exists in the latent stages of AIP. The drawbacks of elevating ALA levels by porphyrinogenic drugs such as AIA, have already been described above.

Accepting that all of the clinical manifestations of AIP can be explained on a neuropathological basis (Goldberg 1959) it is worthwhile considering what new evidence is available from the above studies to implicate ALA in such a neurological disturbance.

The results obtained from the blood-brain barrier and behavioural studies show that ALA can

readily enter brain tissue and that it can affect animal behaviour. The lack of any evident peripheral involvement in the animals used in the behavioural studies, (e.g. limb weakness or paralysis, or loss of withdrawal reflexes in the chronic ALA animals) suggests that the effects seen here could quite possibly be centrally mediated. So too the convulsive effect of isonicotinyl hydrazide is centrally mediated, and the fact that ALA exerted a protective effect against INH compared with AIA treated animals, might be taken as evidence that ALA has central effects.

From the results of the peripheral studies it is difficult to ascertain whether or not ALA could have any effect on neural function. The studies from the 'in vivo' cardiovascular experiments in general, and the pithed rat experiments in particular, indicated that ALA when given in single doses did not affect sympathetic nerve transmission. So too the results from the field stimulated guinea pig ileum experiments, suggested ALA was similarly ineffective on cholinergic nerve transmission.

Other results, however, were suggestive of ALA having an effect on neurotransmission. Thus the occasional potentiation of the 'in vitro' artery sympathetic response by ALA, and the results from the acetylcholine release and ATPase studies, indicated that under certain conditions, a neurological

effect of ALA might be evident. Once more it might be pertinent to suggest that chronic nerve exposure to ALA may produce such changes in a much more readily recognisable, and experimentally verifiable, form.

In conclusion then it may be worthwhile to put forward a tentative hypothesis concerning the course of acute porphyria, with particular reference to ALA as a possible aetiological factor in the production of the signs and symptoms.

——— In the latent stages of the disease, there is a genetically mediated increase in the hepatic biosynthesis of ALA. There are two direct results of this: the first is that it causes a slight depletion in other tissues of the cofactors necessary for the synthesis of ALA and, in particular, of pyridoxal phosphate (Cavanagh and Ridley 1967), and it results in an increase in the blood concentration of ALA. Much of this ALA is excreted by the kidney, (Haeger 1958) but the 'steady-state ALA', that is the difference between the amount produced and the amount metabolised and excreted, reaches an equilibrium situation between the blood and the other tissues. Thus, effectively, ALA passes into various tissues including brain and nervous tissue (McGillion et al, 1974).

In the various tissues ALA can exert its pharmacological activity. Thus in the nervous tissues it causes inhibition of neuronal ATPase and membrane sodium transport; (Becker et al, 1971; Eales et al 1971). So too chemical or metabolic products of ALA, such as kryptopyrrole (Krischer and Pfeiffer 1973) may possibly affect, particularly the central nervous system.

The result of this is that progressive neurological changes develop in the peripheral and central nervous systems. The exact structural changes which occur in the nerves initially are ill defined (Gray 1966) but functionally the effects are not too evident at this stage. The essential change present is that the nervous system is potentially more susceptible to other neurally active agents, and is less co-ordinated, with perhaps a disco-ordination of homeostatic mechanisms in particular.

Thus this is the situation in the latent phase of the disease and it remains as such until some other factor stimulates the predisposed system into attack. Again hypothetically, it is felt that a prime feature of the precipitation of attacks could be the involvement of steroids.

When the porphyric subject, thus predisposed takes a significant amount of a porphyrinogenic drug such as a barbiturate, (or is exposed to other

precipitating agents) two things happen:- there is a further increase in ALA synthesis, with the consequent further depletion of tissue pyridoxal and increase in circulating ALA, and possibly a centrally mediated release of ACTH from the adeno-hypophysis. This latter statement perhaps requires some justification.

-----It is well known that many drugs can increase the rate of release of ACTH from the adenohypophysis, although relatively large doses are required systemically to do this (McCann 1957). It is quite possible however, that even quite small doses of drugs with this ability could release ACTH in acute porphyria as lesions of the hypophysial tract have been reported in the disease. Many of the drugs which precipitate attacks of porphyria (deMatteis 1967) are capable of ACTH release: and indeed, it is possible that many stimulate ALA synthetase activity indirectly via steroid thus released from the adrenal cortex.-----

The resultant increase in circulating ALA, and neural cofactor depletion brings the effects on the nerves above 'threshold', that is the nerve damage is now expressed functionally: and the whole process is exacerbated by the steroids and other associated metabolic disorders. The 'latent' damage of nervous regulatory and feed-back mechanisms

now becomes evident and we have the 'nervous explosion' typical of the acute porphyric attack.

What functional disturbances are present in the peripheral motor and central nerves at this stage is obscure, however it is possible that in the peripheral autonomic nerves there is increased transmitter release (cf.Section IV above) with a sympathetic dominance, and an increased output from the adrenal medulla, both of which may well be exacerbated by the excessive circulating levels of steroids affecting catecholamine uptake mechanisms (Iversen 1970).

These changes in the autonomic nerves and the degenerative changes which would occur in the motor and central nerves, could account for most of the signs and symptoms of AIP.

It is worth pointing out at this stage, that once ALA had produced its neuropathological changes, the progression of the attack would become independant of the blood ALA concentration, in as much as a decrease in circulating ALA during attack would be unlikely to affect its course except perhaps, in terms of time. However the severity and form of attack, that is whether mild, or severe, may well depend on the degree of neural damage produced by ALA and this may be related to the blood concentration of ALA.———

The above hypothesis is highly speculative and, to an extent, simplistic. It by no means offers an explanation for all the diverse aspects of acute porphyria, and may be justifiably criticised accordingly. It is, however, a working hypothesis, using experimentally verified facts, and assumptions which are not unreasonable: and it provides a basis for further experimental procedures which can verify or falsify these assumptions.

It is hoped that as a result of this work some progress has been made towards understanding the course of, and ultimately finding a cure for, acute intermittent porphyria.

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PUBLICATIONS AND COMMUNICATIONS

TO LEARNED SOCIETIES

PUBLICATIONS

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Cardiovascular Actions of △ Aminolaevulinic Acid.
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COMMUNICATIONS TO SOCIETIES

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